Detection of Ammonia-Oxidizing Archaea in Fish Processing Effluent Treatment Plants

A. Devivaraprasad Reddy · Gangavarapu Subrahmanyam ·
Girisha Shivani Kallappa · Iddya Karunasagar ·
Indrani Karunasagar

Received: 29 April 2014 / Accepted: 23 June 2014 / Published online: 12 July 2014
© Association of Microbiologists of India 2014

Abstract Ammonia oxidation is the rate limiting step in nitrification and thus have an important role in removal of ammonia in natural and engineered systems with participation of both ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB). However, their relative distribution and activity in fish processing effluent treatment plants (FPETPs) though significant, is hitherto unreported. Presence of AOA in sludge samples obtained from FPETPs was studied by amplification and sequencing of thaumarchaeal ammonia monooxygenase subunit A (AOA-*amoA*) gene. Different primer sets targeting 16S rRNA and AOA-*amoA* gene were used for the detection of AOA in FPETPs. Phylogenetic analysis of the gene revealed that the AOA was affiliated with thaumarchaeal group 1.1a lineage (marine cluster). Quantitative real time PCR of *amoA* gene was used to study the copy number of AOA and AOB in FPETPs. The AOA-*amoA* and AOB-*amoA* gene copy numbers of sludge samples ranged from 2.2×10^6 to 4.2×10^8 and 1.1×10^7 to 8.5×10^8 mg⁻¹ sludge respectively. Primer sets Arch-*amoA*/Arch-*amoA* and 340F/1000R were found to be useful for the sensitive detection of AOA-*amoA* and Archaeal 16S rRNA genes respectively in FPETPs. Their presence suggests the widespread occurrence and possible usefulness in

removing ammonia from FPETPs which is in line with reports from other waste water treatment plants.

Keywords Ammonia oxidation · Ammonia oxidizing archaea · AOA-*amoA* gene · 16S rRNA gene · Thaumarchaeota

Introduction

Residual ammonia is considered to be a persistent problem in effluent treatment plants dealing with protein-rich wastes. The sustainable mechanism for ammonia removal from effluent treatment plants is biological nitrification in which microorganisms have an important role [1] involving ammonia (NH₃) oxidation to nitrate (NO₃⁻) via nitrite (NO₂⁻) [2]. Monophyletic chemolithotrophic ammonia oxidizing bacteria (AOB) are generally believed to be solely responsible for aerobic ammonia oxidation (AO) being the first and rate limiting step of nitrification [2]. However, this general perception has undergone change since the discovery of ammonia monooxygenase subunit A (*amoA*) homologous genes in archaeal scaffold and cultivation of ammonia oxidizing archaea (AOA) [3, 4]. Further, AO coupled with CO₂ fixation (autotrophy) by AOA has been recently demonstrated [5, 6].

By using culture independent DNA based techniques targeting *amoA* gene, AOA has been identified from diverse natural environments such as marine sediment, hot springs, soils, estuary, freshwater lakes, seawater and in aquarium biofiltration system [6, 13]. Most of these studies reveal the dominant role of AOA over AOB in nitrification and suggest the potential involvement of AOA in nitrogen cycle [6]. There are few studies that show the presence of AOA-*amoA* genes in wastewater treatment

Electronic supplementary material The online version of this article (doi:10.1007/s12088-014-0484-6) contains supplementary material, which is available to authorized users.

A. Devivaraprasad Reddy · G. Subrahmanyam ·
G. Shivani Kallappa · I. Karunasagar · I. Karunasagar (✉)
Department of Fisheries Microbiology, College of Fisheries,
UNESCO-MIRCEN for Marine Biotechnology, Karnataka
Veterinary, Animal and Fisheries Sciences University,
Mangalore 575 002, India
e-mail: karuna8sagar@yahoo.com

plants (WWTPs) and activated sludge bioreactors [8, 9] which recognize AOA as potential ammonia oxidizers in WWTPs; however such reports are lacking in other engineering systems such as fish processing effluent treatment plants (FPETPs) and industrial waste effluent treatment plants.

Disposal of waste water from fish processing industries presents a major problem because of their high protein and ammonia contents. The industry is in dire need of efficient system of wastewater treatment, which covers the mechanisms and processes used to treat protein and ammonia rich waters prior to its release into the environment. Biological treatment based on nitrification–denitrification is the best option for nitrogen removal from such waters. Traditionally AOB is being used in nitrification reactors; however realizing the potential of AOA, we explored AOA mediated nitrification in nitrogen removal from fish processing effluent by looking for their genes. AOA in FPETPs has been detected and is being reported for the first time which shows promise of isolation and their utilization for various downstream applications targeted towards ammonia removal. During the course of this study, different sets of primers for 16S rRNA, and AOA-*amoA* genes were evaluated for the detection of archaea and AOA in sludge samples collected from fish processing effluent plants.

Materials and Methods

Sample Collection

Seventeen sludge samples were collected in clean 1 l plastic containers which included 12 from effluent treatment plants of surimi processing unit located at Ratnagiri, Maharashtra, India and five from fish-meal production unit located near Mangalore, Karnataka, India. The samples were brought to the laboratory in an insulated container with gel ice packs and were processed in triplicate for DNA extraction.

Community DNA Extraction, PCR Amplification and Sequencing

Community DNA from the samples was extracted using HiPurA™ Soil DNA Kit (HiMedia, India) as per the manufacturer's protocol and purity checked in 0.8 % agarose gel by electrophoresis. This was quantified using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) and diluted one in ten for PCR using several sets of primers targeting 16S rRNA gene of archaea and *amoA* of AOA. Additionally, a primer set designated M-AOA-F and M-AOA-R specific for marine Group 1.1a (AOA) was designed (Table S1). The PCR was performed

in 30 µl volumes containing 3.0 µl of 10× PCR buffer (100 mM Tris–HCl, pH 8.3, HiMedia, Mumbai, India), 20 mM MgCl₂, 50 mM KCl, 0.1 % BSA, 200 µM of each of the four dNTP, 0.2 µmol/l of each primer and 0.8 U of *Taq* polymerase (HiMedia, Mumbai, India). Approximately 10–20 ng of DNA was used for the reaction and the annealing temperatures for the different primers were standardized by gradient PCR (DNA Engine, BioRad, M.J. Research Inc., USA). The PCR products were resolved in 1–2 % agarose gels, the concentration used depending on amplicon size, stained with ethidium bromide (5 ng ml^{−1}) and analyzed using a gel documentation system (Gel Doc™ XR⁺, BioRad, USA). Confirmation of the product was done by sequencing as follows. PCR positive *amoA*-AOA gene product (with primers Arch-*amoA*F/Arch-*amoA*R) was purified using PCR Product Purification Kit (Roche, Germany) and ligated to pDrive vector (Qiagen clone kit, USA) as per the manufacturer's protocol. Ligated products were transformed to *E. coli* DH5-α cells. Three of the positive clones were sequenced using both M13 forward and reverse primers in an automated ABI 3100 Genetic analyser using fluorescent label dye terminators (Bioserve, Hyderabad, India).

Quantification of AOA-*amoA* Gene from Sludge Samples

For each sample, quantitative real-time PCR (q-PCR) was performed with triplicate sets of extracted DNA. The quantification of archaeal *amoA* genes and bacterial *amoA* genes was performed using the primer sets Arch-*amoA*F/Arch-*amoA*R [10] and *amoA* 332F/*amoA*822R [11]. q-PCR was carried out in triplicate with a FastStart Universal SYBR Green Master Mix (Roche, Germany) in an Applied Biosystems Step One Plus™ Real-Time PCR System (AB Systems, USA). The PCR mixture with a volume of 15 µl contained 7.5 µl of the q-PCR master mix, 0.5 µl of each primer (0.4 µM), and 1 µl of each sample. The PCR conditions for the quantification of AOA was 95 °C for 3 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 53 °C, 60 s at 72 °C, with data capture for each cycle at 80 °C for 20 s and followed by melt curve. For AOB, the PCR condition were 94 °C for 3 min, followed by 40 cycles of 30 s at 94 °C, 30 s at 55 °C, 45 s at 72 °C, with data capture for each cycle at 80 °C for 20 s and followed by melt curve.

Sequence Analysis

Sequence of *amoA* gene was analyzed using bioinformatic tools. Pairwise sequence analysis was performed using BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST>) and multiple sequence alignment by Clustal W. The

Fig. 1 Amplification of archaeal 16S rRNA and AOA-*amoA* genes from sludge samples taken from FPETPs. **A** Archaeal 16S rRNA amplification with six different set of primers; **B** thaumarchaeal AOA-*amoA* gene amplification with four different set of primers

phylogenetic tree was constructed by the neighbor-joining method (MEGA 5). Bootstrap analysis was performed using 500 data replicates. The sequence of the AOA-*amoA* gene was submitted to GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>).

Results and Discussion

The optimum annealing temperature for all the primer sets is given in Table S1. PCR was positive for archaeal 16S rRNA gene in samples from fish processing sludge with six sets of archaeal primers (Fig. 1A) which generated the expected amplicons. The primer set Arc 340F and Arc 1000R was found to be superior to others as 14 of 17 samples were positive for total archaea (Table S1). Primer sets Arc344F/Arc915R, Arc349F/Arc806R and Arc24F/Arc329R detected archaea in ten, nine and eight samples respectively. Crenarchaeal primer set Cren270F/Cren750R gave positive reaction in four samples.

In the present study, amplification of thaumarchaeal AOA-*amoA* gene with the expected amplicon size and sequence is evidence enough to the presence of AOA in fish processing effluent plants (Fig. 1B). Among all the primers targeted to AOA-*amoA* genes, Arch-*amoA*F/Arch-*amoA*R were found to be useful as they detected AOA in ten samples (Table S1). The primer pairs *AmoA*1F/*AmoA*2R and *amoA*-79F/*amoA*-479R gave positive results for AOA-*amoA* gene in six and four samples respectively. Primers designed in this study M-AOA-F and M-AOA-R detected three samples of AOA Group 1.1a, suggesting that the AOA belongs to marine cluster in FPETPs. This was further confirmed by cloning and sequencing of AOA-*amoA* gene; wherein all the three AOA-*amoA* sequences were

identical and consequently were assigned to thaumarchaeal group 1.1a (Fig. 2). The sequence showed 90 % nucleotide similarity with *Nitrosopumilus* spp. and the AOA-*amoA* gene sequence was assigned accession number KC750159 by GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>).

Quantitative real-time PCR showed that the abundance of AOA-*amoA* gene varied considerably among the samples (Table S2). AOA-*amoA* gene ranged from six orders of magnitude ($2.2 \times 10^6 \text{ mg}^{-1}$ sludge) to the levels of eight orders of magnitude ($4.2 \times 10^8 \text{ mg}^{-1}$ sludge) in the effluent samples. AOB primer set, *amoA* 332F/*amoA*822R can able to detect 10 of 17 samples and quantitative real-time PCR showed that the abundance of AOB-*amoA* gene varied considerably among the samples (Table S2). AOB-*amoA* gene ranged from seven orders of magnitude ($1.1 \times 10^7 \text{ mg}^{-1}$ sludge) to the levels of eight orders of magnitude ($8.5 \times 10^8 \text{ mg}^{-1}$ sludge) in the effluent samples.

Our results are in agreement with Gantner et al. [12] who found the newly designed primer set ARC 340F and ARC 1000R to be the best and highly specific to archaea as it was able to cover 93, 97 and 70 % of Crenarchaeota, Euryarchaeota and Korarchaeota sequences respectively reported in the SILVA data base (<http://www.arb-silva.de/>). Furthermore they observed that, in silico tests of this primer combination revealed at least 38 % higher coverage for total archaea compared to other reported primers. Primer set ARC 344F/ARC915R was also found to be good as it could detect 52 and 86 % of total archaeal sequences by using probe match [12]. The majority of archaeal primers were designed prior to the differentiation of archaea as Nanoarchaeota, Geoarchaeota and Aigarchaeota sub-divisions [13] and hence archaeal specific primers need to be reassessed in the light of these novel taxonomic groups. Very few studies have reported archaea in activated sludge waste water treatment plants [14, 15].

Recent research exemplify that archaeal habitats are not restricted to extreme environments and the present findings of archaea in FPETPs support the observation. Culture independent molecular techniques including PCR-based amplification of 16S rRNA indicate a wide distribution of uncultured archaea in normal habitats, such as ocean waters, lake waters, agricultural soils, sediments, rhizosphere and waste water treatment plants [13–16]. Global initiatives by researchers reveal that archaea present well-defined community patterns across broad environmental gradients and habitats [17]. Further, 16S rRNA based phylogenetic studies show salinity rather than temperature as the principal driving force for their distribution [17]. Although, archaea constitute a considerable fraction of the microbial biomass on earth, their significant ecological role in nature is being gradually unravelled.

q-PCR results were relatively higher than earlier reports where AOA-*amoA* gene copies were ranged from 1.7×10^2

Fig. 2 The phylogenetic relationship among deduced amino acid sequence of AOA-*amoA* gene retrieved from sludge sample. Clone sequence is shown with triangle. Bootstrap values (>50 %) are indicated at branch points. The scale bar represents 10 % estimated

sequence divergence. Clades are labeled with thaumarchaeal groups and their frequently represented environment. The tree was rooted with sequences affiliated to marine cluster, thaumarchaeal group 1.1a

to 1.7×10^5 copies in a nitrogen-removing reactor and WWTPs [14, 15]. This would be due to higher ammonia concentrations of nitrogen-removing reactor and WWTPs than FPETPs. Limpiyakorn et al. [15] found that the effluent of WWTPs contained the higher levels of ammonium resulted in less than the limit of detection (LOD) of AOA-*amoA* genes (1.70×10^5 copies l^{-1} of sludge) in industrial WWTPs. But in municipal WWTPs, the ammonia concentrations were low and resulted in significant numbers of AOA-*amoA* gene with more than eight orders magnitude ($1.05 \times 10^8 \pm 6.74 \times 10^7$ to $7.48 \times 10^{11} \pm 2.08 \times 10^{10}$ copies l^{-1} of sludge) [15].

Archaea possibly play crucial roles in biogeochemical cycles of nature as they have evolved a variety of energy metabolisms using organic and inorganic electron donors and acceptors and also have the ability to exhibit autotrophy [13]. In particular, the carbon and nitrogen cycles are greatly influenced by distribution, diversity and activity of distinct archaeal populations [17, 18]. More recently, the role of AOA is being increasingly recognized in AO in the environment [6, 18] and comparative genomics has enabled AOA to be placed in a newly assigned phylum Thaumarchaeota [6, 18]. Recent meta-analysis of 6,200 AOA-*amoA* gene sequences showed unique AOA community distribution patterns along a wide spectrum of physicochemical conditions and habitat types such as soil, freshwater and the sediment therein, estuarine sediment, marine water and the sediment therein, and geothermal system [19]. These results suggest the existence of AOA populations with different evolutionary history in the varied habitats.

Presence of AOA marine group 1.1a has been previously reported in biofilters of recirculating aquaculture system

[7, 20]. Few studies have shown the presence of archaeal *amoA* genes in WWTPs and nitrogen removal reactors [9, 14] suggesting that like in natural habitats [19]. AOA is widespread even in engineered systems such as FPETPs and WWTPs and have a potential role in removing nitrogen from wastewater. Previous phylogenetic studies of AOA-*amoA* gene from WWTPs suggested that although AOA groups 1.1a and 1.1b were both present in WWTPs, the majority belonged to the Thaumarchaeota group 1.1b [8, 9]. Environmental factors are likely to play a role in defining the ecological niches of AOA [6, 21–23]. The dynamics of ammonia oxidizing microbial community and its correlation with pH, dissolved oxygen, ammonia concentration, biochemical oxygen demand and temperature has been adequately reported [7–9, 15, 22]. Isolation of AOA presents a challenge at the present time. Once that is achieved, its application in effluent treatment plants for ammonia removal will be possible on an industrial scale. The increasing number of publications in the field of wastewater engineering in recent years clearly indicates the widespread interest in ammonia oxidizing microbes [9, 23]. The use of salicylic acid to enhance the bioremediating potential of microbes and a consequent reduction of BOD and COD has been studied [24]. Though the potential role of AOA in waste water treatment is discussed, the role of heterotrophic bacteria also seems imminent as seen from the simultaneous nitrification and denitrification performing bacterium *Diaphorobacter* sp. for improved activity in removal of high-nitrogen-containing wastes under aerobic conditions [25]. The relative importance of AOB and AOA community, their dynamics in nutrient removal and consequent water quality improvement is an area of much interest.

Conclusion

The present investigation suggested the presence of both archaea and AOA in FPETPs. It was found that primer pairs ARC 340F/ARC 1000R and Arch-amoA/Arch-amoAR were useful for the sensitive detection of archaeal and AOA-*amoA* genes respectively. AOA was affiliated to *Nitrosopumilus* spp. under group 1.1a (marine cluster) pointing to the potential role of this group in nitrogen removal. Culturing of AOA and their application for effective removal of ammonia from waste effluents from FPETPs is in progress.

Acknowledgments The funding provided by the ICAR- NAIP- National Fund for Basic, Strategic & Frontier Application Research in Agriculture for carrying out the study is gratefully acknowledged.

References

- Keluskar R, Nerurkar A, Desai A (2013) Development of a simultaneous partial nitrification, anaerobic ammonia oxidation and denitrification (SNAD) bench scale process for removal of ammonia from effluent of a fertilizer industry. *Bioresour Technol* 130:390–397. doi:10.1016/j.biortech.2012.12.066
- Kowalchuk GA, Stephen JR (2001) Ammonia-oxidizing bacteria: a model for molecular microbial ecological research. *Annu Rev Microbiol* 55:485–529. doi:10.1146/annurev.micro.55.1.485
- Venter JC, Remington K, Heidelberg JF et al (2004) Environmental genome shotgun sequencing of the Sargasso Sea. *Science* 304:66–74. doi:10.1126/science.1093857
- Könneke M, Bernhard AE, de la Torre J, Walker CB, Waterbury JB, Stahl DA (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* 437:543–546. doi:10.1038/nature03911
- He JZ, Hu HW, Zhang LM (2012) Current insights into the autotrophic thaumarchaeal ammonia oxidation in acidic soils. *Soil Biol Biochem* 55:146–154. doi:10.1016/j.soilbio.2012.06.006
- Stahl DA, Torre RD (2012) Physiology and diversity of ammonia-oxidizing archaea. *Annu Rev Microbiol* 66:83–101. doi:10.1146/annurev-micro-092611-150128
- Urakawa H, Tajima Y, Numata Y, Tsuneda S (2008) Low temperature decreases the phylogenetic diversity of ammonia-oxidizing archaea and bacteria in aquarium biofiltration systems. *Appl Environ Microbiol* 74:894–900. doi:10.1128/AEM.01529-07
- Park HD, Wells GF, Bae H, Criddle CS, Francis CA (2006) Occurrence of ammonia-oxidizing archaea in wastewater treatment plant bioreactors. *Appl Environ Microbiol* 72:5643–5647. doi:10.1128/AEM.00402-06
- Limpiyakorn T, Fühacker M (2013) *amoA*-encoding archaea in wastewater treatment plants: a review. *Appl Microbiol Biotechnol* 97:1425–1439. doi:10.1007/s00253-012-4650-7
- Francis CA, Roberts KJ, Beman JM, Santoro AE, Oakley BB (2005) Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proc Natl Acad Sci USA* 102:14683–14688. doi:10.1073/pnas.0506625102
- Rothauwe JH, Witzel KP, Liesack W (1997) The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl Environ Microbiol* 63: 4704–4712. <http://aem.asm.org/content/63/12/4704>
- Gantner S, Andersson AF, Alono-Saez L, Bertilsson S (2011) Novel primers for 16S rRNA-based archaeal community analyses in environmental samples. *J Microbiol Methods* 84:12–18. doi:10.1016/j.mimet.2010.10.001
- Offre P, Spang A, Schleper C (2013) Archaea in biogeochemical cycles. *Annu Rev Microbiol* 67:437–457. doi:10.1146/annurev-micro-092412-155614
- Jin T, Zhang T, Yan Q (2010) Characterization and quantification of ammonia-oxidizing archaea (AOA) and bacteria (AOB) in a nitrogen-removing reactor using T-RFLP and qPCR. *Appl Microbiol Biotechnol* 87:1167–1176. doi:10.1007/s00253-010-2595-2
- Limpiyakorn T, Sonthiph P, Rongsayamanont C, Polprasert C (2011) Abundance of *amoA* genes of ammonia-oxidizing archaea and bacteria in activated sludge of full-scale wastewater treatment plants. *Bioresour Technol* 102:3694–3701. doi:10.1016/j.biortech.2010.11.085
- Schleper C, Jurgens G, Jonuscheit M (2005) Genomic studies of uncultivated archaea. *Nat Rev Microbiol* 3:479–488. doi:10.1038/nrmicro1159
- Auguet JC, Barberan A, Casamayor EO (2010) Global ecological patterns in uncultured archaea. *The ISME J* 4:182–190. doi:10.1038/ismej.2009.109
- Schleper C, Nicol GW (2010) Ammonia-oxidizing archaea—physiology, ecology and evolution. *Adv Microb Physiol* 57:1–41. doi:10.1016/B978-0-12-381045-8.00001-1
- Cao H, Auguet J, Gu J (2013) Global ecological pattern of ammonia-oxidizing archaea. *PLoS ONE* 8:e52853. doi:10.1371/journal.pone.0052853
- Sakami T, Andoh T, Morita T, Yamamoto Y (2012) Marine genomics phylogenetic diversity of ammonia-oxidizing archaea and bacteria in biofilters of recirculating aquaculture systems. *Mar Genomics* 7:27–31. doi:10.1016/j.margen.2012.04.006
- Erguder TH, Boon N, Wittebolle L, Marzorati M, Verstraete W (2009) Environmental factors shaping the ecological niches of ammonia-oxidizing archaea. *FEMS Microbiol Rev* 33:855–869. doi:10.1111/j.1574-6976.2009.00179.x
- Subrahmanyam G, Hu HW, Zheng YM, Archana G, He JZ, Liu YR (2014) Response of ammonia oxidizing microbes to the stresses of arsenic and copper in two acidic alfisols. *Appl Soil Ecol* 77:59–67. doi:10.1016/j.apsoil.2014.01.011
- Subrahmanyam G, Shen JP, Liu YR, Archana G, He JZ (2014) Response of ammonia oxidizing archaea and bacteria to long-term industrial effluent polluted soils, Gujarat, western India. *Environ Monit Assess* 186:4037–4050. doi:10.1007/s10661-014-3678-9
- Khardenavis AA, Kapley A, Purohit HJ (2010) Salicylic-acid-mediated enhanced biological treatment of wastewater. *Appl Biochem Biotechnol* 160:704–718. doi:10.1007/s12010-009-8538-7
- Khardenavis AA, Kapley A, Purohit HJ (2007) Simultaneous nitrification and denitrification by diverse *Diaphorobacter* sp. *Appl Microbiol Biotechnol* 77:403–409. doi:10.1007/s00253-007-1176-5