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Sequence Similarity based Identification of Nitrifying Bacteria in Coastal Aquaculture for Bioremediation predictability

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Abstract

Chemolithoautotrophic nitrifying bacteria are an essential functional group for detoxification of toxic ammonia into less toxic nitrate. In the first step of nitrification, an autotrophic ammonium-oxidizing bacteria (AOB) oxidize ammonia to nitrite through the intermediate hydroxylamine. In the present study, ammonia monooxygenase (a*moA*) gene is chosen to detect nitrifiers in different environmental samples collected from various coastal aquaculture systems in Tamilnadu and also commercially available bioaugmentors. The *amoA* was detected in most of the environmental samples and bioaugmentors. The *amoA* gene isolated from coastal soil has been sequenced. Sequence alignment revealed homology with that of *amoA* of uncultured beta-proteobacteria, *Nitrosomonas nitrosa* and other *Nitrosomonas sp.* available in the GenBank. The present study has a potential for making bioremediation strategies for ammonia removal from brackishwater aquaculture systems.

Introduction

Coastal aquaculture is associated with generation of toxic ammonia as a results of excess feed and faecal waste, which can adversely effect aquaculture productivity. This metabolite increases blood pH, reduces the oxygen content in the blood, affects gills, creates stress resulting in reduced feeding and strong potential for diseases. Soils are estimated to harbor up to 10^{10} bacteria of about 10^4 different ribotypes per gram, of which more than 95% cannot be cultured by present methods (Amann et al. 1995; Mayr et al. 1999). Nitrification is an oxidative process for waste treatment in ammonia removal (Conrad 1996; Zumft 1997; Herbert 1999; Tamegai et al. 2007), however, nitrifying bacteria occur in low numbers and require much longer time to increase in numbers than typical heterotrophic strains. This makes them difficult to detect in environmental

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samples by traditional methods (Rotthauwe et al. 1997; Ford et al. 1980; Watson et al. 1989; Saraswat et al. 1994). Moreover, culture dependent methods underestimate total bacterial count, often by as much as 2 orders of magnitude and are not accurate in monitoring of natural bacterial populations (Torsvik et al. 1990). Culture - independent methods are therefore used to monitor bacterial populations (Smith and Tiedje 1992). The molecular techniques have been used to detect very low concentrations of nitrifying bacteria in various aquatic environments (Rotthauwe et al. 1997; Scala & Kerkhoff 1998; 1999). In the first step of nitrification, the autotrophic ammonium-oxidising bacteria (AOB) oxidizes ammonia to nitrite through the intermediate hydroxylamine (Hollocher et al. 1981; Hooper et al. 1997; Wood 1986).

$$NH_3 + O_2 + 2e^- + 2H^+ \longrightarrow NH_2OH + H_2O \longrightarrow NO_2 + 5H^+ + 4e^-$$

Ammonia monooxygenase, *amoA*, that catalyzes the oxidation of ammonia to hydroxylamine has higher diversity compared to 16S rRNA genes, hence it is often used to study genetic differences in natural populations of AOB (McTavish et al. 1993; Rotthauwe et al. 1997; Purkhold et al. 2000). The present study has been carried with the objective of examining AOB diversity in Indian coastal environment for predictability of ammonia bioremediation.

Materials and Methods

DNA extraction from environmental soil samples and commercially available bioaugmentors

Composite soil samples were also collected from coastal environment of Tamil Nadu (Devanagari, Nathamedu, Kalpakkam, Kokilamedu, Pooncheri, Kattur) for detection of ammonia oxidizing bacteria. Genomic DNA was extracted from 0.4 g of composite soil samples and commercial bioaugmentors with a FastDNA spin kit for soil (UltaClean Soil DNA Kit, MO Bio laboratories, Carlsbad, California) using bead beating according to the manufacturerís instructions. DNA was also extracted from composite soil samples and bioaugmentors using modified standard procedure. Genomic DNA extracted was quantified using NanoDrop (Agilent) at 260 nm, and purity was determined by measuring the 260 / 280 nm absorbance ratio.

PCR amplification and sequencing of amoA

The polymerase chain reaction was performed on the samples along with negative control (DEPC treated water) with a 50 µl reaction mixture using Eppendorf thermal cycler (Master cycler gradient). The amplification programs were as follows: one cycle consisting of 94°C for 90 sec, followed by 36 cycles consisting of denaturation (94 °C for 45 sec), annealing (58 °C for 45 sec) and elongation (72 °C for 45 sec) and a final

extension step consisting of 72°C for 8 min. Aliquots (10 µl) of the PCR products were electrophoresed and visualized in 1% agarose gels by using standard electrophoresis procedures. Bioanalyzer (Azilent) has also been employed to confirm the size of PCR product.

A modified primer set of forward primer and reverse primer originally constructed by Holmes et al. (1995) and Rotthauwe et al. (1997) respectively has been used to amplify ~ 669 bp fragment of *amoA*. The original forward primer devised by Holmes et al. (1995) has one degeneracy (N = C, G, A, T) at 3rd nucleotide. We used a forward primer (A189-y) with two degeneracy, which contain a mixture of C, G, A and T at 3rd position and Y (= T or C) as the 12th nucleotide. We also used forward primer with less degeneracy. The original reverse primer devised by Rotthauwe et al. (1997) contained 21 bases with two degeneracy at 7th (K= T or G) and 9th (S = G or S) nucleotide. We used reverse primer without any degeneracy at 9th nucleotide (G).

The amplified *amoA* (669 bp) was purified with a gel extraction kit (QIAquick) by following the manufacture′s instructions. The purified PCR product was ligated by using the pGEM-T Easy vector system (Promega) and were transformed into high efficiency competent cells (*E.coli* DH5-·). Three unique clones were identified by *EcoRI* restriction digestion. Plasmid DNA from unique transformants were isolated by using a plasmid isolation kit for sequencing, which was done in an ABI 3100 Genetic Analyzer. Primary sequences were analysed using the BLAST tool of www.ncbi.nlm.nih.gov.

Results and Discussion

Detection of amoA in coastal soil samples and bioaugmentors

In the marine environment, nitrification is important because it detoxify ammonia into nitrate. In the first step of nitrification, *amoA* catalyzed the oxidation of ammonia into hydroxyl amine, and very specific for nitrifying bacteria. Moreover *amoA* encoding ammonia monooxygenase, with about large entries in GenBank has more sequence information than that available for these genes (Rosch & Bothe 2005). The gene encoding *amoA* is largely unique to nitrifying bacteria and has been used for detection of nitrifier specific DNA in environmental samples (Scala & Kerkhof 1998; 1999; Hunter et al. 2006). In the present study, *amoA* has been chosen to characterize nitrifying diversity in coastal aquaculture. 669 bp of the *amoA* gene was amplified (Fig. 1) via PCR, using *amoA* gene-specific primers, originally designed by Holmes et al. (1995) and Rotthware et al. (1997). The original forward primer devised by Holmes et al. (1995) contained degeneracy at 3rd nucleotide (N= C, G, A and T), we also used forward primer with less degeneracy. The original reverse primer devised by Rotthauwe et al. (1997) contained

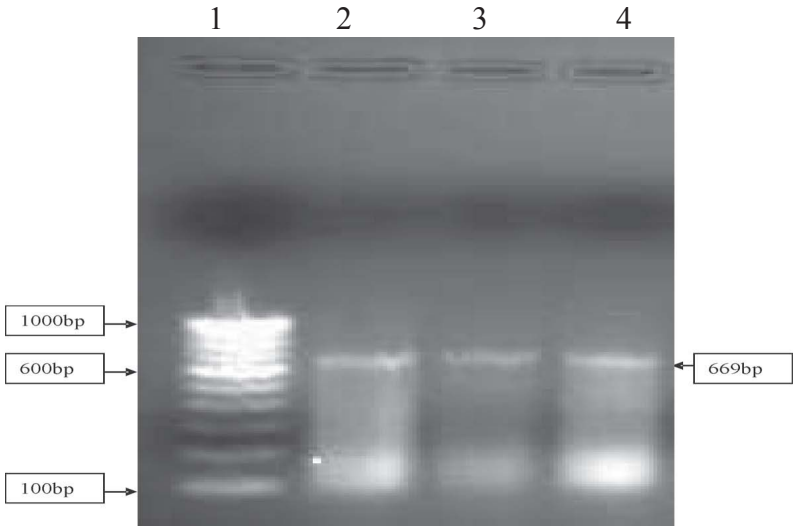

Figure 1. PCR amplification of 669 bp fragment of the *amoA* gene (Lane 1 Marker 100 bp, Lane 2,3,4; 669 bp *amoA*).

degeneracy at two positions, a K at the seventh nucleotide in the primer sequence (K = T or G) and an S at the ninth nucleotide (S=G or C). In this study, we also used a reverse primer with less degeneracy. Modified primers amplified templates with the same or better efficiency.

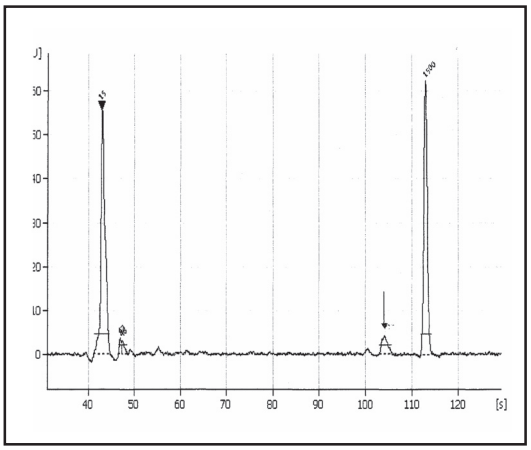

Figure 2: Analysis of 669 bp a*moA* gene using bioanalyzer.

Rotthauwe et al. (1997) and Holmes et al. (1995) reported that the PCR amplification of *amoA* is a very powerful tool for analyzing indigenous ammonia-oxidizing communities because this gene is specific for AOB′s and represents a functional trait rather than a phylogenetic trait. In the present study, PCR product 669 bp products have also been confirmed by bioanalyzer (Fig. 2). A total of 50 coastal soil samples of Tamilnadu and 6 commercially available bioaugmentors were screened for *amoA*. The *amoA* was found in 36 coastal soil samples, which revealed inhabitation of nitrifying organisms in shrimp aquaculture. The *amoA* was found in most of the samples collected from Devanagari and Nathamedu areas followed by Kattur area of Tamilnadu. This indicated that there is a prevalence of indigenous nitrifying bacteria in those coastal aquaculture systems, where biostimulation

approach can very well be applied. Out of six different commercially available bioaugmentors tested, four were *amoA* positive, which revealed the presence of nitrifying bacteria in these products.

Diversity of amoA in coastal environment

Knowledge of microbial ecology is required to predict nutrient fluxes and to analyze the fate of metabolites (Rosch et al. 2002; Scala & Kerkhoff 1998; 1999). Diversity of the ammonia monooxygenase (*amoA*) gene was examined in coastal soil obtained from the coastal envir onment in Tamilnadu. The nucleotide sequences determined in this study have been deposited in the GenBank database. The accession numbers for the 669 bp gene sequences are EU284710 through EU284712. The phylogenetic tree of *amoA* genes was generated by using the maximum likelihood method, based on the alignment of 669 bp of *amoA* gene fragments (Fig. 3).

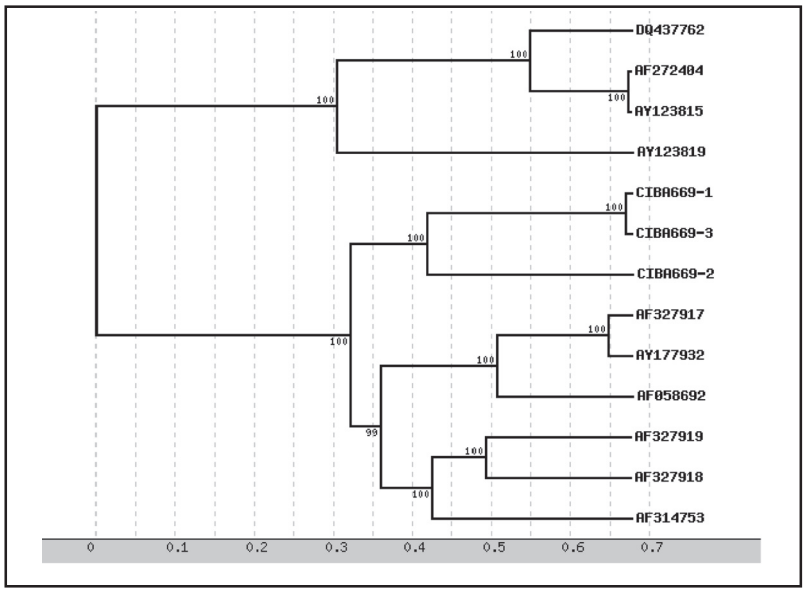

Figure 3. Phylogenetic tree based on *amoA* genes (669 bp) as determined by maximum likelihood method.

For the *amoA* gene clonal library (669 bp), nucleotides and predicted amino acids sequences of the *amoA* genes identified in this study are compared with the sequences available in the GenBank. Sequence alignment revealed homology with that of *amoA* of *Nitrosomonas sp.* available in the GenBank (Table-1). The molecular methods described in this study shed light on the ecology of the uncultured organisms in our coastal environment. The level of similarity between different pairs of *amoA* genes ranged from 80 to 99% (Table 2). This revealed that sequence difference has resulted from actual diversity in the sample and there was no PCR artifact. This has also been confirmed by

Table 1. Similarity of 669 bp sequences to *amoA* nucleotides in GenBank database

	% Similarity		
	CIBA669-1 (EU156172)	CIBA669-2 (EU156173)	CIBA669-3 (EU156174)
Nitrosomonas sp. Y41 (DQ437762)	99	79	98
Nitrosomonas sp. (AL212) (AF327918)	78	84	78
Nitrosomonas sp. JL21 (AF327919)	77	84	77
Nitrosomonas Nm148 (AY123815)	99	79	98
Nitrosomonas sp. Nm86 (AY123819)	75	87	75
Nitrosomonas GH22 (AF327917)	81	77	81
Nitrosomonas nitrosa (AF272404)	99	78	99
Nitrosomonas europaea (AF058692)	81	76	81
Nitrosomonas eutropha (AY177932)	81	76	80
Nitrosomonas cryotolerans (AF314753)	77	83%	77

Table 2. The level of similarity between different pairs of *amoA* genes at nucleotide and amino acids levels

Sequence A	Sequence B	Score	
		Nucleotide level	Amino acid level
CIBA669-1 (EU156172)	CIBA669-2 (EU156173)	80	85
CIBA669-1 (EU156172)	CIBA669-3 (EU156174)	99	98
CIBA669-2 (EU156173)	CIBA669-3 (EU156174)	80	84

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ABV90497        DWDFWMDWKDRQWWPVVTPIVGITYCSTIMYYLWVNYRQPFGATLCVVCLLIGEWLTRYW
ABV90499        DWDFWMDWKDRQWWPVVTPIVGITYCSTIMYYLWVNYRQPFGATLCVVCLLIGEWLTRYW
AAL86636        DWDFWMDWKDRQWWPVVTPIVGITYCSAIMYYLWVNYRQPFGATLCVVCLLIGEWLTRYW
AAO60371        DWDFWMDWKDRQWWPVVTPIVGITYCSAIMYYLWVNYRQPFGATLCVVCLLIGEWLTRYW
AAC38653        DWDFWMDWKDRQWWPVVTPIVGITYCSAIMYYLWVNYRQPFGATLCVVCLLIGEWLTRYW
AAL86637        DWDFWLDWKDRQWWPVVTPIAGIMYCAALMYYLWVNYRLPFGATLCIVCLLVGEWLTRYW
AAL86638        DWDFWLDWKDRQWWPVVTPIVGIMYCAALMYYLWVNYRLPFGATLCIVCLLVGEWLTRYW
AAG60667        DWDFWLDWKDRQYWPVVTPIVGIMYCAAIMYYLWVNYRLPFGATLCIVCLLVGEWLTRYW
ABV90498        DWDFWMDWKDRQWWPVATPIVGVMYCAAIMYYLWVNYRLPYGATLCIVCLLIDEWLTRYW
ABV90497        GFYWWSHYPINFVTPSNMIPGALMLDITLYLTRNWLVTALIGGGFFGLLFYPGNWPIFGP
                *****:******:***.***.*: **:::********* *:*****:****:.*******

ABV90499        GFYWWSHYPINFVTPSIMIPGALMLDITLYLTRNWLVTALIGGGFFGLLFYPGNWPIFGP
AAL86636        GFYWWSHYPLNFVTPGIMLPGALMLDFTMYLTRNWLVTALVGGGFFGLMFYPGNWPIFGP
AAO60371        GFYWWSHYPLNFVTPGIMLPGALMLDFTMYLTRNWLVTALVGGGFFGLMFYPGNWPIFGP
AAC38653        GFYWWSHYPINFVTPGIMLPGALMLDFTLYLTRNWLVTALVGGGFFGLLFYPGNWPIFGP
AAL86637        GFYWWSHYPINFVLPSTMIPGALMLDTIMLLTGDWLITALLGGAFWGLFFYPGNWPIFGP
AAL86638        GFYWWSHYPLNFVLPSTMIPGALMMDTIMLLTGNWLVTALLGGGFFGLFFYPGNWPIFGP
AAG60667        GFYWWSHYPMNFVLPSTMIPGALMLDTVMLLTRSWLVTALVGGGFFGLFFYPGNWPIFGP
ABV90498        GFYWWSHYPINFVLPSTMIPGALMLDTILLLTRNWLVTALIGGGFWGLFFYPGNWPIFGP
                *********:*** *. *:*****:*  : ** .**:***:**.*:**:***********

ABV90497        THLPVVAEGVLLSMADYMGHLYIRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFAAFVSML
ABV90499        THLPVVAEGVLLSMADYMGLLYIRTGTPEYVRLIEQDSLRTFGGHTTVIAAFFAAFVSML
AAL86636        THLPIVVEGTLLSMADYMGHLYVRTGTPEYVRHIEQGSLRTFGGHTTVIAAFFAAFVSML
AAO60371        THLPIVVEGTLLSMADYMGHLYVRTGTPEYVRHIEQGSLRTFGGHTTVIAAFFAAFVSML
AAC38653        THLPIVVEGTLLSMADYMGHLYVRTGTPEYVRHIEQGSLRTFGGHTTVIAAFFSAFVSML
AAL86637        THLPLVVEGVLLSVADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFSAFVSML
AAL86638        THLPVVVEGVLLSIADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFSAFVSML
AAG60667        THLPVVVEGVLLSLADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFAAFVSML
ABV90498        THLPVVVEGVLLSVADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFSAFVSML
                ****:*.**.***:*** * **:********* ***.****************:******

ABV90497        MFVVWWFLGKVYCTAFFYVKGKRGRIVKRDDVTAFGEEGFPEG
ABV90499        MFVVWWFLGKVYCTAFFYVKGKRGRIVKRDDVTAFGEEGFPEG
AAL86636        MFAVWWYLGKVYCTAFFYVKGKRGRIVQRNDVTAFGEEGFPEG
AAO60371        MFAVWWYLGKVYCTAFFYVKGKRGRIVQRNDVTAFGEEGFP
AAC38653        MFTVWWYLGKVYCTAFFYVKGKRGRIVHRNDVTAFGEEGFPEG
AAL86637        MFCVWWYFGKLYCTAFFYVKGERGRISMKNDVTAFGEKGFAQG
AAL86638        MFCVWWYFGKLYCTAFYYVKGERGRISMKNDVTAFGEKGFAQG
AAG60667        MFCVWWYFGKLYCTAFTMLRCKR-QVSMKHDVTAFGEEGFAEG
ABV90498        MFCVWWYFGKLYCTAFYYVKGERGHISMKKDVTAFGEEGFPEG
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Figure 4. Partial alignment of the predicted amino acids encoded by *amoA*. (The residues conserved in CIBA669-1, CIBA669-2 and CIBA669-3 sequences are highlighted with asterisk*).

only 84-98% similarity at the amino acid levels. Partial alignment of the predicted amino acids encoded by *amoA* is shown in Fig. 4. Residues conserved in CIBA669-1 (ABV90497), CIBA669-2 (ABV90498) and CIBA669-3 (ABV90499) sequences are highlighted. A total of 162 of 223 amino acid residues were conserved in all the three CIBA669 sequences.

Conclusion

An understanding of diversity of nitrifying bacteria in the system is very important for enhancing nitrification predictability and reliability. Previous studies have focused mainly on the composition and activities of the soil microbiota in aquaculture, only a few studies have employed molecular tools to understand the diversity of archaeal and bacterial community structures along the coastal aquaculture. In this study, we provide the detailed characterization of nitrifying microbial diversity in coastal environment through analyses of 669 bp fragment sequences of *amoA* gene. The present study based on DNA sequencing of clone library of *amoA* gene supply is a novel sequence information and allow the phylogenetic identification of individual clones. Three unique marine sediment *amoA* genes were identified and sequenced. The marine *amoA* genes were most closely related to the *amoA* genes of uncultured beta-proteobacteria, *Nitrosomonas nitrosa* and other *Nitrosomonas sp.* available in the GenBank. The present study has a potential for making nitrification strategy in coastal aquaculture and also for a more accurate understanding and modeling of the nitrogen fluxes.

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