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ORIGINAL PAPER

Change in the community structure of ammonia-oxidizing bacteria in activated sludge during selective incubation for MPN determination

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Abstract We investigated the changes in the community structure of ammonia-oxidizing bacteria (AOB) in activated sludge during incubation of the sludge in a medium selective for AOB. The number of AOB present in the activated sludge sample was enumerated by the most-probable-number (MPN) method. Both the activated sludge sample and the incubated samples for MPN determination were analyzed by polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE). Universal PCR-DGGE indicated that even after 40-d incubation in a medium selected for AOB, the MPN samples were predominantly composed of heterotrophic bacteria and not AOB. Denitrification by heterotrophic bacteria might lead to the underestimation of the MPN count of AOB. Not dominated in whole bacteria, one species of AOB was detected in both original activated sludge and samples after MPN incubation by PCR-DGGE targeting AOB. Furthermore, two new species of AOB were detected only after incubation. Therefore, the community structure of AOB in the MPN samples partially resembled that in the original activated sludge.

Keywords Ammonia-oxidizing bacteria · Most-probable-number (MPN) method · Polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) · Microbial community structure

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Introduction

Ammonia-oxidizing bacteria (AOB) play an important role in nitrogen removal in wastewater treatment processes and in the nitrogen cycle in aquatic environments. The most-probable number (MPN) method has been commonly used as the standard method for enumerating AOB in Japan [1]. In this method, samples are incubated in a selective growth medium containing NH_4^+. The presence of AOB is identified by the generation of NO_2^-, and the number of AOB is determined by a statistical procedure.

By using the MPN method, one can only enumerate live AOB. This method can detect AOB even in samples with low numbers of this bacterium (low detection limit). However, the enumeration of AOB by this method involves the preparation of a lot of dilution series and an incubation period of more than 1 month. Moreover, enumeration given by MPN method is known to have the possibility of underestimation in case in which the ammonia concentration, salinity, and pH of the incubation media have not been well chosen [2]. The MPN value represents only the number of AOB incubated under the given conditions. Few studies have investigated about whether the dominant AOB in original samples is still dominant after MPN incubation. Kowalchuk et al. investigated the AOB community structure in the highest positive MPN dilutions by polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) hybridization [3, 4]. They reported that the AOB community structure in these dilutions did not differ significantly with that in the original sample. However, their analysis targeted only AOB and was performed using the positive MPN samples.

Therefore, we investigated the bacterial community structure in both an activated sludge sample and in samples incubated for MPN estimation by using PCR-DGGE to

ascertain whether the AOB community structure in the highest positive MPN dilution reflects that in the original environmental sample. Samples at the highest dilution stage, including MPN-positive samples, were subjected to PCR, which targeted not only AOB but also the other bacteria present in the sample.

Methods

Sampling site

In November 2006, an activated sludge sample was collected from the membrane activated sludge reactor at the Field Science Center, Graduate School of Agriculture Science, Tohoku University, Japan. The reactor consisted of an approximately 3 m^3 aerobic tank. The hydraulic retention time (HRT) and sludge retention time (SRT) were set at 3 d and 23 d, respectively. On sampling day, the concentration of the mixed-liquor suspended solids (MLSS) of the activated sludge from the reactor was estimated as 4,900 mg/L. The influent wastewater from a milking parlor contained approximately 40 mgN/L of ammonium; however, the ammonium was not detected in the effluent.

AOB in the activated sludge sample were enumerated by using the MPN method. Further, 0.5 mL of the activated sludge sample was centrifuged at 17,860×*g* for 15 min. The supernatant was discarded, and the pellet was used for DNA extraction and PCR amplification.

MPN culture and preparation of the DNA template for molecular analysis

MPN counts of AOB were determined according to the protocol formulated by the Japan Sewage Works Association [1], with modifications in the method used to identify MPN positive/negative tubes. The activated sludge sample was divided into three aliquots. A 10-fold dilution series of each aliquot was prepared using sterilized saline, and 1 mL of each dilution was added to 9 mL of the sterilized growth medium in plastic tubes (five replicates per dilution stage). The tubes were then incubated at 28°C. The growth medium contained 30 mg/L $(NH_4)_2SO_4$, 100 mg/L KH_2PO_4, 6 mg/L EDTA-Fe, 50 mg/L $MgSO_47H_2O$, 20 mg/L $CaCl_22H_2O$, 200 mg/L $NaHCO_3$, 2 mg/L$CaCO_3$, and a small amount of quartzose sand.

After incubating the tubes for 40 days, they were centrifuged at 17,860×*g* for 15 min. Contents of each tube were harvested as a pellet, and the amount of NO_2^- and NO_3^- in the supernatant was measured using an ion chromatography system (Dionex Corporation, Sunnyvale, USA). The MPN tubes were scored as positive or negative based on the presence or absence of nitrite and/or nitrate. The MPN values were calculated using the table prepared by the Japan Sewage Works Association. After the MPN values were calculated, the pellets from the highest positive MPN dilution stage tubes were used for DNA extraction and PCR amplification (referred to as MPN sample hereafter).

According to the protocol described by the Japan Sewage Works Association, nitrite production in the positive tube is identified by using Griess-Romijn (GR) reagent and zinc powder. The detection limit of nitrite production by using the GR reagent was previously estimated as 0.03 mgN/L (data not shown); therefore, in this study, tubes in which more than 0.03 mgN/L of nitrite (NO_2^-) and/or nitrate (NO_3^-) was measured by using an ion chromatography system were recorded as MPN positive tubes.

DNA extraction from activated sludge and enrichment cultures

Genomic DNA was extracted from the pellets of the activated sludge or MPN samples by using the benzyl chloride method [5] as follows. Each pellet was shaken with a DNA extraction buffer (250 µl; 100 mmol/L of Tris-HCl, 40 mmol/L of EDTA [pH 9.0]), benzyl chloride (200 µl), and 10% sodium dodecyl sulfate (50 µl) at 50°C for 30 min. After adding 3 M sodium acetate (150 ml), the mixture was centrifuged, and the supernatant was precipitated with isopropanol. The precipitate was washed with 70% ethanol and then dried, and then dissolved in Tris-EDTA buffer (100 µl; 10 mmol/L of Tris-HCl and 1 mmol/L of EDTA [pH 7.5]). The extract was purified using MagExtractor-Genome (Toyobo, Japan).

PCR conditions

DNA extracted from the activated sludge sample or MPN samples was amplified by nested PCR performed using the following primer sets: 27f-1492r (primary) [6], 968f-1401r (secondary) [7], CTO189f-CTO654r (secondary) [8], amoA2f-amoA5r (primary) [9], and amoA1f-amoA2r (secondary) [9]. The primer sets of 27f-1492r and 968f-1401r are specific for the amplification of universal bacterial 16S rRNA, and the CTO189f-CTO654r primer set, for the amplification of the 16S-rRNA β-subgroup ammonia oxidizers. The primer sets of amoA2f-amoA5r and amoA1f-amoA2r target the *amoA* gene of the β-subgroup ammonia oxidizers. For PCR-DGGE, the GC-rich sequence (GC-clamp) was added to the primers 968f, CTO189f, and amoA1f.

Amplification was performed in a total reaction volume of 50 µl by using iCycler (Bio-Rad laboratories Inc., USA). The reactions were carried out in a solution containing 1× PCR buffer (0.025 U/L of Ex Taq (Takara, Japan)), 1× Ex Taq buffer, 0.5 mM of each dNTP, and 0.25 mM of each

primer. Table 1 lists the PCR primers and the PCR conditions used in this study. The PCR products were examined by standard agarose gel electrophoresis (1.2% agarose, 1× Tris-acetate-EDTA (TAE)) followed by ethidium bromide staining to confirm the product size.

DGGE

DGGE was performed using the D-code system (Bio-Rad laboratories Inc., Foster City, USA) according to the manufacturer's instructions. PCR products were loaded onto 8% (w/v) polyacrylamide gels in 1.0× TAE buffer (40 mM Tris-HCl, 40 mM acetic acid, 1 mM EDTA; pH 8.4). The polyacrylamide gels were prepared with a denaturing gradient ranging from 30% to 60% (100% denaturant contains 7 M urea and 40% formamide). Table 2 lists the DGGE conditions used in this study. After electrophoresis, the gels were soaked for 15 min in the GelStar nucleic acid gel stain (dilution, 1:10000; Takara, Japan).

Table 2 DGGE condition used in this study

Secondary PCR primer sets	Temperature (°C)	Electrical pressure (V)	Time (h)
968f-1401r	60	160	8
CTOf-CTOr	60°	65	16
amoA1f-amoA2r	60°	100	8

Sequencing

DGGE bands were cut from the gel and placed overnight in vials containing 100 µl of sterilized water for extraction. Extracted DNA from the bands was used as template DNA in the PCR performed using non-GC clamp primers. After amplification, the PCR products were purified using the MagExtractor-PCR & Gel Clean up kit (Toyobo, Japan) and used as template DNA in a sequencing reaction performed using the BigDye Terminator v1.1 Cycle Sequence Kit (Applied Biosystems, USA) according to the manufacturer's instructions. The DNA fragments were sequenced using a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, USA).

The sequences were compared with known sequences listed in the GenBank nucleotide sequence databases. The basic local alignment search tool (BLAST) search option of the National Center for Biotechnology Information (NCBI) internet site (http://www.ncbi.nlm.nih.gov) was used to search for close evolutionary relatives in the GenBank database.

Result

MPN enumeration

The positive/negative identification of MPN tubes and the calculated MPN values are shown in Table 3. Nitrite was not detected in all the MPN tubes. In the case of aliquot 1, all five 10^{-1} diluted MPN tubes were positive, i.e., nitrate was detected; only two of the five 10^{-2} diluted samples were positive; and all five 10^{-3} diluted samples were negative, i.e., nitrate was not detected. In the case of aliquots

Table 1 Summary of phylogenetic primers, functional primers, and PCR conditions used in this study

Primer[ab]	Target gene	Nested-PCR amplification stage	Thermocycling program
27f-1492r	16S rRNA	Primary	5 min at 94°C; followed 35 cycles of 30 s at 94°C, 30 s at 55°C, and 100 s at 72°C; followed by a 5 min final extension at 72°C
968f-1401r	16S rRNA	Secondary	5 min at 94°C; followed 35cycles of 30 s at 94°C, 30 s at 57°C, and 40 s at 72°C; followed by a 5 min final extension at 72°C
CTO189f-CTO654r	16S rRNA	Secondary	5 min at 94°C; followed 35 cycles of 30 s at 94°C, 30 s at 57°C, and 45 s at 72°C; followed by a 5 min final extension at 72°C
amoA2f-amoA5r	amoA	Primary	5 min at 94°C; followed 35 cycles of 30 s at 94°C, 50 s at 45°C, and 60 s at 72°C; followed by a 5 min final extension at 72°C
amoA1f-amoA2r	amoA	Secondary	5 min at 94°C; followed 35 cycles of 30 s at 94°C, 30 s at 55°C, and 40 s at 72°C; followed by a 5 min final extension at 72°C

[a] f, Forward primer; r, Reverse primer

[b] Primer 968f, CTOf, and amoA1f had the GC clamp for DGGE

Table 3 Nitrate concentration in the MPN samples (mgN/L) and MPN count(MPN/mL)

Dilution	Sample														
	Aliquot 1					Aliquot 2					Aliquot 3				
	1-1	1-2	1-3	1-4	1-5	2-1	2-2	2-3	2-4	2-5	3-1	3-2	3-3	3-4	3-5
10^{-1}	0.10	0.034	0.22	0.61	0.16	0.28	1.9	0.74	1.7	0.63	1.6	0.21	0.97	1.6	2.2
10^{-2}	N.D.	N.D.	N.D.	0.17	0.37	0.079	1.4	0.69	1.2	0.92	3.2	1.7	0.69	3.0	2.7
10^{-3}	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
MPN count	4.9×10 (MPN/mL)					2.4×10^2 (MPN/mL)					2.4×10^2 (MPN/mL)				

two and three, all the 10^{-1} and 10^{-2} diluted samples were positive and all five 10^{-3} diluted samples were negative. Based on these findings, the MPN values of aliquots one, two, and three were calculated as 4.9×10, 2.4×10^2, and 2.4×10^2, respectively. Additionally, nitrate concentration varied widely even in samples at the same dilution stage.

PCR-DGGE

The activated sludge sample and the 10^{-2} diluted MPN samples each of the three aliquots were analyzed by PCR-DGGE. Fig. 1 shows the patterns of the nested PCR-DGGE bands. PCR-DGGE was performed using the universal bacterial 16S rRNA sets 27f-1492r (primary), 968f-1401r (secondary). The DGGE banding patterns of the MPN samples were different from that of the activated sludge sample. Four bands were obtained from the activated sludge sample and eight bands from the 10^{-2} diluted MPN samples; three of these eight bands had the same sequence. The results of the sequencing and the BLAST analysis of the bands are shown in Table 4. The sequences of bands S-U1 to S-U4, which were obtained from the activated sludge sample, were similar to those of known bacteria of the phylum Proteobacteria; these bacteria are not AOB. Further, the sequences of bands M-U1 to M-U6, which were obtained from the MPN samples, were similar to those of known bacteria of four phyla (Chlamydiae, Verrucomicrobia, Bacteroidetes, and Proteobacteria); these bacteria are also not AOB. Thus, the microbial community structure in the original environmental sample was altered during the selective incubation of this sample for MPN enumeration. Although the growth medium was selective for AOB, it was observed that AOB were not the dominant species in the activated sludge and MPN samples.

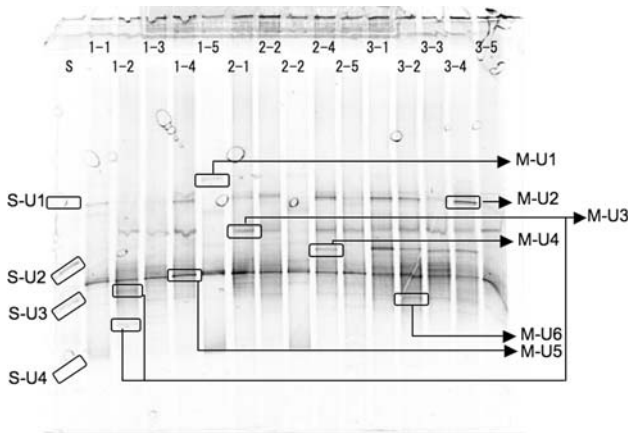

Fig. 1 PCR-DGGE profile of amplified 16S rRNA fragment by universal primer set (27f-1492 [primary], 968f-1492r [secondary]). Samples shown are S (activated sludge sample), 1-1 to 1-5 (MPN samples of aliquot 1 on 10-2 dilution stage), 2-1 to 2-5 (MPN samples of aliquot 2 on 10-2 dilution stage), 3-1 to 3-5 (MPN samples of aliquot 3 on 10-2 dilution stage). Since three DGGE bands of MPN samples had the same sequence, five sequences were obtained from MPN samples. Four sequences were obtained from activated sludge sample

DNA from the activated sludge sample was amplified by nested PCR performed using the primer sets amoA2F-amoA5R (primary) and amoA1F-amoA2R (secondary); however, PCR products were not detected from the MPN samples. PCR-DGGE of the DNA extracted from the activated sludge sample yielded three bands. The results of the sequencing and BLAST analysis of the three bands are shown in Table 5. The detected partial *amoA* gene sequences of AOB are similar to those of known bacteria of the genus *Nitrosospira*.

Figure 2 shows the PCR-DGGE band pattern obtained using the 27f-1492r (primary) and CTO189f-CTO654r (secondary) primer sets targeting the 16S rRNA of the β-subgroup ammonia oxidizers. One band was obtained from the activated sludge sample, and seven bands were obtained from the positive MPN samples, i.e., 1-4 to 1-5, 2-1 to 2-5, 3-1 to 3-5. Furthermore, even from the negative MPN samples, i.e., 1-1 to 1-3, DNA was amplified, and the DGGE bands were obtained at the same position as those from the positive MPN samples. The sequences of five bands were entirely the same; therefore, three sequences were obtained from seven bands. The results of the sequencing and the BLAST of the bands are shown in Table 6.

Band CTO-2 was obtained from both the activated sludge sample and the MPN samples, and its sequence was entirely the same as those of Nitrosospira sp. Nsp5, *Nitrosospira* sp. Nsp12, Nitrosospira sp. EnWyke2, Nitrosospira

Table 4 sequence analysis of the DGGE-band using universal primer set

Band	Closest species			
	Name	Identity	Acession no.	Phylum
S-U1	*Bdellovibrio bacteriovorus*	400/416 (96%)	AF263832	Proteobacteria
S-U2	*Alkanindiges hongkongensis*	355/375 (94%)	AY251391	Proteobacteria
S-U3	*Xanthomonas axonopodis*	383/395 (96%)	AB101447	Proteobacteria
S-U4	*Xanthomonadaceae* bacterium	396/423 (93%)	AY673181	Proteobacteria
M-U1	*Parachlamydia acanthamoebae*	295/318 (92%)	Y07556	Chlamydiae
M-U2	*Prosthecoba*cter vanneervenii	373/384 (97%)	U60013	Verrucomicrobia
M-U3	*Pseudom*onas fluorescens	394/394 (100%)	EF424136	Proteobacteria
M-U4	*Fluviicol*a taffensis	200/213 (93%)	AF493694	Bacteroidetes
M-U5	*Pseudomonas fluorescens*	393/394 (99%)	EF424136	Proteobacteria
M-U6	*Pseudomonas* sp. J10	389/394 (98%)	DQ289551	Proteobacteria

Table 5 Sequence analysis of the DGGE-bands using amoA primer set

Band	Closest species		Identity
	Name	Accession no.	
amo-1	*Nitrosospira* sp. Nsp5	411/415 (99%)	AY123834
amo-2	*Nitrosospira* sp. Nsp5	411/415 (99%)	AY123834
amo-3	*Nitrosospira* sp. Nsp12	402/413 (97%)	AY123823

sp. 40KI, Nitrosospira sp. Is148. Different sequences of these strains were amplified with the *amoA* primers; however, when the CTO primers were used, the same sequences of these strains were amplified.

Band CTO-1 and CTO-3 were obtained only from the MPN samples. The sequences of band CTO-1 and CTO-3 were similar to the 16S rRNA sequences of *Nitrosomonas* sp. NL7 and *Nitrosospira* sp. Ka3, respectively. AOB, which were not detected before incubation were detected after the samples were incubated in a selective growth medium.

Discussion

The number of nitrifiers in the activated sludge of wastewater treatment plants (WWTPs) has been estimated as 1×10^7–10^8 MPN/mL [1]. In Heidelberg, Germany, 1×10^4–10^5 MPN/mL of AOB was detected in a municipal WWTP [10]. Moreover, 1×10^5–10^7 MPN/mL of AOB was detected from a municipal WWTP in Tokyo, Japan [11]. In this study, the average number of AOB in the activated sludge sample was 1.8×10^2 MPN/mL, which was less than that previously reported. However, since approximately 40 mg/L of influent ammonium nitrogen (NH_4^+-N) was not detected in the effluent, it was considered that complete nitrification was achieved.

The results of the PCR-DGGE analysis possibly indicated that even after incubating the MPN samples in a growth medium selective for AOB for 40 days, the samples predominantly composed of heterotrophic bacteria and not AOB. Although the growth medium did not contain organic

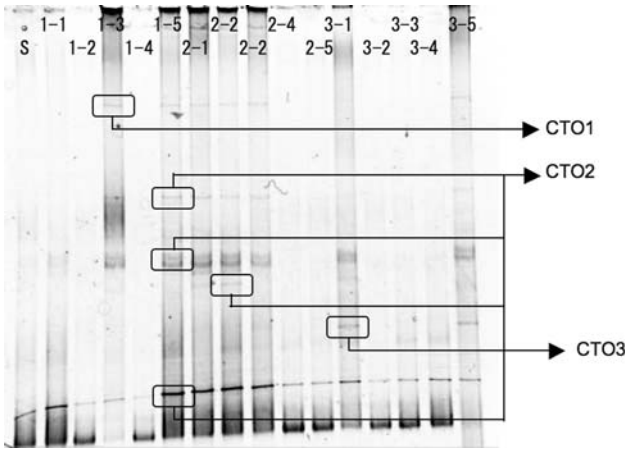

Fig. 2 PCR-DGGE profile of amplified 16S rRNA fragment by specific primer set for AOB (27f-1492 [primary], CTOf-CTOr [secondary]). Samples shown are S (activated sludge sample), 1-1 to 1-5 (MPN samples of aliquot 1 on 10-2 dilution stage), 2-1 to 2-5 (MPN samples of aliquot 2 on 10-2 dilution stage), 3-1 to 3-5 (MPN samples of aliquot 3 on 10-2 dilution stage). Since five DGGE bands of MPN samples had the same sequence, three sequences were obtained from MPN samples. One sequence was obtained from activated sludge sample

Table 6 sequence analysis of the DGGE-band using CTO primer set

Band	Closest species		
	Name	Identity	Accession no.
cto-1	Nitrosomonas sp. NL7	407/423 (96%)	AY958677
cto-2	Nitrosospira sp. Nsp5	424/424 (100%)	AY123793
	Nitrosospira sp. Nsp12	424/424 (100%)	AY123801
	Nitrosospira sp. EnWyke2	424/424 (100%)	EF175095
	Nitrosospira sp. 40KI	424/424 (100%)	X84656
	Nitrosospira sp. Is148	424/424 (100%)	AJ621030
cto-3	Nitrosospira sp. Ka3	420/424 (99%)	AY123806

substrates, 10^{-2} MPN tubes contained organic matter (one part per thousand) derived from the activated sludge sample. There is a possibility that incubated AOB was used as carbon source by heterotrophic bacteria. It has been reported that the ecosystem started from AOB was constructed in a carbon-limited autotrophic nitrifying biofilm fed only NH_4^+ as an energy source and composed of 50% heterotrophic bacteria [12].

Not dominated in whole bacteria, the same sequence that was similar to the genus *Nitrosospira* was detected from both the activated sludge sample and MPN samples using PCR-DGGE targeting AOB. However, two 16S rRNA sequences of AOB, which were detected from the MPN samples, were not detected from the activated sludge sample. One sequence was similar to that of bacteria belonging to the genus *Nitrosospira*, and another sequence was similar to the genus *Nitrosomonas*. Kowalchuk et al. also reported that 16S rRNA sequences similar to those of *Nitrosomonas* cluster 7 were detected from the highest positive MPN dilutions of a compost sample by PCR-DGGE hybridization; however, these sequences were not detected in the sample prior to its incubation for MPN enumeration [3]. These results seem to indicate that the numbers of AOB, which was less than the detection limit prior to incubation, increased under appropriate culturing conditions to a detectable numbers. PCR is known to have high detection sensitivity. However, it has been reported that the initial template DNA ratio in a sample obtained from a mixed population may affect the detection of bacterial species that have low levels of abundance by genus-specific PCR-DGGE [13, 14]. Thus, when the concentration of the DNA template of a particular bacterial species is significantly higher than that of another species, the species with a lower concentration of the DNA template may not be detected by PCR-DGGE even if this "low" concentration is higher than the detection limit of single PCR. Zhang et al. have reported that the minimum ratio was between 1:20 and 1:50 when the lower DNA was not detected in the PCR-DGGE using two bacterial DNA as the template [14]. Bollmann et al. also performed a similar experiment and have shown the ratio at which the lower DNA would not be detected to be between 1:29 and 1:99 [13]. The results suggested that undetectable number of AOB species such as CTO-1 and CTO-3 at the beginning of the incubation did grow more efficiently than the other AOB during the 40-d incubation period.

AOB that were dominant in the original activated sludge sample were detected after incubation of the sample for MPN enumeration. However, in addition to the AOB that were originally present in the activated sludge sample, two new species of AOB were detected. Therefore, the community structure of AOB in the MPN samples only partially reflected that of the original activated sludge.

The PCR-DGGE of the activated sludge sample yielded three bands: amo-1∼amo-3, with the *amoA* primer and one band: CTO-2 with the CTO primer. It might be because the sequence region amplified with *amoA* primers has higher sequence variability between AOB than that amplified with CTO primers. Band CTO-2 was obtained from both the activated sludge sample and the MPN samples, which indicates that AOB in the activated sludge were also present in the MPN samples. However, bands amo-1∼amo-3 were not obtained from the MPN samples. These results were attributed to the higher amplification efficiency of the 16S rRNA gene than the *amoA* gene and not to the copy number of the template DNA. The copy number of bacterial 16S rRNA genes varies from 1 to 15 copies. All investigated b-subgroup AOB have only one copy of the 16S rRNA gene [15]. However, some AOB have been reported to have more than two copies of the *amoA* gene [16, 17].

In this study, AOB similar to those belonging to the genus *Nitrosospira* were detected even in the negative MPN samples 1-1, 1-2, and 1-3 (dilution level, 10^{-2}). This result might be due to the bacteria identified as those belonging to genus *Pseudomonas* or genus *Prosthecobacter* by universal bacterial PCR-DGGE. Most bacteria referred to these genera have the ability of denitrification under anoxic conditions with organic materials. It may be that in MPN samples 1-1, 1-2, and 1-3, under partial anoxic conditions, NO_3^- generated by the AOB was consumed for denitrification; therefore the MPN samples showed false negative. Negative MPN tubes (samples 1-1, 1-2, and 1-3 at the 10^{-2} dilution stage and all 15 samples at the 10^{-3} stage) were analyzed. An average of 9.1 mgN/L of NH_4^+ was detected in all 10^{-3} diluted MPN tubes, except for 1-1. Less than 0.2 mgN/L of NH_4^+ was detected in 10^{-2} diluted tubes of sample 1-1, 1-2, and 1-3 and the 10^{-3} diluted tube of sample 1-1 (data not shown). From these results, it is possible that NO_3^- was generated by AOB and then consumed for denitrification; thus it was not detected in 10^{-2} diluted tubes of sample 1-1, 1-2, and 1-3 (and in the 10^{-3} diluted tube of sample 1-1). In the case that the AOB numbers in environmental samples are low like in this study, MPN positive/negative determinations are done on the low dilution stage. Then, the MPN tubes might contain other non-AOB bacteria, affecting MPN result into underestimation.

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