Detection and quantification of expression of amoA by competitive reverse transcription-PCR


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Abstract Ammonia oxidation by chemolithotrophic ammonia-oxidizing bacteria is an important step in the biological nitrogen removal process. The first conversion step, the oxidation of ammonia to hydroxylamine is catalyzed by ammonia monooxygenase (AMO). To investigate the activity of ammonia oxidation, mRNA (designated as amoA) encoding a subunit of AMO was quantified by competitive reverse transcription (RT)-PCR. As a result, it was possible to detect and quantify amoA expression in cultured Nitrosomonas europaea and even complex microbial communities such as nitrifying bacterial aggregates by competitive RT-PCR. It was estimated that amoA concentration in cultured N. europaea was $2.3 \times 10^8$ copies·ml$^{-1}$. Additionally, it was calculated that the copy number of amoA in nitrifying bacterial aggregates was $1.0 \times 10^{12}$ copies·ml$^{-1}$ (5.1 $\times 10^{10}$ copies·mg$^{-1}$-dry weight). On the other hand, amoA expression in the natural activated sludge in a household Gappei-Johkaso was undetectable, whereas 16S rRNA of ammonia-oxidizing bacteria was detected by RT-PCR. Then, four days cultivation of this sludge in inorganic artificial wastewater resulted in increasing amoA expression to a quantifiable amount by competitive RT-PCR. In conclusion, the competitive RT-PCR was effective to investigate the expression of amoA as an indicator of ammonia oxidation activity by autotrophic ammonia-oxidizing bacteria.

Keywords Ammonia monooxygenase; ammonia-oxidizing bacteria; amoA; nitrogen removal; reverse transcription-PCR

Introduction

Nitrogen compounds, which cause eutrophication in enclosed water bodies such as lakes and inland seas, have to be removed in the modern wastewater treatment facilities to preserve water environment resource. In general, the biological nitrogen removal process consists of biological nitrification and denitrification. Because the biological denitrification is principally carried out by heterotrophic bacteria that grow rapidly, the process usually progresses smoothly. On the other hand, biological nitrification, which consists of two successive oxidation processes of ammonium ($\text{NH}_4^+$) to nitrite ($\text{NO}_2^-$) and nitrite to nitrate ($\text{NO}_3^-$), is principally carried out by the chemolithoautotrophic ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). Since the growth rate of those nitrifying bacteria is extremely slow, the nitrification process is a key step in the series of the nitrogen removal process. Although the nitrification process has been intensively studied during the past several decades, it is still recognized as being consequently difficult to maintain in engineering systems. Therefore, in order to construct and maintain the advanced nitrogen removal process stably, it is necessary to promote growth of nitrifying bacteria, and to regulate the optimum condition that is most suitable for nitrification. For this purpose, it is essential for understanding the optimum condition for nitrifying bacteria to evaluate microbial growth, community and metabolic activity of nitrifying bacteria in the bioreactor which has been regarded as a black box.
Traditional cultivation-dependent methods such as a viable plate count and Most-Probable-Number (MPN) methods have been frequently used to estimate the number and activity of nitrifying bacteria in environmental samples. However, due to the slow growth of nitrifying bacteria, cultivation-dependent methods take a long time to get the results and require complicated operation. Furthermore, cultivation-dependent methods are inadequate to comprehend microbial community and nitrification activity, because those are extremely affected by fluctuation of pH and temperature and toxic shocks. Moreover, it has been pointed out as a serious problem that most members of microbes in environmental samples are hardly detected by present cultivation techniques. It is suggested that only 20% of naturally existing bacteria have been isolated from their habitats and can be grown and studied in laboratory conditions (Ward et al., 1990). For example, the culturability determined as a percentage of culturable bacteria in total counted cells counts was estimated at 0.001–0.1% in seawater (Kogure et al., 1979, 1980; Ferguson et al., 1984), 1–15% in normal activated sludge (Wagner et al., 1993, 1994), and 0.3% in soil (Torsvik et al., 1990). The reason for this is, that for the majority of the microorganisms in the environment it is still impossible to mimic the natural conditions required for their growth, and they cannot be handled under laboratory conditions. Therefore, it is considered that the results obtained by traditional cultivation-dependent methods are not usually reflected by actual microbial community and activity in environment.

The recent development of immunological techniques such as ELISA (enzyme-linked immunosorbent assay), or molecular biological techniques based on 16S rDNA and rRNA such as FISH (fluorescence in situ hybridization) and PCR-DGGE (denaturing gradient gel electrophoresis) has enabled scientists to avoid the bias of traditional cultivation-dependent methods. Many research groups reported that these methods offer the possibility of rapid, sensitive and extremely specific detection, quantification and community analysis of nitrifying bacteria without cultivation (Mobarry et al., 1996; Kowalchuk et al., 1997; Ikuta et al., 2000; Noda et al., 2000). However, these methods are unable to estimate microbial metabolism such as ammonia oxidation activity. For a better understanding of the behavior and function of natural microorganisms not only their presence, but also their activity should be studied. Therefore, it is important to study at the level of mRNA, which reflects activity of metabolism such as ammonia oxidation. Ammonia oxidation associated with chemolithoautotrophic bacteria is mediated by two kinds of enzymes, i.e. ammonia monooxygenase (AMO) catalyzing the oxidation of NH₃ to hydroxylamine (NH₂OH), and hydroxylamine oxidoreductase (HAO) catalyzing the oxidation of NH₂OH to NO₂⁻ (Wood, 1986). In this study, mRNA (designated as amoA) encoding a subunit of AMO was quantified by competitive reverse transcription (RT)-PCR. In particular, detection and quantification of amoA expression in cultured Nitrosomonas europaea and complex microbes such as nitrifying bacterial aggregates or activated sludge were investigated. The purpose of this study is to demonstrate employment of amoA-targeted competitive RT-PCR to environmental samples and our final goal is acquisition of new biological knowledge for effective nitrification activity.

Methods
Bacterial strain and culture condition
Nitrosomonas europaea cells as representative ammonia-oxidizing bacteria were cultured in a nitrification medium with slight modification (Ikuta et al., 2000; Noda et al., 2000). NO₂⁻ formation and NH₃ consumption were used as indicators of metabolic activity. About 14 days after, cells were harvested by centrifugation, and immediately proceeded to RNA extraction as described below.
Environmental samples
The environmental samples were collected from two types of wastewater processes. One is the bench-scale reactor where inorganic artificial wastewater containing high-concentration ammonium (0.8 kg·m$^{-3}$·day$^{-1}$) was continuously fed. The other one is the advanced and compact household Gappei-Johkasou that possesses high ability of nitrogen removal. Actual domestic wastewater containing low-concentration ammonium (approximately 0.15 kg·m$^{-3}$·day$^{-1}$) was fed to this Johkasou where a membrane separation unit was utilized in an aeration tank for sludge separation from the effluent water. The ammonium in the effluent was not detected in both processes. Activated sludge collected from this Johkasou was also cultured at 30°C to increase amoA expression. Inorganic artificial wastewater (700 ml) containing 150 mg·l$^{-1}$ of ammonium was used for the cultivation of activated sludge (70 ml) that had been previously centrifuged and washed. Air was supplied at a rate sufficient to provide excess dissolved oxygen. Collected samples from two types of wastewater processes and cultured activated sludge were immediately frozen by liquid N$_2$ and stored at –80°C in a deep freezer.

RNA extraction and purification
All reagents were made in either sterile, disposable labware or glassware that had been combusted at 450°C for 4 h. All solutions were made with water that had been treated with diethylpyrocarbonate (DEPC). Samples harvested by centrifugation were divided into 0.05 g wet weight each and collected in 2 ml screw-cap microcentrifuge tubes. For guanidinium isothiocyanate-phenol-sarcosyl (GIPS) extraction, 0.5 ml of a solution containing 4 M guanidinium isothiocyanate, 0.5% sarcosyl, 25 mM sodium citrate (pH 7.0), 0.1 M mercaptoethanol, 50 µl of 2 M CH$_3$COOHNa (pH 4.0), 0.5 ml of water saturated phenol, 0.1 ml of chloroform-isoamyl alcohol (49:1), and 1 g of glass beads (diameter, 0.1 mm; Biospec Products) were added to each tube. The tubes were placed in ice for 15 min followed by 2 min of bead beating (5,000 rpm) using a Mini-beadbeater (Biospec Products), and then microcentrifuged for 20 min. After the aqueous layer was removed, the remains were reextracted in the same way as described above, except that water saturated phenol was not added. The aqueous extracts were combined, and the RNA was precipitated with equivalent volume of isopropanol to aqueous extracts at –20°C for 2 h. The RNA-containing pellet was collected by microcentrifugation, washed with ice-cold 75% ethanol and dissolved in TNMC buffer (20 mM Tris-HCl, 10 mM NaCl, 6 mM MgCl$_2$, and 0.1 mM CaCl$_2$). To eliminate contaminating genomic DNAs, 10 U of RNase-Free DNase I (TaKaRa) was added to each RNA extraction before incubation at 37°C for 1 h. Excess DNase I was removed by phenol-chloroform extraction followed by ethanol precipitation, and then purified RNA samples were stored at –80°C.

RT-PCR
The amoA in the extracted total RNA was transcribed into first-strand cDNA and amplified by two-step RT-PCR system (ReverTra Ace (TOYOBO) and KOD plus (TOYOBO)) with amoA-1F/amoA-2R primer set (Rotthauwe et al., 1997). This primer set was designed to amplify a partial stretch of the amoA from a wide range of autotrophic ammonia oxidizers belonging to b subclass of the Proteobacteria. In the first step, the purified total RNA and 0.5 pmol of reverse primer were mixed in the PCR tube (total volume; 11 µl) and by heating at 70°C for 10 min. After heat denaturation, the PCR tube was placed in ice for 1 min or more, and 1 µl of RNase inhibitor (10 units/µl), 4 µl of 5×RT buffer, 2 µl of dNTP Mixture (10 mM each) and 1 µl of ReverTra Ace (MLMV Reverse Transcriptase RNase H$^-$; 100 units/µl) were added to the tube. After reverse transcription was carried out at 60°C for 50 min, RTase was inactivated at 94°C for 2 min. One µl of this first-strand cDNA mixture
was used as a following PCR template and 49 µl of PCR buffer containing 5 µl of 10× buffer, 1 mM MgSO₄, 0.4 µM of primers, 0.2 mM of dNTPs and 1 µl of KOD plus DNA polymerase (1.0 units/µl) were added. PCR was carried out with 30 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min; and a final 4 min extension step at 72°C. The 16S rRNA of autotrophic ammonia oxidizers belonging to the β subclass of the Proteobacteria was reversely transcribed and amplified by one-step RT-PCR system (One Step RNA PCR Kit (TaKaRa)) with CTO189f / CTO654r primer set (Kowalchuk et al., 1997). One µl of RNA template was added to the one-step RT-PCR components containing 24 µl of RNase free H₂O, 1 µl of RNase inhibitor (40 units/µl), 5 µl of 10× one step RNA PCR buffer, 10 µl of 25 mM MgCl₂, 0.2 pmol of primer, 5 µl of 10 mM dNTP mixtures, 1 µl of AMV RTase XL (5 units/µl) and 1 µl of AMV-optimized Taq (5 units/µl). RT-PCR was carried out in a GeneAmp PCR System 9700 (PE Biosystem) on the following cycle program: one cycle of 60°C for 50 min and 94°C for 4 min; 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; and a final 4 min extension step at 72°C. Then, 8 µl of each mixture was analyzed by electrophoresis in a 2% agarose gel which was subsequently stained with ethidium bromide. The gel was visualized with UV light and photographed.

**Competitive RT-PCR**

The RNA heterologous competitor was constructed using a Competitive DNA Construction Kit (TaKaRa) and Competitive RNA Transcription Kit (TaKaRa). A four-hundred base RNA competitor was designed to distinguish from the target (amoA; 491 bases). The copy number of the RNA competitor was estimated by measuring the absorbance at 260 nm. Competitive RT-PCR was set up as described above, except that known amounts of RNA competitor were added. After electrophoresis and photographing of the gel, the fluorescent intensity of each band was detected and expressed numerically. In determination of competition equivalence point (EP), the log of the intensity ratio of the competitor to the target was plotted as a function of the log of the initial amount of the competitor. By linear regression analysis, the fluorescent intensity of the competitor band should be equal to that of the target band at the EP where their initial amount ratio should be equal to 1. Interpolation on the plot for a vertical axis value of zero (log 1 = 0) gives the copy number of the target presented in the sample.

**Results and discussion**

**Detection and quantification of amoA in N. europaea**

The clear band was obtained by two-step RT-PCR system with the amoA-1F/amoA-2R primer set from the RNA template extracted from the cultured N. europaea (data not shown). Genomic DNAs contamination during the RNA preparation would lead to false-positive and ambiguous results in RT-PCR. Therefore, an accurate detection and quantification with RT-PCR and competitive RT-PCR substantially relies on the RNA purification step. Since the RNA was sufficiently purified by DNase I and phenol-chloroform extraction, no band was obtained by only PCR. Thus, no genomic DNA contamination in the purified RNA template was confirmed, and thus the band obtained by RT-PCR obviously showed existence of amoA mRNA. To ensure a successful analysis of gene expression, the amplification efficiencies of the target and the competitor were examined. The equal amounts of the competitor RNA and the template RNA extracted from cultured N. europaea were mixed in 6 reaction tubes, and reverse transcription and several cycles (15, 20, 25, 30, 35, 40 cycles) of PCR were carried out. As a result, the amplification efficiencies of the target and the competitor were almost the same in each reaction tube, and their fluorescent intensity ratios were constant in any cycles (Figure 1). Therefore, it was regarded that the target/competitor ratio in the amplified products reflected the ratio.
of initial concentration, which allowed us to calculate the copy number of amoA in the sample.

The expression of amoA in cultured *N. europaea* was examined by competitive RT-PCR with amoA-1F/amoA-2R primer set and running for 30 PCR cycles. As shown in Figure 2A, fluorescent intensity of amoA band increased by decreasing the concentration of added competitor (lanes 1 to 5), which means the target and the competitor competed with each other. The copy number of amoA was calculated by determining the competition equivalence points in linear regression plots (Figure 2B). The correlation coefficient ($r^2$) of the line determined by least-square regression analysis was 0.99. The copy number of amoA in 1 ml of the cultured *N. europaea* was $2.3 \times 10^8$.

**Expression of amoA in environmental samples**

*Complex nitrifying bacterial aggregates in the bench-scale reactor.* The expression of amoA in complex nitrifying bacterial aggregates in the bench-scale reactor was examined. Total RNA extracted from 0.5 g wet weight of bacterial aggregates was used as the template for competitive RT-PCR with amoA-1F/amoA-2R primer set. As a result, Figure 3A suggested that detection and quantification of amoA in the complex microbial community by
competitive RT-PCR was possible and useful to evaluate ammonia oxidation activity. Expression of \textit{amoA} at 1.0 \times 10^{12} \text{ copies·ml}^{-1} (5.1 \times 10^{10} \text{ copies·mg}^{-1} \text{·dry weight}) was detected in this reactor (Figure 3B), which was 4 orders of magnitude higher than the obtained value from cultured \textit{N. europaea}. Inorganic artificial wastewater containing high concentration of ammonium (0.80 kg m$^{-3}$ day$^{-1}$) was supplied to this reactor, and consequently ammonia-oxidizing bacteria were observed as the dominant population in the bacterial aggregates by FISH analysis (data not shown). It was considered that plenty of \textit{amoA} was expressed in these ammonia-oxidizing bacterial aggregates. Therefore, it was suggested that the bacterial aggregates are composed of complex nitrifying bacteria species possessing high ammonia-oxidation ability and greatly contributed to oxidation of the influent ammonium.

\textit{Activated sludge in advanced and compact household Gappei-Johkasou.} Detection of \textit{amoA} in RNA template extracted from 2 g wet weight of activated sludge in an advanced and compact household Gappei-Johkasou was also examined. However, \textit{amoA} was unable to be detected probably due to its limited expression. Then, it was tried to reverse transcribe and amplify ammonia-oxidizing bacterial 16S rRNA by one-step RT-PCR system with CTO189f / CTO654r primer set. As a result, 465 bp amplification product was observed (data not shown), which suggested that total RNA was successfully extracted and ammonia-oxidizing bacteria securely existed in the activated sludge. In this Johkasou, actual domestic wastewater containing low concentration ammonium (approximately 0.15 kg·m$^{-3}$·day$^{-1}$) was fed. Compared with the bench-scale reactor described above, the ammonium concentration of the influent in this Johkasou was relatively low, which might result in a lower amount of \textit{amoA} expression.

Then, to increase \textit{amoA} expression, activated sludge collected from this Johkasou was cultured in the inorganic artificial wastewater containing high concentration of ammonium (150 mg·l$^{-1}$). Consequently, \textit{amoA} expression increased to be detectable by RT-PCR with \textit{amoA}-1F/\textit{amoA}-2R from template RNA extracted from 0.05 g wet weight of the activated sludge after 95 h cultivation. As a result of competitive RT-PCR, \textit{amoA} in 1 ml of activated sludge after cultivation for four days was estimated as 1.1 \times 10^{8} \text{ copies (1.5 \times 10^{8} \text{ copies·mg}^{-1} \text{·dry weight}) (Figure 4).}

As has been noted, \textit{amoA} expression in the bench-scale reactor provided with high concentration of ammonium and activated sludge after cultivation for short time was detectable and quantifiable by competitive RT-PCR with \textit{amoA}-1F/\textit{amoA}-2R primer set. Consequently, it was found that we could use this method to estimate ammonia oxidation
activity in environmental samples. Nevertheless, the copy number of amoA in cultured activated sludge will not indicate real ammonia oxidation activity in the Johkasou, and thus improvement of the detection limit was necessary to be used for the practical monitoring of the domestic wastewater treatment process.

Conclusions
1. Through the competitive RT-PCR with amoA-1F/amoA-2R primer set, it was possible to detect and quantify amoA in cultured N. europaea.
2. The amplification efficiencies of the target and the competitor were same, which allowed us to calculate the copy number of amoA as an indicator of ammonia oxidation activity by competitive RT-PCR.
3. The expression of amoA in a complex microbial community such as a bench-scale reactor provided with high concentration of ammonium was quantifiable and the copy number of amoA in this reactor was calculated as $1.0 \times 10^{12}$ copies·ml$^{-1}$ (5.1 $\times 10^{10}$ copies·mg$^{-1}$-dry weight).
4. The expression of amoA in the activated sludge could be quantified after cultivation for 4 days, whereas it was impossible to directly detect amoA in the activated sludge collected from Johkasou due to the detection limit. Therefore, it was important to raise the detection limit of RT-PCR method through improvement of RNA extraction and rearrangement of the primer set.

References


