Direct Detection by In Situ PCR of the *amoA* Gene in Biofilm Resulting from a Nitrogen Removal Process

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Ammonia oxidation is a rate-limiting step in the biological removal of nitrogen from wastewater. Analysis of microbial communities possessing the *amoA* gene, which is a small subunit of the gene encoding ammonia monooxygenase, is important for controlling nitrogen removal. In this study, the *amoA* gene present in *Nitrosomonas europaea* cells in a pure culture and biofilms in a nitrifying reactor was amplified by in situ PCR. In this procedure, fixed cells were permeabilized with lysozyme and subjected to seminested PCR with a digoxigenin-labeled primer. Then, the amplicon was detected with an alkaline phosphatase-labeled antidigoxigenin antibody and HNPP (2-hydroxy-3-naphthoic acid-2′-phenylanilide phosphate), which was combined with Fast Red TR, and with an Alexa Fluor 488-labeled antidigoxigenin antibody. The *amoA* gene in the biofilms was detected with an unavoidable nonspecific signal when the former method was used for detection. On the other hand, the *amoA* gene in the biofilms was detected without a nonspecific signal, and the cells possessing the *amoA* gene were clearly observed near the surface of the biofilm when Alexa Fluor 488-labeled antidigoxigenin antibody was used for detection. Although functional gene expression was not detected in this study, detection of cells in a biofilm based on their function was demonstrated.

The biological removal of nitrogen compounds is an integral part of most modern wastewater treatment facilities to preserve environmental water resources. In this process, ammonia oxidation is a rate-limiting step, where autotrophic ammonia conversion into hydroxylamine is catalyzed by ammonia monooxygenase. Thus, analysis of microbial communities possessing the *amoA* gene, which encodes ammonia monooxygenase, is important for controlling nitrogen removal.

On the other hand, fluorescent in situ hybridization (FISH) (4, 9) and denaturing gradient gel electrophoresis (10, 17) based on 16S ribosomal DNA and rRNA for molecular analysis have been used in various fields to determine the genetic diversity of a microbial community and to identify individual members. In particular, in situ hybridization with fluorescence-labeled oligonucleotide probes has been widely used for in situ analysis of microbial communities, such as a biofilm in a wastewater treatment process (5, 18, 22, 29). This method relies on the presence of many target sequences within an individual cell. Therefore, bacterial cells containing insufficient rRNA cannot be detected by this approach. Moreover, this taxonomic identification approach cannot be used to detect the presence of single-copy functional genes or their expression at the single-cell level. Hence, in situ hybridization cannot estimate a specific metabolic activity such as ammonia oxidation.

Recently, in situ PCR was developed to amplify and detect functional genes and their expression inside a single cell, thus making it possible to detect a single copy of a functional gene. This method was first developed to amplify and detect a DNA virus inside a cell (11), and Nuovo et al. (21) and Bagasra et al.

(6) developed this method for molecular pathology. In environmental microbiology, in situ PCR and in situ reverse transcription-PCR protocols have been used to detect the presence and expression of *nahA* (12) and *todC1* (8) in *Pseudomonas* cells, *lac* in *Salmonella enterica* serovar Typhimurium (28), and *slt-I* and *slt-II* in *Escherichia coli* O157 (27). However, the in situ PCR protocol has been applied only to a dispersed sample of a model microbial community in seawater and river water and has never been used for the analysis of a biofilm. For this reason, little is known about the distribution of a functional gene in biofilms. To determine the distribution of functional genes and their expression in a biofilm is a primary objective in the fields of microbial ecology and wastewater treatment. The purposes of this study were to establish an original in situ PCR protocol for the detection of the *amoA* gene in a biofilm and to examine the distribution of a microbial community possessing the *amoA* gene in a biofilm for nitrogen removal.

MATERIALS AND METHODS

Samples and cell fixation. *Nitrosomonas europaea* (IFO 14298) as a representative ammonia-oxidizing bacterium was cultured in a nitrification medium according to the method of Watson and Mandel (30) and Bock et al. (7) with minor modifications. The culture was incubated at 28 to 30°C in the dark, and then cells were collected by centrifugation and washed with PBS solution (137 mM NaCl, 8.10 mM $Na_2HPO_4 \cdot 12H_2O$, 2.68 mM KCl, 1.47 mM KH_2PO_4; pH 7.4). Biofilms were collected from a laboratory-scale aerobic up-flow nitrifying reactor that treated inorganic-ammonia-rich wastewater when the ammonia load was about 1.5 kg of N/m^3/day. The granule-like biofilms were collected through a sampling port and were settled for a few minutes to separate them from the bioreactor liquid phase. The samples were suspended in 4% paraformaldehyde in PBS solution for 16 h (for the pure culture) and 20 h (for the biofilm) at 4°C for fixation and were then washed with PBS solution. The fixed biofilm samples were embedded in Tissu Mount (Chiba Medical, Saitama, Japan) and rapidly frozen at −25°C. Then, the biofilms were cut with a cryostat (CM1850; Leica Microsystems, Nussloch, Germany) into 10-μm-thick sections.

DNA extraction. DNA extraction was carried out according to the methods of Smalla (26) with minor modifications. DNA was extracted from a 0.5-g (wet

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weight) biofilm pellet. The biofilms were harvested by centrifugation at 10,000 × g for 10 min. The harvested cells were sonicated with an ultrasonic disrupter (type UR-20P; probe diameter, 2 mm; TOMY Seiko Co., Ltd., Tokyo, Japan) at 20 W and 28 kHz for 30 s in 5 ml of sucrose lysis buffer (0.3 M sucrose, 0.7 M NaCl, 40 mM EDTA, 50 mM Tris-HCl) and then centrifuged at 2,000 × g for 10 min. The supernatant was incubated at 55°C in the presence of sodium dodecyl sulfate (0.5%), proteinase K (0.1 mg/ml), and hexadecylmethylammonium bromide (1%). DNA was purified by applying phenol, chloroform, and isoamyl alcohol and precipitated by the addition of ethanol and sodium acetate.

Oligonucleotides. The following three primers were used: *amoA*-1F (5′-GGG GTTTCTACTGGTGGT-3′), *amoA*-2R (5′-CCCCTCKGSAAAGCCTTCTT C-3′ [K = G or T; S = G or C]) (25), and *amoA*-1FF (5′-CAATGGTGGCCG GTTGT-3′). These primers target stretches corresponding to positions 332 to 349, 802 to 822, and 187 to 203 of the open reading frame published previously for the *amoA* gene sequence of *N. europaea*, respectively. The *amoA*-1FF primer was designed specifically for ammonia-oxidizing bacteria belonging to the β subclass of *Proteobacteria*. Specificity was examined using FASTA (24) and BLAST (1) programs to compare the primers with the complete sequence data registered in GenBank. As for FISH analysis, the oligonucleotide probe Nso190 (16) labeled with CY3 was used for in situ detection of ammonia-oxidizing bacteria. Nso190 is a probe specific for a region in the 16S rRNA of ammonia-oxidizing bacteria.

Cell wall permeabilization. Cell wall permeabilization and in situ PCR were carried out according to the method of Hodson et al. (12) with minor modifications. The washed samples were resuspended in PBS solution. A 30-μl aliquot was spotted onto amino alkylsilane-coated slides (Applied Biosystems, Foster City, Calif.) and dried in an oven at 50°C for 5 min. Prior to cell wall permeabilization, the fixed samples were dehydrated sequentially in 50, 80, and 100% ethanol. The dehydrated samples were treated with the lysozyme solution (0.5 mg [for the pure culture] and 1.0 mg [for the biofilm] of lysozyme per ml, 100 mM Tris-HCl [pH 8.2], and 50 mM EDTA) for 30 min at room temperature. Lysozyme was removed by consecutive washes with PBS solution. Further permeabilization was carried out by treatment with proteinase K at a final concentration of 0.1 μg/ml for 10 min at room temperature, followed by heat inactivation of proteinase K at 94°C for 3 min. Then, the samples were washed with the PBS solution and dehydrated sequentially in 50, 80, and 100% ethanol. The samples on the slides are ready for the addition of PCR mixture.

In situ PCR procedures. A GeneAmp in situ PCR core kit (Applied Biosystems) was used for amplification of the *amoA* gene in cells. Then, the following seminested PCR protocol was used. First, a 50-μl aliquot of PCR buffer (10 mM Tris-HCl, 50 mM KCl; pH 8.3) containing 0.6 μM (each) *amoA*-1FF and *amoA*-2R, 2.5 mM MgCl$_2$, 0.6 mM (each) deoxynucleoside triphosphates, and 10 U of AmpliTaq DNA polymerase, IS (Applied Biosystems), was added to each sample spot on the slide and covered with an AmpliCover disk and AmpliCover clip (Applied Biosystems). The concentrations of polymerase and MgCl$_2$ were higher than those used in the conventional PCR because polymerase and MgCl$_2$ tend to adhere to a glass slide or a coverslip (20). PCR was performed with the following temperature profile using a GeneAmp in situ PCR system 1000 (Applied Biosystems): initial denaturation at 94°C for 90 s and then 20 cycles of denaturation at 94°C for 40 s, annealing at 53°C for 30 s, and extension at 72°C for 40 s. The slide was then washed with 0.5× SSC (750 mM NaCl, 75 mM trisodium citrate; pH 7.0) at 45°C for 10 min. In the second PCR, the reaction mixture was the same as that for the first amplification except that *amoA*-1FF was replaced by digoxigenin-labeled *amoA*-1F.

Detection of the amplified gene with Alexa Fluor 488-labeled antidigoxigenin antibody. A digoxigenin-labeled amplicon was detected with an Alexa Fluor 488 (Molecular Probes Inc., Eugene, Oreg.)-labeled antidigoxigenin antibody. Antidigoxigenin Fab fragments (Roche Diagnostics, Mannheim, Germany) were labeled with an Alexa Fluor 488 protein labeling kit (Molecular Probes) according to the manufacturer's instructions. First, 50 μl of a blocking buffer (30 μg of bovine serum albumin per ml in PBS solution) was spotted onto the samples and incubated for 30 min at room temperature. Then, 25 μl of the buffer was replaced by 25 μl of alkaline phosphatase-conjugated antidigoxigenin Fab fragments (Roche Diagnostics) diluted to 68 nM (for biofilm) or 0.27 μM (for pure culture) in a dilution buffer (100 mM Tris-HCl [pH 7.5], 150 mM NaCl), and incubated at 37°C for 1 h. After that, the slides were washed with buffer I (100 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Tween 20) three times and then washed with buffer II (100 mM Tris-HCl [pH 8.0], 100 mM NaCl, 10 mM MgCl$_2$) twice. Then, the samples were counterstained with TO-PRO-3 (Molecular Probes) for 5 min. For preparation of a working solution, TO-PRO-3 was diluted with double-distilled water (ddH$_2$O) 1,000-fold. After washing, the samples were mounted in FluoroGuard antifade reagent (Bio-Rad, Richmond, Calif.) for observation under a confocal laser scanning microscope (TCS4D; Leica Lasertechnik, Heidel-

berg, Germany) equipped with an Ar-Kr ion laser (488, 568, and 647 nm). Confocal images were obtained with a PL FLUOTAR ×10/0.30 objective (total magnification, ×100) and PL APO ×63/1.40 oil objective (total magnification, ×630). Figures were composed using Adobe Photoshop 6.0 (Adobe Systems, San Jose, Calif.) on a Macintosh PowerPC (Apple Computer Co., Cupertino, Calif.).

Detection of the amplified gene with alkaline phosphatase-labeled antidigoxigenin antibody. The detection of the amplicon with an alkaline phosphatase-labeled antidigoxigenin antibody and 2-hydroxy-3-naphthoic acid-2′-phenylanilide phosphate (HNPP), which was combined with Fast Red TR according to the methods of Yamaguchi et al. (31) with minor modifications. HNPP is converted to HNP by alkaline phosphatase. Then, HNP combines with Fast Red TR and produces a bright red fluorescent material, HNP-TR (13). The procedure was almost the same as that for detection using the Alexa Fluor 488-labeled antidigoxigenin antibody. After blocking, 25 μl of the buffer was replaced by 25 μl of alkaline phosphatase-labeled antidigoxigenin antibodies diluted to 60 in the dilution buffer and incubated at 37°C for 1 h. After the slides had been washed, the samples were counterstained with TO-PRO-3 for 5 min. After being washed with buffer II, the samples were incubated in HNPP-Fast Red TR (Roche Diagnostics) solution (100 μg of HNPP per ml and 250 μg of Fast Red TR per ml in buffer II) for 15 min at room temperature, and the slides were washed with ddH$_2$O. The samples were mounted in MacIlvaine buffer (53.2 mM citric acid and 93.6 mM Na$_2$HPO$_4$ [pH 4.5]) for observation under a confocal laser scanning microscope.

FISH targeting 16S ribosomal DNA. Hybridization was performed according to the standard protocol described by Amann (2) at 46°C for 2.5 h in a hybridization buffer containing NaCl (0.9 M), formamide (40%) (5, 16), Tris-HCl (20 mM, pH 7.4), and sodium dodecyl sulfate (0.01%). The probe concentration was 0.5 ng/μl. Hybridization was followed by a stringent washing step at 48°C for 20 min in a washing buffer containing Tris-HCl (20 mM, pH 7.4), NaCl (35 mM) (5, 16), and sodium dodecyl sulfate. The washing buffer was removed by rinsing the slides with ddH$_2$O. Then, the samples were counterstained with TO-PRO-3, mounted in FluoroGuard antifade reagent (Bio-Rad), and observed under the confocal laser scanning microscope.

PCR and agarose gel electrophoresis. To confirm specificity, extracted DNA was amplified by PCR with *amoA*-1F, *amoA*-2R, and *amoA*-1FF. Extracted DNA was added to 50 μl of a PCR buffer (5 mM Tris-HCl, 5 mM KCl, 10 μM EDTA, 0.1 mM dithiothreitol, 0.0001% Tween 20, 0.0001% Nonidet P-40) containing 0.5 μM (each) primers, 200 μM deoxynucleotide triphosphate, 1 mM MgSO$_4$, and 1 U of KOD DNA polymerase (Toyobo Co., Ltd., Osaka, Japan). PCR was carried out in a GeneAmp PCR system 9700 (Applied Biosystems) using the same cycle program as the in situ PCR cycle program. PCR products were electrophoresed in a 2% agarose gel and visualized by ethidium bromide staining.

RESULTS

Amplification of *amoA* from extracted DNA. Amplification of the *amoA* gene from extracted DNA of the biofilms using the oligonucleotide primer set *amoA*-1FF and *amoA*-2R resulted in a 636-bp DNA fragment with a slight smear (Fig. 1, lane 2). The 636-bp amplicon was reamplified using the seminested primer set *amoA*-1F and *amoA*-2R, and as a result, only a 491-bp amplicon was generated (Fig. 1, lane 1).

***amoA* gene detection with in situ PCR.** To establish the in situ PCR protocol, the *amoA* gene in *N. europaea* was amplified by in situ PCR. At first, detection of the amplicon generated by *amoA*-1F and *amoA*-2R was attempted by in situ hybridization with the fluorescein isothiocyanate-labeled probe. However, no signal was observed under the confocal laser scanning microscope. An insufficient amount of amplicon or problems related to hybridization were thought to be the cause of this failure. To solve these problems, the seminested PCR protocol has been used to increase the specificity and sensitivity of in situ PCR (12, 17, 28). The *amoA*-1F and *amoA*-2R primers were used in the first PCR, and *amoA*-1F and a fluorescein isothiocyanate-labeled primer that was complementary to a region internal to *amoA*-1F and *amoA*-2R were used in the second PCR. As a result, although the level of sensitivity was certainly increased, the level of signal intensity was still very
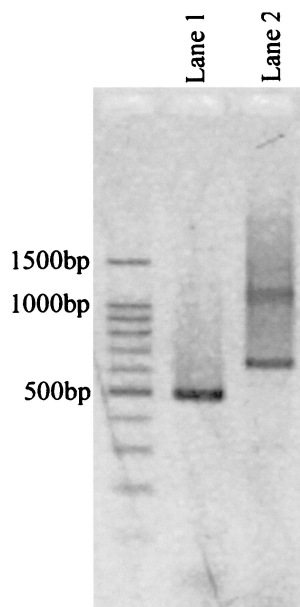
FIG. 1. Gel electrophoresis of *amoA* gene products amplified from extracted DNA of the biofilms. Lane 1, 491-bp *amoA* gene fragment amplified by primers *amoA*-1F and *amoA*-2R; lane 2, 636-bp *amoA* gene fragment amplified by primers *amoA*-1FF and *amoA*-2R

low. To increase the sensitivity further, a digoxigenin-labeled primer was used in the second PCR, and the amplicon was detected with the alkaline phosphatase-labeled antidigoxigenin antibody and HNPP combined with Fast Red TR. The level of sensitivity was increased significantly, and thus, this method was applied to the subsequent in situ PCR procedure.

First, the *amoA* gene in an *N. europaea* cell was amplified and detected. In situ PCR requires that the polymerase be able to penetrate into the cells and act on the nucleic acid in cells without the PCR amplicon diffusing away from the cells. Therefore, to determine the optimal pretreatment conditions, the time of fixation with 4% paraformaldehyde was varied from 4 to 20 h and the lysozyme concentration was varied from 0.05 to 5.0 mg/ml. As a result, the *amoA* gene was successfully detected inside intact *N. europaea* cells that had been fixed for 16 h and permeabilized with 0.5-mg/ml lysozyme buffer (Fig. 2A). Moreover, the protocol was applied to the biofilm that was collected from the up-flow aerobic nitrifying reactor. The fixation time was varied from 4 to 32 h, and the lysozyme concentration was varied from 0.05 to 5.0 mg/ml. The *amoA* gene was detectable in the sample fixed for 20 h and permeabilized with 1.0 mg of lysozyme per ml. However, an unavoidable nonspecific signal was particularly observed in the case of the biofilm (Fig. 2B and C).

To reduce the intensity of the nonspecific signal, the digoxigenin-labeled amplicon in the cell was detected with antidigoxigenin antibodies labeled with Alexa Fluor 488. As a result, the *amoA* gene was detected inside *N. europaea* cells without the nonspecific signal (Fig. 3A). Moreover, the *amoA* gene in the biofilm was also detected with a weak nonspecific signal, as shown in Fig. 3B and C. The green fluorescence in Fig. 3C (upper right) is the supposed nonspecific signal caused by adsorption of the antibodies. However, it was clear that cells
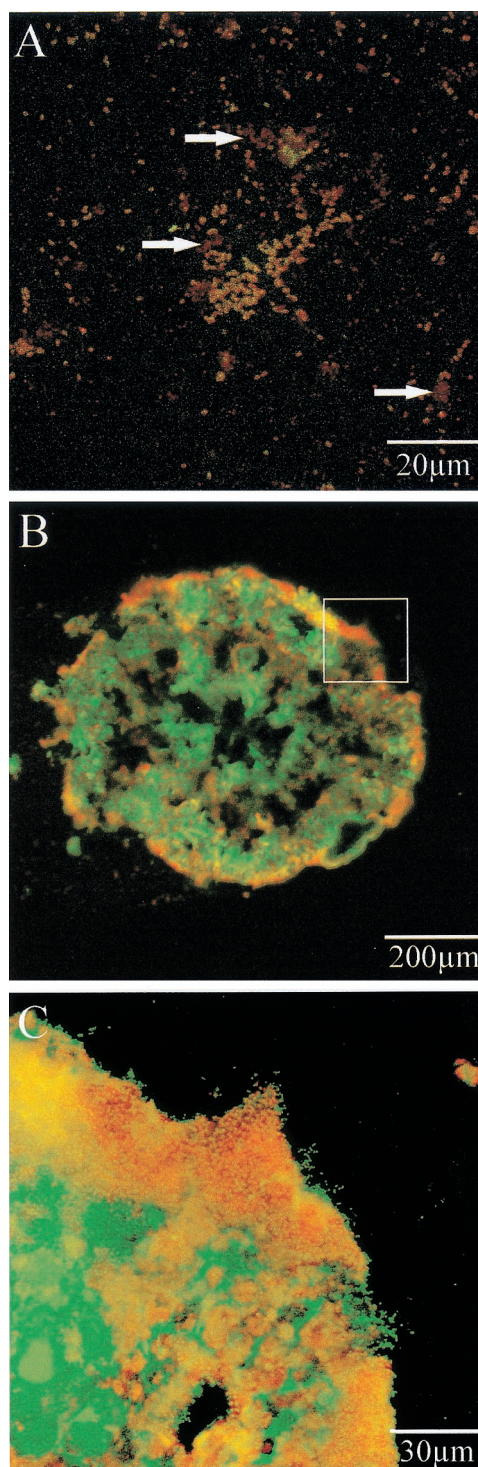

FIG. 2. Detection of ammonia-oxidizing bacterial cells possessing *amoA* gene in pure culture (A) and in the biofilm (B and C), with alkaline phosphatase-labeled antidigoxigenin antibody. Panels B and C show the same biofilm. The yellow color indicates positive cells, while the green shows negative cells (arrows indicate the nonspecific signal). The portion of panel B that was magnified to produce panel C is indicated with a white box.
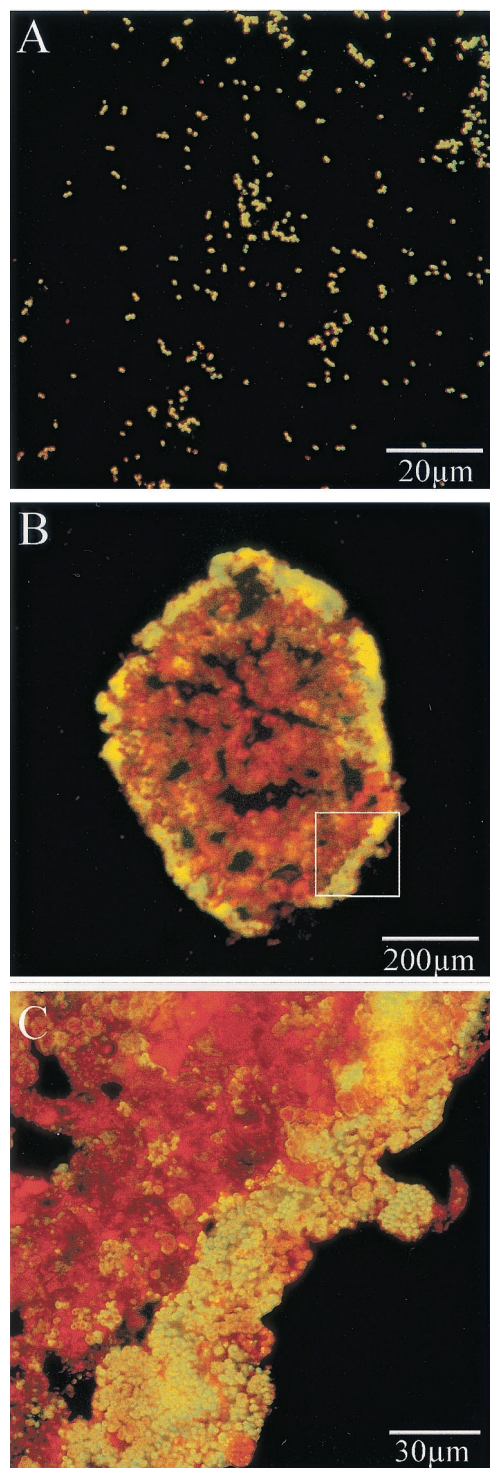
FIG. 3. Detection of the cells possessing *amoA* in pure culture (A) and in the biofilm (B and C), with Alexa Fluor 488-labeled antidigoxigenin antibody. Panels B and C show the same biofilm. The yellow color indicates positive cells, while the red shows negative cells. The portion of panel B that was magnified to produce panel C is indicated with a white box.
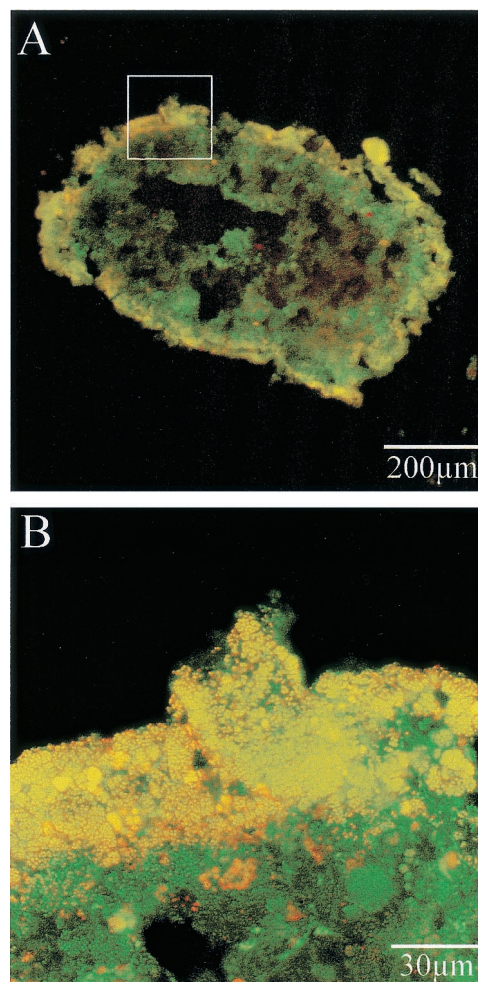

FIG. 4. Detection of ammonia-oxidizing bacteria with a CY3-labeled Nso190 probe. Panels A and B show the same biofilm. The yellow color indicates ammonia oxidizers, while the green shows other bacteria. The portion of panel A that was magnified to produce panel B is indicated with a white box.

possessing the *amoA* gene were found near the surface of the biofilm.

FISH. To confirm the validity of microbial communities detected by in situ PCR, ammonia-oxidizing bacteria in the biofilm were visualized by FISH analysis (Fig. 4). The yellow shows ammonia-oxidizing bacteria, and the green shows other bacteria. It was observed that ammonia-oxidizing bacteria were the dominant population on the surface of the biofilm.

DISCUSSION

At first, the *amoA* gene in the *N. europaea* cells (pure culture) and the biofilm (mixed culture) was detected with an alkaline phosphatase-labeled antidigoxigenin antibody; however, an unavoidable nonspecific signal was observed, which has also been reported by Tani et al. (27). This was probably due to the fluorescent HNP-TR diffusing out of the cells.

To reduce the intensity of the nonspecific signal, a new strategy of detecting the amplicon in the cells with an Alexa Fluor 488-labeled antidigoxigenin antibody was proposed.

First, detection of the *amoA* gene in the cell was conducted completely under the same conditions as for the alkaline phosphatase-labeled antidigoxigenin antibody. However, a sufficient signal intensity was not obtained, which might be due to the lower penetration efficiency and/or the lower sensitivity of the antibody compared with the alkaline phosphatase-labeled antidigoxigenin antibody. Therefore, several concentrations (0.035, 0.07, and 0.14 μM) were attempted to increase signal intensity. In consequence, when the concentration of the antibody was increased to 0.14 μM, for the *N. europaea* cells, the *amoA* gene was detected without the nonspecific signal. As for the biofilm, the *amoA* gene was successfully detected with a low background signal, although the concentration of the antibody was increased to 35 nM. These results indicate that the use of Alexa Fluor 488-labeled antibody enables the construction of a promising detection system that can be applied to in situ PCR.

The images obtained both by in situ PCR analysis with the Alexa Fluor 488-labeled antibody and by FISH analysis show that the ammonia-oxidizing bacteria were found near the surface of the biofilms. This result suggests that the bacteria possessing the *amoA* gene were successfully detected by in situ PCR. Although FISH analysis is a very important tool in microbial ecology, cells with an insufficient ribosomal content cannot be detected by this method. Practically, a fluorescent signal was observed only near the surface of the biofilms by FISH analysis with EUB338 (a probe targeting all bacteria except *Archaea*), which was probably due to the low activity of the bacteria living in a deep part of the biofilm (data not shown). In spite of extensive attempts to increase the intensity of the fluorescent signal from FISH by sample enrichment (25) and using a single probe with multiple fluorochromes (4) and multiple probes (3), low-activity cells are difficult to detect. In contrast, a few copies of the *amoA* gene in the cells were successfully detected by in situ PCR. This indicates that cells possessing a functional gene can be detected by in situ PCR regardless of its activity. Actually, when the biofilm cultivated without ammonia for a month was examined by FISH and in situ PCR, ammonia-oxidizing bacteria were not detected by FISH at all, whereas the cells possessing the *amoA* gene were detected near the surface of the biofilms by in situ PCR (data not shown).

However, the images obtained by in situ PCR analysis are less distinct than those obtained by FISH analysis. This was probably because cells were damaged by permeabilization and/or thermal cycling, as well as (i) diffusion of the labeled amplicon and its adhesion to negative cells, (ii) adhesion of the digoxigenin-labeled primer to negative cells, and (iii) adhesion of the Alexa Fluor 488-labeled antibody to negative cells.

Cases 2 and 3 are practically inconceivable because no signal was detected when the negative control without polymerase was examined. As for case 1, this diffusion artifact has been reported as a problem of in situ PCR (14, 20). On the other hand, Nuovo (19) reported that the movement of DNA segments was restricted not by the pore size but rather by biochemical forces, such as hydrogen bonding and ionic charges. Therefore, under optimal fixation and permeabilization conditions, there is minimal migration of the amplicon from its site of origin, and diffusion artifacts are significantly reduced by reduction of the PCR cycle ($\leq$30), generation of longer PCR

products, or incorporation of biotinylated nucleotides to generate bulkier and thus less diffusible PCR products (15, 23). In this study, to increase the level of sensitivity, the seminested PCR protocol was used, and the total number of thermal cycles was 40 cycles. This led to the destruction of the cell morphology, which probably caused the diffusion artifacts. However, taking into account that fewer cycles caused a very low signal, it is important to optimize these parameters very carefully and to simplify the PCR protocol to the furthest extent possible.

In this study, the cells possessing the *amoA* gene were successfully detected by in situ PCR with the Alexa Fluor 488-labeled antibody. As a result, bacteria could be detected by in situ PCR regardless of its rRNA content and could be detected based on their function. On the other hand, problems that are not observed when in situ PCR is applied to dispersed samples were revealed when in situ PCR was applied to biofilms. However, the fact that the spatial distribution of a functional gene in the biofilm becomes detectable represents significant progress in this field. This in situ PCR is expected to be a valuable method in cases such as those in which the ammonia oxidation rate has dropped although the number of ammonia-oxidizing bacteria is not changed, and it will be essential for explaining such phenomena. It is anticipated that in future studies, functional gene expression (mRNA) will be detected by in situ PCR, and consequently the spatial distribution of the "actually functioning" bacteria in a biofilm from the natural environment or from a wastewater treatment system will be elucidated.

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