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Quantification of anaerobic ammonium-oxidizing bacteria in enrichment cultures by quantitative competitive PCR

HAO Chun[1,2], WANG Huan[1], LIU Qinhua[1], LI Xudong[1,*]

1. Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu 610041, China. E-mail: haochun@cib.ac.cn
2. Graduate University of the Chinese Academy of Sciences, Beijing 100039, China

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Abstract

The anaerobic ammonium-oxidizing (ANAMMOX) bacteria were enriched from a sequencing batch biofilm reactor (SBBR). A quantitative competitive polymerase chain reaction (QC-PCR) system was successfully developed to detect and quantify ANAMMOX bacteria in environmental samples. For QC-PCR system, PCR primer sets targeting 16S ribosomal RNA genes of ANAMMOX bacteria were designed and used. The quantification range of this system was 4 orders of magnitude, from 10^3 to 10^6 copies per PCR, corresponding to the detection limit of 300 target copies per mL. A 312-bp internal standard was constructed, which showed very similar amplification efficiency with the target *amxC* fragment (349 bp) over 4 orders of magnitude (10^3–10^6). The linear regressions were obtained with R^2 of 0.9824 for 10^3 copies, 0.9882 for 10^4 copies, 0.9857 for 10^5 copies and 0.9899 for 10^6 copies, respectively. Using this method, ANAMMOX bacteria were quantified in a shortcut nitrification/denitrification-anammox system which was set for piggery wastewater treatment.

Key words: anaerobic ammonium oxidation; 16S rRNA approach; quantitative competitive PCR

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Introduction

The anaerobic ammonium-oxidizing (ANAMMOX) process is emerging as one of the efficient and cost-effective ways to remove ammonia from ammonia-rich wastewater. The advantages of this process are lower oxygen demand and no requirement for external carbon sources compared to the conventional combination of nitrification and denitrification processes. In this process, ammonium and nitrite are directly oxidized to nitrogen gas under anoxic conditions (Mulder *et al*., 1995; Jetten *et al*., 1999). The ANAMMOX process has been recognized as being difficult to apply for practical wastewater treatments due to long start-up period and strictly anaerobic and autotrophic enrichment condition (Abma *et al*., 2005; Strous *et al*., 1998). However, ANAMMOX bacteria still have not been isolated in pure culture. To implement the ANAMMOX process as a useful wastewater treatment technology, a better understanding of the physiology and kinetics of ANAMMOX bacteria is obvious of great importance.

Quantification of ANAMMOX bacteria has been attempted by using the fluorescence *in situ* hybridization (FISH) (Schmid *et al*., 2005; Isaka *et al*., 2006) and real-time polymerase chain reaction (PCR) (Tsushima *et al*., 2007). FISH is time consuming and sometimes difficult to be used in environmental sampling due to low contents of rRNA molecules per microbial cell and the formation of dense microbial clusters (Third *et al*., 2001). The real-time PCR, based on continuous monitoring of fluorescence intensity throughout the PCR reaction, is a fast and sensitive method to count uncultured bacteria. However, real-time PCR needs special, expensive instrument and reagent. QC-PCR is one of the most widely used approaches for quantitation of nucleic acids. The assay is based on competitive coamplification of a specific target sequence together with known amounts of an internal standard (IS) in one reaction tube. QC-PCR has also been successfully used to quantify specific groups of bacteria from environmental samples (Hallier *et al*., 1996; Johnsen *et al*., 1999; Kondo *et al*., 2004; Leser *et al*., 1995; Mendum *et al*., 1999; Mesarch *et al*., 2000; Rudi *et al*., 1998; Stephen *et al*., 1999; Watanabe *et al*., 1998). QC-PCR has the same sensitive as real-time PCR (< 5 copies of input targent) (Zentilin and Giacca, 2007) but is more cost-effective. However, quantification of ANAMMOX bacteria by QC-PCR has not been reported to date, and reliable quantification method is needed to determine kinetic parameters of ANAMMOX bacteria.

The goal of this study is to develop a QC-PCR system to quantify the copy numbers of 16S rRNA gene of ANAMMOX bacteria from environmental samples. At first, ANAMMOX bacteria were enriched from biofilm in a sequencing batch biofilm reactor (SBBR). Thereafter, the

* Corresponding author. E-mail: lixd@cib.ac.cn

QC-PCR system was developed and applied to evaluate its feasibility and to determine the populations of the enriched ANAMMOX bacteria.

1 Materials and methods

1.1 Sequencing batch biofilm reactor

A shortcut nitrification/denitrification-ANAMMOX system was used to remove nitrogen in piggery wastewater with low C/N in SBBR. The activated sludge from a municipal sewage treatment plant was employed at first to adopt biofilm on the soft fibrous fillers. Then the fillers with biofilm were put into the SBBR. Three enrichment cultures were carried out in 2-L SBBR containing a synthetic nutrient medium for ANAMMOX bacteria in the dark at (30 ± 2)°C under anoxic condition. The pH was not adjusted. After 155 d running, ANAMMOX activity and ANAMMOX bacteria could be detected. Piggery wastewater was pre-treated by a shortcut nitrification/denitrification system, then the effluent of the shortcut nitrification/denitrification system was set as influent of the ANAMMOX system at day 155. After another 30 d running, the shortcut nitrification/denitrification-ANAMMOX system was stable and the microorganism in this system was composed of ANAMMOX bacteria, ammonium oxidizer, nitrite oxidizer and denitrification bacteria, and ANAMMOX bacteria was the main functional bacteria for nitrogen removal in this system (data not shown).

1.2 Nucleic acids extraction and purification

Total DNA was extracted from enrichment culture samples with a sodium dodecyl sulfate (SDS)-based method (Zhou *et al*., 1996). Plasmid DNA was prepared using a UNIQ-10 spin column plamid mini-preps kit (Sangon, China) and measured spectrophotometrically and verified on agarose gel. The diluted plasmid DNA was measured by SmartSpec Plus Spectrophotometer (Bio-Rad, USA).

1.3 Primer design

The forward primer PLA46F (5'-GGATTAGGCATGCAAGTC-3') (Sangon, China) (Neef *et al*., 1998) for Planctomycetales and the reverse primer AMX368R (5'-CCTTTCGGGCATTGCGAA-3') (Sangon, China) (Schmid *et al*., 2003) for all known ANAMMOX bacteria were used in this study.

1.4 PCR amplification

The PCR amplification condition was first optimized for primer and $MgCl_2$ concentrations. All PCR amplifications were accomplished using the optimal conditions in a 50-μL volume containing 1 × Taq DNA polymerase buffer (20 mmol/L Tris-Cl, 20 mmol/L KCl, 10 mmol/L $(NH_4)SO_4$, pH 8.4), 1.25 mmol/L $MgCl_2$, 200 μmol/L deoxynucleotide triphosphates, 15 pmol of each primer, and 2 U Taq DNA polymerase (Biomed, China). The PCR reaction was performed in an automated thermal cycler (DNA Engine® PTC-200, Bio-Rad, USA) with an initial denaturation at 95°C for 5 min, followed by various cycles of 94°C for 30 s, 60°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 5 min.

1.5 Specificity and sensitivity test

To test the specificity of the primer sets, PCR amplifications with the primers were performed with plasmid DNAs that carry target and closely related but non-target clone sequences, which were obtained previously during the cloning analysis of the SBBR biomass. Genomic DNA (gDNA) of *Escherichia coli* DH5α and *Bacteroides fragilis* were used as negative controls. Ten nanograms of gDNA and plasmid DNA were used for PCR amplification with 35 cycles. The PCR products were resolved in 2% of agarose gel (Sangon, China) and detected by ethidium bromide staining.

The sensitivity of the primers was evaluated using a 10-fold serial dilution of cloned *amxC* (from 3×10^8 to 300 copies) in the presence of 10 ng of non-specific target DNA (*B. fragilis* genomic DNA) to evaluate the detection limit. All reactions were performed in triplicates.

1.6 Construction of *amxC* internal standard

The IS of *amxC* was generated using an approach described by Zentilin and Giacca (2007). Briefly, low stringency conditions (annealing at 45°C) were used in the amplification with composite primers PLA46ISF (5'-GGATTAGGCATGCAAGTCcgcaagggtagataatgcatag-3') (Sangon, China) and AMX368R (5'-CCTTTCGGGCATTGCGAA-3') (Sangon, China). These specific primer (cgcaagggtagataatgcatag) was targeted to nucleotides 83–104 of 16S rDNA gene of ANAMMOX bacteria. Amplified IS (312 bp) and *amxC* fragments were cloned to a pMD 18-T simple vector using a TA cloning kit (TaKaRa, Japan), and confirmed by PCR with PLA46F and AMX368R primers.

The amplification efficiency of IS was evaluated using a cloned *amxC*. Equal molar quantities of *amxC* and IS (from 10^3 to 10^6 copies of each) were mixed and subjected to PCR amplifications. The total cycle number varied with the template concentrations. A total of 30 cycles was used for 2×10^5 and 2×10^6 copies of templates, 34 cycles with 2×10^4 copies and 36 cycles with 2×10^3 copies of templates. The PCR products were examined every 2 cycles after 20 cycles. Amplified *amxC* and IS were resolved in a 2.5% TAE agarose gel. The intensity of each band was analyzed using Quantity One software (Bio-Rad, USA). A standard containing equal amount of *amxC* and IS was run in parallel with assaying samples to calibrate the integrated intensity. The correction factor based on the ratio of *amxC* to IS from standards was used to normalize the intensity of the each band.

1.7 Accuracy and quantitation limit

The accuracy and quantitation limit of competitive PCR were evaluated by using certain amount of cloned *amxC* fragments, ranging from 10^3 to 10^6 copies. A two-fold dilution series of IS were used to co-amplify with constant amount of *amxC*. To evaluate the effect of heterogeneous templates on the accuracy of quantification, all reactions

included 10 ng genomic DNA from *E. coli* DH5α. All amplifications were maintained within exponential phase which measured in the amplification efficiency test step. Linear regression of IS to *amxC* ($\log_2$) ratio and added IS ($\log_2$) was obtained for all assays.

1.8 Competitive PCR

The PCR amplification was the same as described. Ten-fold serial dilutions of IS co-amplified with the sample DNA (10–100 ng environmental sample DNA). The dilution of IS which gave roughly equal amplification with the target sample was used as a reference point for making 2-folds serial dilutions of IS. Two-fold serial dilutions of IS were then coamplified with the sample DNA. The initial ratios of IS to *amxC* in the sample DNA were calculated by software and based on the calibrated intensity (the same as above) of amplified IS and *amxC* fragments on the agarose gel. The $\log_2$ of each ratio was plotted as a function of the $\log_2$ of the added IS. The interpolation on the plot for a *Y* value of 0 gives the number of *amxC* in the sample DNA.

2 Results and discussion

2.1 Specificity and sensitivity tests

The specificities of the primer combination of PLA46F and AMX368R were experimentally evaluated by PCR using plasmid DNAs, which contains the *amxC* fragments (AMXC operational taxonomic unit 1, AMXC OUT 1) obtained from the enrichment cultures in this study and the clones previously obtained from the SBBR biomass (SBBR 3, 4, 14, and 20 in Table 1). The 349-bp PCR amplicons were observed for all Planctomycetales related plasmids including AMXC OTU 1, SBBR 3, 4, 14, and 20. No specific amplification was observed in *E. coli* DH5α and *B. fragilis* related plasmids.

Detection limit was evaluated using a dilutionseries of cloned *amxC*. Specific amplification was observed from 1 fg plasmid DNA, which was equivalent to 300 copies of *amxC*.

Table 1 Specificity test for PLA46F and AMX368R primers

Plasmid DNA	Closest strain	Similarity (%)	PCR amplification with PLA46F and AMX368R primers
AMXC OUT 1	Planctomycetales	95	+
SBBR3	Planctomycetales	90	+
SBBR4	Planctomycetales	90	+
SBBR14	Planctomycetales	92	+
SBBR20	Planctomycetales	94	+
Negative control 1	*E. coli* DH5α	100	–
Negative control 2	*B. fragilis*	100	–

+: visible band of expected size; –: no visible band.
SBBR3, 4, 14, 20: these clones were obtained from the SBBR biofilm previously.

2.2 Internal standard of *amxC*

The amplification efficiency was examined by co-amplifying equal molar quantities of IS and the target *amxC* fragment. Similar amplification efficiencies were obtained with the template over 4 orders of magnitude examined (Fig. 1). Competitive PCR should be performed within exponential phase (Wiesner *et al*., 1993). Thus, the cycle when the PCR reactions reached plateau periods was measured. The PCR reactions reached plateau periods after 26, 30, 32 and 34 cycles, respectively, when 10^6, 10^5, 10^4 and 10^3 copies of each IS and *amxC* were used as templates. Thus, competitive PCR for quantitation of 10^6, 10^5, 10^4 and 10^3 copies of *amxC* should be performed for less than 26, 30, 32 and 34 cycles, respectively.

2.3 Accuracy and quantitation limit

The accuracy and quantitation limit were examined by co-amplifying known amount of cloned *amxC* fragments, ranging from 10^3 to 10^6 copies, with a two-fold dilution series of IS. Linear regression of IS to *amxC* ($\log_2$) ratio and added IS ($\log_2$) was obtained for all assays with a R^2 of 0.9824 for 10^3 copies of *amxC*, 0.9882 for 10^4 copies of *amxC*, 0.9857 for 10^5 copies of *amxC* and 0.9899 for 10^6 copies of *amxC* (Fig. 2). When there was less than 10^3 copies of *amxC* fragment in the competitive PCR reaction, no good linear regression was achieved ($R^2 < 0.9$). Thus,

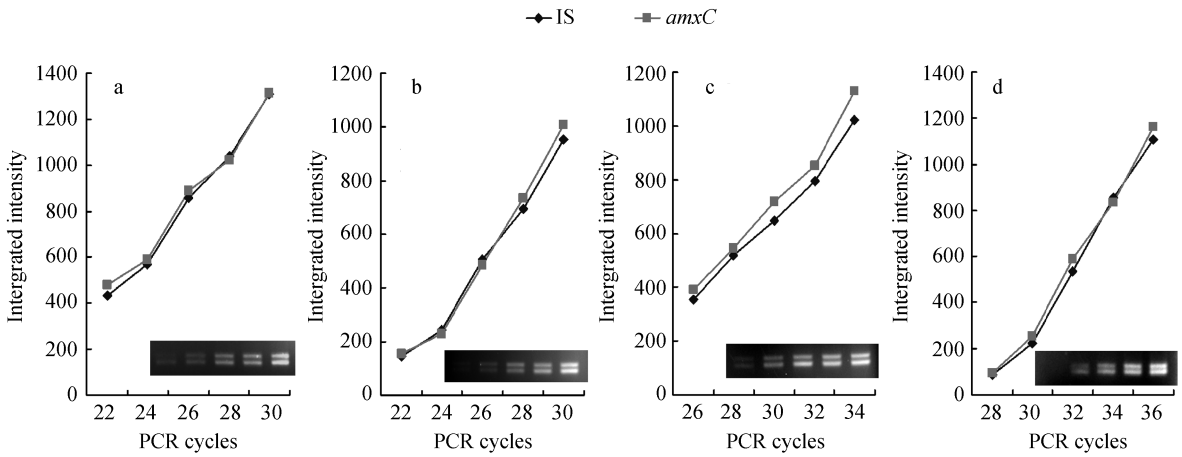

Fig. 1 Co-amplification of *amxC* and IS using 10^6 (a), 10^5 (b), 10^4 (c), and 10^3 (d) copies of *amxC* and IS . Similar plot patterns were observed in replicate experiments.

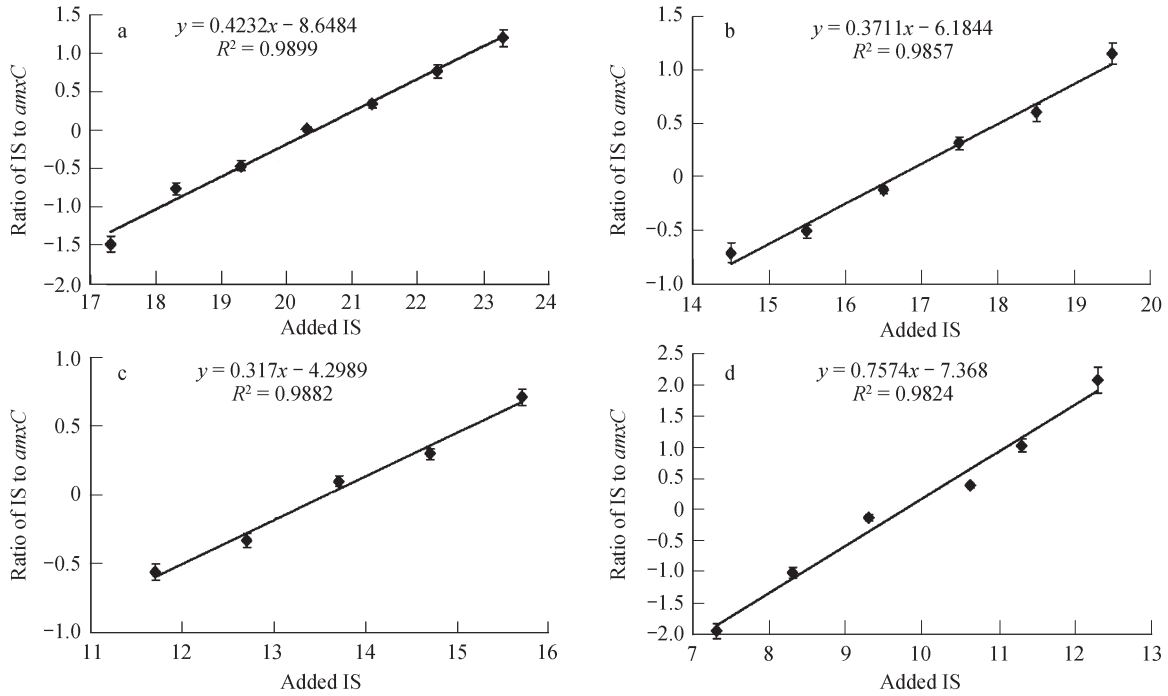

Fig. 2 Accuracy of competitive PCR. Quantitation of plasmid *amxC* by competitive PCR in presence of 10 ng gDNA from *E. coli* DH5α. The pictures showed two-fold serial dilution of IS co-amplified with 10^6 copies of *amxC* for 25 cycles (a), 10^5 copies of *amxC* for 27 cycles (b), 10^4 copies of *amxC* for 31 cycles (c) and 10^3 copies of *amxC* for 33 cycles (d).

we were unable to quantify less than 10^3 *amxC* by using this system.

2.4 Quantification of ANAMMOX bacteria from environmental samples

In this three enrichment cultures, clear ANAMMOX reaction could be observed after 115 d running period. The copy numbers of 16S rRNA gene of ANAMMOX bacteria were quantified and the nitrogen removal rates were measured every ten days from day 115 to 185. The effluent of the shortcut nitrification/denitrification system was set as influent of the ANAMMOX system at day 155 (Fig. 3).

The phenomenon indicated that ANAMMOX bacteria increased about 440 times from day 115 to day 155 and kept stable from day 155 to day 185. Based on these results, the doubling time of ANAMMOX bacteria was estimated to be (4.5 ± 0.5) d for the former 40 days. The stability of ANAMMOX bacteria in the late stage was probably due to the organic matter which was introduced by the effluent of the shortcut nitrification/denitrification system.

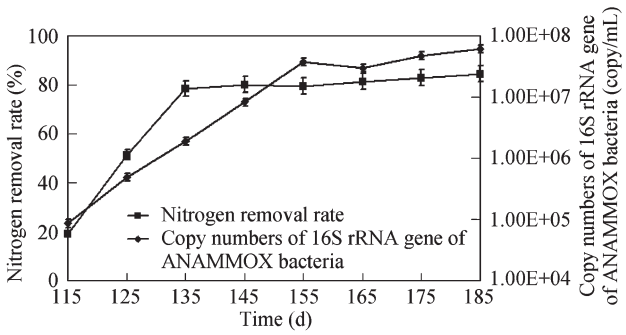

Fig. 3 Time course of nitrogen removal rate and the copy numbers of 16S rRNA gene of ANAMMOX bacteria in the enrichment batch culture determined by QC-PCR.

Furthermore, 16S rRNA gene copies cannot be directly converted into cell counts, because ANAMMOX bacteria might have different 16S rRNA operon copy numbers from species to species, and the 16S rRNA operon of ANAMMOX bacteria are not presently known.

3 Conclusions

We obtained the enrichment culture of ANAMMOX bacteria from a sequencing batch biofilm reactor. In addition, we successfully developed the competitive PCR assay for quantification of 16S rRNA gene of ANAMMOX bacteria. Using this real-time PCR assay, the doubling time of the ANAMMOX bacteria enriched in this study was estimated to be 4.5 d. The competitive PCR assay is a useful tool to quantify very slow-growing ANAMMOX bacteria in various environmental and engineering samples. This assay will be used to screen appropriate seed sludges with high abundance of ANAMMOX bacteria for rapid and efficient start-up of ANAMMOX bioreactors in the future study.

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