The abundance and diversity of ammonia-oxidizing bacteria in activated sludge under autotrophic domestication

Qiang Li, Chao Ma, Shifang Sun, Hui Xie, Wei Zhang, Jun Feng and Cunjian Song*
Key Laboratory of Molecular Microbiology and Technology for Ministry of Education, Nankai University, Tianjin, 300071, P.R. China.
*Corresponding Author email: songcj@nankai.edu.cn

Abstract
Ammonia-oxidizing bacteria (AOB) play a key role in nitrogen-removal wastewater treatment plants (WWTPs) as they can transform ammonia into nitrite. AOB can be enriched in activated sludge through autotrophic domestication although they are difficult to be isolated. In this study, autotrophic domestication was carried out in a lab-scale sequencing-batch-reactor (SBR) system with two activated sludge samples. The ammonia removal capacity of the sludge samples increased during the domestication, and pH exhibited a negative correlation with the ammonia removal amount, which indicated that it was one important factor of microbial ammonia oxidation. The count of AOB, measured by the most probable number (MPN) method, increased significantly during autotrophic domestication as ammonia oxidation efficiency was enhanced. We investigated the changes in the community structure of AOB before and after domestication by amoA clone library and T-RFLP profile. It showed that AOB had been successfully enriched and the community structure significantly shifted during the domestication. Two groups of AOB were found in sludge samples: Nitrosomonas-like group remained predominant all the time and Nitrosospira-like group changed obviously. Simultaneously, the total heterotrophic bacteria were investigated by MPN and Biolog assay. The metabolic diversity of heterotrophs had changed minutely, although the count of them decreased significantly and lost superiority of microbial communities in the sludge.

Key words
Autotrophic domestication, Ammonia-oxidizing bacteria, AmoA, PCR-based fingerprinting

Introduction
Ammonia nitrogen is one of the main pollutants in the water environment because it can cause eutrophication and be toxic to aquatic organisms (Egli et al., 2003). Currently, removal of ammonia nitrogen from wastewater treatment plants (WWTPs) mostly depends on microbial ammonia oxidation (Prosper et al., 2006), i.e., oxidation of ammonia to nitrite is carried out by ammonia-oxidizing bacteria (AOB) in the activated sludge (Purkhold et al., 2000).

The oxidation of ammonia to hydroxylamine is catalyzed by ammonia monoxygenase (AMO) (Hollocher et al., 1981). In all known AOB, the genes encoding the enzyme AMO belong to an operon with the structure amoCAB (Chain et al., 2003; Klotz et al., 2006). Despite the potential of using the whole amoCAB operon for molecular studies, only a section of the gene amoA, about 491 bp, has generally been used as a molecular marker to study the diversity of AOB (Rotthauwe et al., 1997), which has the advantages of a higher specificity than 16S rRNA gene (Junier et al., 2010).

Since the publication of the first amoA sequence in Nitrosomonas europaena (McTavish et al., 1993), the number of molecular ecology researches on amoA has increased significantly and many effectual methods have been used, such as amoA gene clone libraries (Rotthauwe et al., 1997), terminal restriction fragment length polymorphism (T-RFLP) (Horz et al., 2000), denaturing gradient gel electrophoresis (DGGE) (Oved et al., 2001), fluorescence in situ hybridization (FISH) (Persson et al., 2002), and quantitative polymerase
chain reaction (qPCR) (Hoefel et al., 2005). However, only a few articles were about amoA in activated sludge of WWTPs, among which most focused on AOB community structure in the reaction tanks of WWTPs in situ (Lydmark et al., 2007; Wells et al., 2009; Ziembinska et al., 2009; Jin et al., 2010) while few has expounded the effect of manual intervention on the abundance and composition of AOB in activated sludge.

The chemoaautrophic bacteria have a slow growth rate and are highly sensitive to several environmental factors such as temperature, salinity, pH, dissolved oxygen, ammonium concentration and hydraulic retention time (Okabe et al., 1999; Limpiyakorn et al., 2007; Lydmark et al., 2007), which makes AOB a model for microbial molecular ecology (Kowalchuk et al., 2001) and difficult to be isolated, especially from artificial ecosystem (Koops et al., 2003; Purkhold et al., 2003; Campbell et al., 2011).

Rich ammonium nutrient can promote AOB growth (Di et al., 2009; Webster et al., 2005), and autotrophic growing environment can inhibit NH$_4^+$-assimilating heterotrophic bacteria (Geets et al., 2006). Therefore, autotrophic domesticating activated sludge rich ammonium medium seems to be a more feasible way to obtain bacterium suspension of AOB than isolating pure culture, and has a bright prospect in the field of bioaugmentation (Zhang et al., 2009; Jiao et al., 2011). However, few reports have touched upon the following questions: Whether autotrophic domestication has effect on changing bacterial community of AOB and heterotrophic bacteria while changing the abundance of AOB in activated sludge? How is the change of bacterial community coupled with the ammonia removal capability of activated sludge before and after autotrophic domestication? Therefore, the objective of the present study was to study the change law of the community and metabolism on both autotrophic and heterotrophic bacteria during the domestication.

For this, a lab-scale autotrophic domestication system was carried out with two activated sludge samples. To characterize the abundance and composition of bacterial community in the sludge samples during autotrophic domestication, the most probable number (MPN) method was used to count AOB and total heterotrophic bacteria, and the diversity of AOB and heterotrophs were analyzed using clone library, T-RFLP and biolog assay.

**Materials and Methods**

**Sampling**: Two activated sludge samples, designated as D and S, were collected from two waste water treatment plant (WWTPs) located in Shandong (38.01°N115.32'E and 37.26°N 116.17'E) China during September 2010. The wastewater treatment systems were operated under sequencing-batch-reactor (SBR) and anaerobic–anoxic–oxic (A2/O) processes. Sludge D was sampled from the wastewater treatment system under SBR process during the aerobic stage, while Sludge S was sampled from the aerobic tank of A2/O process. Samples were taken in sterile polyethylene bottles, transported to the laboratory on ice, and stored at 4°C.

**Autotrophic domestication**: Autotrophic domestication was operated in 3 l SBR (Fig. 1). 50 g sludge was added to 2 l domestication medium. The composition of medium was as follows: 4.0 g (NH$_4$)$_2$SO$_4$(approximately 400 mg l$^{-1}$ NH$_3$-N), 2.0 g Na$_2$CO$_3$, 2.0 g NaCl, 2.0 g K$_2$HPO$_4$·3H$_2$O, 0.1 g MgSO$_4$·7H$_2$O, 0.2 g CaCl$_2$, 2000 ml water, pH 7.2 (Zhang et al., 2009).

The hydraulic retention time (HRT) was controlled at 24 hr, 23 hr for the aerobic running stage, 0.5 hr for the precipitate stage, and 0.5 hr for the medium-change stage, respectively. The temperature and aeration rate were controlled at 30°C and 0.2 m$^3$ hr$^{-1}$.

The content of ammonia nitrogen and pH in the effluent were measured by electrode method and Nessler reagent spectrophotometry every 2$^{nd}$ day and the ammonia nitrogen removal amount was calculated by subtracting ammonia nitrogen content in the effluent from that in the influent.

**Bacterial count**: Autotrophic domestication lasted for 16 days. Four sludge samples designated as DB, SB (before domestication) and DA and SA (after domestication) were collected using centrifugation at 8000 g.

The MPN method was used to count AOB and total heterotrophic bacteria. The dilution series of sludge samples (usually tenfold diluted until 10$^{-6}$) was added to tubes with 5 ml autotrophic domestication medium for AOB and 5 ml yeast extract medium (6 g l$^{-1}$ tryptone, 3 g l$^{-1}$ yeast extract) for heterotrophs, shaking at 30°C for 3 days (Fuchsluger et al., 2011; Hirooka et al., 2009). Three replicates were made.
The positive tubes were checked by Griess reagent coloration and optical density measurement. The computation of the MPN was carried out according to the US Food and Drug Administration (Blodgett, 2006).

**Biolog assay**: Sludge samples were washed twice with 0.85% (w/v) NaCl solution and then diluted to a cell density with an optical density at 590 nm (OD590) of 0.08 ± 0.02. Then, 150 μl of the cell suspension were inoculated into each well of an EcoPlate (Biolog), which contained 31 individual carbon sources in triplicate and 3 negative control (without carbon source) in a 96-well plate format. The plates were incubated at 25°C for 60 hr. During this time, the OD590 values were measured every 12 hr using a plate reader (Biolog). The patterns of sole carbon source utilization were then expressed as an index of the net OD590 value in each well by subtracting the reading of the control well. The community metabolic diversity (CMD) was calculated as the sum of positive carbon responses (purple-colored wells with threshold ODs of 0.25) observed over the incubation time (Garland and Mills, 1991).

**DNA extraction**: Genomic DNA was extracted in triplicate from all samples using a SoilGen DNA Kit (CWBio) according to the manufacturer’s instructions. DNA integrity was checked by 0.8% agarose gel electrophoresis. The purity and the quantity of extracted DNA were determined by UV spectrophotometry at 260 and 280 nm and stored at −20°C.

**Clone library construction**: For the construction of clone library, bacterial amoA gene was PCR-amplified in triplicate from the genomic DNA using the primers amoA-1F (5'-GGGGTTTCTACTGGTGTTG-3') and amoA-2R (5'-CCCTCKGSAAA GCCTTCTTC-3') (Rotthauwe et al., 1997). The DNA extracts were 10-fold diluted and used as template with a final content of 1–10 ng in each reaction mixture, and bovine serum albumin (BSA) was added. A 25 ml reaction mixtures also included 1 × PCR buffer, 2.0 mM MgCl2, 200 mM each dNTP, 2.5 U HotStarTaq DNA polymerase except for the primers (He et al., 2007). The PCR program was as follows: 5 min at 95°C, followed by 40 cycles of 60 sec at 94°C, 45 sec at 57°C and 45 sec at 72°C, and finally 10 min at 72°C (Okano et al., 2004). The PCR products were separated by 1% agarose gel electrophoresis. The bands with expected size were excised and purified with a QIAquick gel extraction kit (Qiagen). The purified PCR products were cloned into pMD19-T vector by TA cloning kit (TaKaRa) and transformed into *E. coli* DH5α competent cells. White colonies in blue-white screening plates were selected to conduct colony PCR with the vector-specific primers M13-47 and RV-M. 130 positive clones were selected for sequencing.

The sequences were blasted with sequences from the GenBank database (http://blast.ncbi.nlm.nih.gov/).

Similarity analysis was performed with DNAMAN version 6, and sequence with > 98% similarity was included. Phylogenetic tree was conducted by ClustalX version 1.83 and MEGA version 3.0, using neighbour-joining method and Kimura 2 parameter distance with 1000 replicates to produce Boot-strap values (Kumar et al., 2004).

All amoA gene sequences were submitted to Gen Bank database and accession numbers were obtained HQ821876-HQ822005.

**T-RFLP analysis**: For T-RFLP analysis, PCR amplification of amoA gene was conducted the same as that for library construction, except that the forward primer amoA-1F was 5’-end labeled with FAM. Three replicate PCR products for each sample were purified using a QIAquick PCR purification kit (Qiagen) and then digested with the restriction enzyme *Hpa*I for 3 hr at 37°C in a mixture containing 8 μl of purified PCR products, 1 μl of buffer and 1 μl of restriction enzyme (10 U μl). Aliquots (2 μl) of the digest were mixed with 2 μl of deionized formamide, 0.5 μl of loading buffer, and 0.5 μl of a DNA fragment length standard (GS-500LIZ, ABI). The mixture was denatured at 94°C for 5 min and snap-cooled on ice. The fluorescent labeled terminal restriction fragments (T-RFs) were detected by electrophoresis on 7% polyacrylamide gel for 10 hr at 2,250 V on an automated ABI 3730XL DNA sequencer in the GeneScan mode. T-RFLP profiles were analyzed using GeneMarker V1.8 software (Jin et al., 2010). Because of the detection range of internal marker GS500, T-RFs smaller than 50 bp were excluded from further analysis. The relative abundance of T-RFs was determined by calculating the ratio between the peak height of each peak and the total peak height of all peaks within one sample. The T-RFs with a relative abundance of less than 2% were regarded as background noise and excluded from the analysis.

**Results and Discussion**

**Autotrophic domestication**: The lab-scale domestication lasted for 16 days until the system and parameters became stable. In order to evaluate the effect of domestication, pH and the ammonia nitrogen removal amount were measured and calculated every two days as shown in Fig 2.

After domestication, the ammonia nitrogen removal amount of S system increased from the initial value of 81.5 to 134.7 mg l⁻¹, as well as pH of the effluent decreased from 7.28 to 5.17 continuously. The increase of ammonia nitrogen removal amount meant that the activity of domesticated sludge had risen due to the metabolism of AOB. The pH is another important factor to measure the ammonia oxidation reactions, because the ammonia oxidation by AOB is an acid-producing process (shown as equation 2 above). The decrease of pH in the effluent of S system demonstrated the
enhancement of the ammonia oxidation reactions. A negative correlation was found between pH in the effluent and the ammonia nitrogen removal amount \((r = 0.9224, n = 8, P < 0.05)\), which indicated that it was an important factor in microbial ammonia oxidation.

For D system, the change of pH and the ammonia nitrogen removal amount was approximately consistent with S system, decreasing from pH 7.32 to 5.83 and increasing from 80.4 to 139.1 mg l\(^{-1}\) respectively. However, in the initial period of domestication, pH rose to 8.41 until 10\(^{th}\) day. Correspondingly, the ammonia nitrogen removal amount was slower than that in S system. After 10\(^{th}\) day, pH decreased rapidly and the ammonia nitrogen removal amount increased remarkably. Large amount of NH\(_4^+\)-assimilating heterotrophic bacteria could be found in activated sludge (Geets et al., 2006) and survived using carbon source in sludge and from bacterial lysis. The metabolism of these heterotrophic bacteria may lead to the erratic change of pH. However, pH resumed in negative correlation with ammonia nitrogen removal amount \((r=0.828, n=4, P < 0.05)\) after 10\(^{th}\) day.

Besides pH and the concentration of N-substrate, other factors can also affect ammonia oxidation reactions. Groeneweg (1994) found that temperature had an even stronger effect on the rate of ammonia oxidation than the availability of NH\(_4^+\)-N. Bae (2001) found that the rate of ammonia oxidation rose as temperature rose from 10 to 30\(^{\circ}\)C in wastewater-bioreactors. In this study, temperature was set at 30\(^{\circ}\)C for an ideal rate of ammonia oxidation reactions. DO was significant for the growth of AOB and low DO could also inhibit ammonia oxidation reactions (Geets et al., 2006; Lydmark et al., 2007). The aeration rate was controlled at 0.2 m\(^3\) h\(^{-1}\) to maintain DO above 2.0 mg l\(^{-1}\).

The AOB count in S sludge increased from \(2.05 \times 10^7\) to \(3.25 \times 10^7\) MPN g\(^{-1}\), and from \(7 \times 10^6\) to \(1.3 \times 10^7\) MPN g\(^{-1}\) in D sludge, respectively. Simultaneously, heterotrophic bacterial count in Sludge decreased from \(3.9 \times 10^6\) to \(1.65 \times 10^6\) MPN g\(^{-1}\), and from \(7.25 \times 10^7\) to \(7.8 \times 10^6\) MPN g\(^{-1}\) in D sludge, respectively (Fig. 3).

The AOBs are the major performer in ammonia oxidation. The ammonia oxidation reactions enhanced as the count of AOB and the copies of amoA increased (He et al., 2009). Through autotrophic domestication, AOB in S and D sludge obtained enrichment and increased by 18.6 and 159 times, and ammonia removal capacity of the sludge samples rose correspondingly.

It is assumed that AOB have a lower affinity for ammonium and oxygen than NH\(_4^+\)-assimilating heterotrophs, and hence are weaker competitors for ammonium. The assimilation of ammonia by heterotrophs happened in preference to nitrification, and reduced the available ammonia for AOB (Geets et al., 2006). It has been clearly indicated that the addition of organic matter, which promoted the growth of heterotrophs, could inhibit ammonia oxidation (Hanaki et al., 1990). Autotrophic domestication, which can inhibit the growth of heterotrophs, is favourable for the enrichment of AOB. Through autotrophic domestication, heterotrophs in S and D sludge decreased by 23.6 and 9.3 times loosing superiority and hence providing growing space for AOB.
The diversity of bacterial community may significantly affect the diversity of metabolism and substrate utilization. Sole-carbon-source tests (Biolog) have become very popular for metabolically fingerprinting microbial communities (Garland and Mills, 1991). In this study, Biolog microplates were inoculated with the cell suspension of activated sludge to quantify the metabolic diversity of the heterotrophic community. The CMD curve obtained by Biolog assay showed the carbon source utilization of heterotrophs in activated sludge samples before and after domestication (Fig 4). The CMD increase of DA and SA was lower than that of DB and SB due to the difference of heterotrophic count (Fig 3).

Among the 31 kinds of carbon source in the microplates (Table 1), the four samples could utilize 30, 30, 29 and 28 kinds, respectively. The substrate which couldn’t be utilized by the original sludge was 2-hydroxybenzoic acid and the decreased available substrates after autotrophic domestication were L-threonine for D sample; α-ketobutyric acid and phenylethylamine for S sample. The change of CMD represented the change of the heterotrophic community (Garland and Mills, 1991). Only a little change about the carbon source utilization of heterotrophs in D and S samples represented the change of the heterotrophic diversity was tiny, even though heterotrophs had decreased obviously and lost superiority through autotrophic domestication. This suggested that organic carbon source limitation was necessary when the domesticated sludge was used for ammonia removal. Verhagen et al. (1992) reported that autotrophic ammonia-oxidizing activity was repressed by heterotrophic microorganisms at a C/N ratio higher than 10.

To investigate the detailed information of AOB diversity after domestication, two amoA clone libraries were constructed. In total, 62 and 68 sequences were obtained from D and S samples respectively. Phylogenetic tree was built by the sequences with less than 98% similarity. In the phylogenetic analysis, the sequences from this study fell into two clusters (Fig. 5), which corresponded to the Nitrosospira and Nitrosomonas groups. Most of the sequences from D clone library (45 out of 62 sequences) fell into Nitrosospira group (cluster 1), whereas most of the sequences from S clone library (58 out of 68 sequences) fell into Nitrosomonas group (cluster 2). However, no sequences fell into Nitrosococcus group.

So far, AOB has been reported to belong to several different species. One of them involves strains of Nitrosococcus sp. within Gamma-Proteobacteria, and the other contains the genera Nitrosomonas and Nitrosospira within Beta-Proteobacteria (Kovalchuk et al., 1997). In particular, Nitrosomonas and Nitrosospira are considered as the most important AOB in activated sludge (Purkhold et al., 2000). In this study, two groups of Nitrosomonas and Nitrosospira were both enriched as the phylogenetic analysis showed and Nitrosococcus was absent.

The AOB T-RFLP profiles showed that four T-RFs of 70, 85, 106 and 150 bp were found in both D and S samples before and after domestication. The T-RFs matched along with the sequences in the clone libraries, which could show the shift of AOB during domestication. The T-RF of 85 bp was significantly dominant and accounted for 32–61% of the total T-RFs, corresponding to D72, D76, S2, S8, S69, et al (cluster 2b of Nitrosomonas group) in the phylogenetic tree (Fig 5). The T-RF of 301 bp was only found in the samples after domestication, corresponding to D4, D9, D69, S47, S52, et al (cluster 1b of Nitrosospira group) in the phylogenetic tree. The T-RF of 449 bp was only found in D sludge, corresponding to D19, D43, et al (cluster 1a of Nitrosospira group) in the phylogenetic tree. For other T-RFs, there were no related sequences in clone libraries.

Through T-RFLP fingerprinting analysis, Nitrosomonas occupied the dominant position in the AOB group all along, even in the total bacteria community after domestication. However, the diversity of Nitrosospira was remarkably changed. Although Nitrosospira and Nitrosomonas were two main groups of AOB in activated sludge, Nitrosospira was not as easily detected as Nitrosomonas (Wells et al., 2009; Ziembinska et al., 2009; Jin et al., 2010). Some studies have indicated that high ammonia loadings can enrich different species of Nitrosomonas and Nitrosospira (Limpiyakorn et al., 2007; Jin et al., 2010). Other studies have also shown high nitrogen environments may be beneficial for the enrichment of Nitrosospira (Horz et al., 2004; Di et al., 2009).

In this study, after autotrophic high-ammonia-loading domestication two main groups of AOB Nitrosomonas and Nitrosospira were enriched in two activated sludge samples, and the diversity increased. The

| Table 1: Chemical properties of sludge samples from two sewage treatment plants |
|---------------------------------|----------|----------------|----------------|----------------|
| Properties                      | Sludge Samples | D             | S             |
| Solid content (%)               | 1.2±0.01   | 1.48±0.01     | Drying in 378 K |
| pH (H2O)                       | 7.2±0.08   | 6.83±0.09     | Potentiometric |
| Organic carbon (g kg⁻¹ DS)     | 351±3.67   | 380±4.16      | K, Cr, O₃ titration method |
| TN (g kg⁻¹ DS)                 | 22.55±1.81 | 10.61±0.54    | UV–vis         |
| TP (g kg⁻¹ DS)                 | 18.03±4.51 | 7.02±0.46     | UV–vis         |
| TK (g kg⁻¹ DS)                 | 6.91±0.27  | 5.83±0.24     | AAS            |
| SO₄²⁻ (g kg⁻¹ DS)              | 0.51±0.03  | 0.35±0.03     | UV–vis         |

TN : total nitrogen, TP : total phosphorus, TK : total potassium and DS : dry sludge. Values are mean of three replicates ± SD.
Fig. 5: Neighbor-joining phylogenetic tree of amoA gene sequences for members of clone libraries. Clones obtained from constructed libraries are shown in bold fonts, while references are shown in italic fonts, both followed by an accession number in parenthesis. The tree was constructed based on the Kimura 2 parameter model and the neighbor-joining algorithm using the MEGA 3.0 package. Bootstrap values from 1000 replicates are indicated at the nodes of branches. The bar represents 0.05 substitutions per nucleotide position.
enhancement of their quantity and metabolism increased the ammonia removal capability of the sludge. Simultaneously, the count and diversity of heterotrophs in the system decreased correspondingly and heterotrophs lost superiority, which proved favorable for the enrichment of AOB. It is always desirable to cultivate the existing endogenous population rather than to add a potential non-representative type of nitrifier (Bouchez et al., 2000). Therefore, AOB-rich sludge obtained through suitable domestication has a bright prospect in the field of bioaugmentation and may improve nitrogen removal efficiency of the activated sludge process in WWTPs.

Acknowledgments

This work was supported by National High Technology Research and Development Program of China (863 program) 2012AA021505; Natural Science Foundation of China (Grant nos. 31070309, 51073081) and Scientific Project of Tianjin, China 11JCYBJC09500.

References


