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Community structure of β-Proteobacterial ammonia-oxidizing bacteria in prawn farm sediment

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Abstract

To examine the community structure of β-Proteobacterial ammonia-oxidizing bacteria (AOB) in prawn farm sediment, the 16S rRNA gene library was constructed with β-Proteobacterial AOB-specific primers. The library was screened by PCR-restriction fragment length polymorphism (RFLP) analysis and clones with unique RFLP patterns were sequenced. Two groups of β-Proteobacterial AOB, the *Nitrosomonas* and the *Nitrosospira,* were detected. The *Nitrosomonas* occupied an absolute dominant position, accounting for more than 90% of total clones in the clone library, while the *Nitrosospira* accounting for 5.48%. *Nitrosomonas*-affiliated clones were grouped into the *Nitrosomonas marina* and the *Nitrosomonas* sp. Nm143 clusters, and *Nitrosospira*-affiliated clones were grouped into the *Nitrosospira* cluster 1. No other clusters of β-Proteobacterial AOB were found. The results enriched our knowledge of AOB diversity in the prawn farm sediment and provided important foundational data for further functional studies of these microbes in mariculture environments.

Keywords: Ammonia-oxidizing bacteria; Prawn farm sediment; Phylogenetic diversity; 16S rRNA gene

1. Introduction

With the rapid development of aquaculture and continuous increase of intensive management, fisheries water is being polluted seriously. In particular, nitrogenous compounds, which may accumulate due to uneaten feed, feces and plankton die-offs in aquaculture ponds, can be toxic to aquatic animals and cause environmental problems such as eutrophication. Nitrification, the oxidation of ammonia to nitrate via nitrite, is central to the cycling of nitrogen in the environment and, when coupled with denitrification, alleviates the effects of eutrophication through removal of nitrogen to the atmosphere as nitrous oxide or dinitrogen gas [1]. The ammonia-oxidizing bacteria (AOB) are responsible for the first rate-limiting step in nitrification, the oxidation of ammonia to nitrite, and are generally members of the β-subdivision of the class Proteobacteria except for the marine genus *Nitrosococcus*, which belongs to the γ-subdivision [2]. Traditionally, the study of ammonia-oxidizing bacteria was mainly through isolation and cultivation methods. However, low maximum growth rates and growth yields of AOB render cultivation-based analysis of their environmental diversity extremely time-consuming and tedious. Furthermore, all culture techniques are potentially selective and thus bear the risk of incomplete coverage of the actually existing bacterial diversity [3]. The application of molecular techniques, in particular analysis of 16S rRNA genes, provides new opportunities for the assessment of ammonia-oxidizing populations in natural environments. Using cultivation-independent molecular techniques, many new lineages of AOB have been identified from a number of environments, including soils, sand dunes, biofilms, fluidized bed reactors, lakes, wastewater,

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and seawater [3–9]. Relatively few reports are regarding the AOB in aquaculture environments. Using culture enrichments and culture-independent molecular methods, Burrell et al. [10] revealed four major strains related to the *Nitrosomonas marina* cluster, the *Nitrosospira* cluster, or the *Nitrosomonas europaea–Nitrosococcus mobilis* cluster of β-Proteobacterial AOB in freshwater aquaria. McCaig et al. [11] studied the community structure of AOB in the underlying sediment of a polluted marine fish farm, and selected a novel *Nitrosomonas* subgroup from polluted fish farm sediments, they reported that the relative abundance of this group was influenced by the extent of pollution.

Prawn farming environment is a typical organic-enriched, intensive marine aquaculture environment. At present, the sulfur- and phosphorous-conversion bacteria have been investigated [12] in shrimp-culturing sediments, and the phylogenetic diversity of archaea has also been revealed [13]. However, no data is available about the nitrogen cycling related bacteria in such environments. Here we examined the phylogenetic diversity of β-Proteobacterial AOB through analysis of the 16S rRNA gene clone library for the purpose of studying the AOB community structure and their potential ecological function in prawn farm sediment.

2. Materials and methods

2.1. Sample collection

Three prawn farm ponds for the experiment are located in Xiamen, Fujian Province. The temperature, pH value and the salinity of the farming ponds were 27.5–31.2 °C, 8.0–8.2 and 24–28 ppt, respectively. Three surface sediment (0–2 cm) samples from each pond were collected in July 2005. The sediments were transported to the laboratory on ice and 0.5 g from each sediments were thoroughly mixed and stored at −20 °C until nucleic acid extraction.

2.2. DNA extraction, PCR amplification and library construction

Total DNA was extracted and purified according to the previously described method [14] and was used for PCR amplification of the 16S rRNA genes. In order to achieve an initial increase in template concentration, a nest PCR was performed. The first PCR was performed using eubacterium-universial primers 27F (5′-AGAGTTTGATCA TGGCTCAG-3′) and 1492R (5′-GGTACCTTGTTACG ACTT-3′). The PCR program was the same as described by Regan [15]. A 1:10 dilution of the first PCR products was reamplified by using the primers specific for β-subgroup ammonia-oxidizing bacteria, namely, NITA (5′-CTTAAGTGGGG AATAACGCATCG-3′) and NITB (5′-TTACGTGTGAAGCCCTACCCA-3′). The PCR program was the same as described by Voytek and Ward [16]. The amplified products were gel-purified and ligated into the pMD18-T vector (TaKaRa Co., Dalian, China) and then transformed into competent cells of *E. coli* DH5α. About 90 ampicillin-resistant clones from clone library were randomly picked and screened for inserts by performing colony PCR with M13 primers (Invitrogen, Shanghai, China). In total, 73 clones with inserts of expected lengths from the clone library were obtained for subsequent restriction digest analysis.

2.3. PCR-RFLP screening and statistical analysis of the library

To examine the RFLP patterns in the library, a 1:10 dilution of the colony PCR products amplified by the M13 primers was reamplified by using the primers NITA and NITB. Two aliquots of 8–10 μl PCR products were separately digested with the two restriction endonucleases *HhaI* and *AfaI*. Restriction fragments were resolved by electrophoresis on 2.5% agarose gel. Phylotype diversity in the clone library obtained through PCR-RFLP analysis was subjected to Coverage [17] calculation and rarefaction analysis. Coverage value is derived from the equation: Coverage = 1–(N/Individuals), where N is the number of clones that occurred only once. The rarefaction curves [18] were calculated and analyzed by PAST software (PAlaeontological STatistics, ver. 1.34, http://folk.uio.no/ohammer/past).

2.4. DNA sequencing and phylogenetic analysis

Clones with unique RFLP patterns were sequenced. The sequencing was carried out on an ABI model 377 automated DNA sequence analyzer (Applied Biosystems, Perkin-Elmer) using the primer NITA. All the nucleotide sequences were checked for putative chimeras by the RDP CHIMERA_CHECK [19] and reliable sequences were compared to known 16S rDNA sequences in the database using the BLASTN search (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences obtained and their neighbors retrieved from the database, combined with representative sequences from different marine AOB groups, were aligned using the program ClustalX 1.80 [20]. The neighbor-joining tree was constructed by the software MEGA3 [21]. Bootstrap values were obtained with 100 resamplings. The 16S rDNA sequences from the γ-Proteobacterial AOB, *Nitrosococcus oceani* and *Nitrosococcus halophilus*, were used as outgroups.

2.5. Nucleotide sequence accession numbers

Clone sequences have been deposited in GenBank under the accession numbers of EU155063–EU155078.

3. Results

3.1. PCR-RFLP screening, pattern frequency and statistical analysis of the library

In total, 73 clones were subjected to PCR-RFLP screening using the endonucleases *HhaI* and *AfaI*, and 16 differ-

ent RFLP patterns were obtained. Details on the frequency of RFLP patterns for different clones are shown in Fig. 1. The high coverage value of 92% indicated that the clone numbers screened in the library were enough for the diversity analysis, and the rarefaction curve, as shown in Fig. 2, had approached an asymptote, suggesting that the clonal diversity was well represented. Among the 16 unique RFLP patterns detected in these clones, four patterns were most abundant; they occurred 14, 13, 12 and 9 times, respectively (Fig. 1), together these dominant patterns accounted for 25% in total patterns and 65.75% of the total clones. Six RFLP patterns were secondly dominant. They occurred two to four times, respectively; together they comprised 37.5% of the total patterns and 26.03% of the total clones. The remaining six RFLP patterns were observed only once (Fig. 1), they accounted for 37.5% in total patterns and 8.22% of the total clones. These results indicated that the phylogenetic diversity of AOB was not very high and relatively few phylotypes dominated the prawn farm sediment.

3.2. Phylogenetic analysis of the 16S rDNA sequences of AOB in prawn farm sediment

All unique RFLP patterns in the AOB 16S rDNA clone library were subjected to sequencing and a total of 16 sequences were obtained. Comparisons with the GenBank database by BLAST searches showed that all sequences were homologous with known 16S rDNA sequences of β-Proteobacterial AOB. In general, the most close relatives to our sequences were from intertidal muddy sediments, coastal marine sediments, and coastal seawater, and the sequences similarities ranged from 95% to 99%.

A phylogenetic tree was constructed from the sequences obtained in this study and their close relatives and some representative sequences of β-Proteobacterial AOB using the neighbor-joining method (Fig. 3). All main branches of the tree possess a high bootstrap value indicating high confidence of the tree topology. Phylogenetic and 16S rDNA sequence similarity analysis showed that the clones fell within two major groups of β-Proteobacterial AOB, *Nitrosomonas* spp. and *Nitrosospira* spp. The *Nitrosomonas*

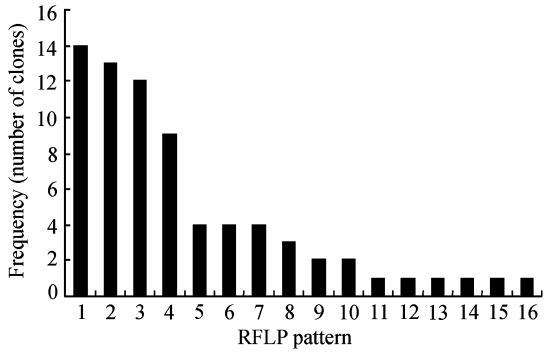

Fig. 1. RFLP pattern frequency in the ammonia-oxidizing bacterial 16S rDNA clone library in prawn farm sediment. Frequency values are ranked in the decreasing order.

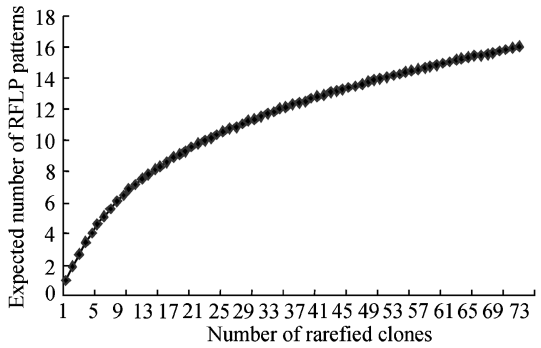

Fig. 2. Rarefaction curve of observed RFLP richness of all clones screened in the clone library. The expected number of RFLP patterns is plotted versus the number of clones.

occupied an absolute dominant position, accounting for more than 90% of total clones in the library, while *Nitrosospira* accounting for just a minor component (about 5%, Fig. 4), suggesting that the β-Proteobacterial AOB in the prawn farm sediment were mainly members within the *Nitrosomonas* group.

Clones affiliated *Nitrosomonas* were further subdivided into two clusters, the *N. marina* cluster and the *N.* sp. Nm143 cluster [22]. Clones within the *N. marina* cluster were more abundant than those within the Nm143 cluster. They comprised nine phylotypes and occupied 64.38% of total clones in the library (Fig. 4), and were most closely related with clone MZS-2 obtained from intertidal muddy sediments (GenBank description) and marine bacteria C-45 and Nm51, isolated from seawater [2,23]. The Nm143 cluster comprised five phylotypes and accounted for 28.77% of the total clones (Fig. 4). Clones from prawn farm sediment within this cluster were most closely related with clones TLBs244r and C24s44r recovered from coastal marine sediment [24]. One additional clone A-49 within the *Nitrosomonas* genera could not be grouped with any known cluster, indicating that the clone probably represented a novel *Nitrosomonas* cluster not previously recognized.

Only one phylotype (containing four clones) grouped into the *Nitrosospira*, this phylotype (represented by clone B-32) was most closely related with clone LD1-A1, isolated from anoxic marine sediment [25]. No other *Nitrosospira*-affiliated clusters were found, suggesting that even though there was some *Nitrosospira* spp. in the prawn farm sediment, the abundance and the diversity were very low.

4. Discussion

A previous study [2] showed that *Nitrosomonas* can be subdivided further into five phylogenetically well-defined lineages, while in the present study, only two *Nitrosomonas* clusters (*N. marina* cluster and Nm143 cluster) were found, other *Nitrosomonas*-affiliated clusters were absent, indicating that the distribution of some AOB clusters is environment specific. *N. marina* cluster was

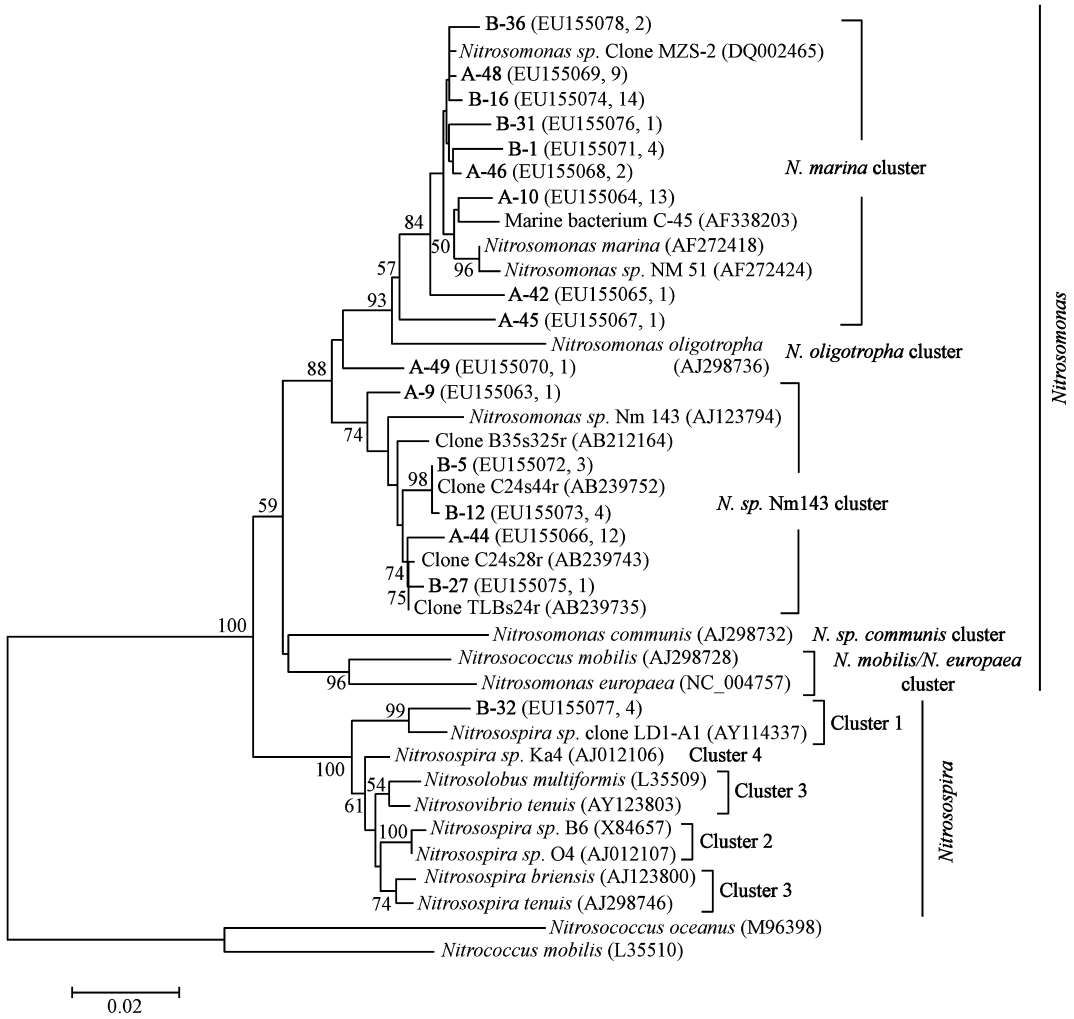

Fig. 3. Neighbor-joining tree generated from alignments of 16S rDNA sequences of β-Proteobacterial AOB from the prawn farm sediment and representative references retrieved from GenBank. Accessions numbers are shown in parentheses. Clones from this study are indicated in boldface. Numbers in brackets that follow the accession numbers indicate the occurrence frequency of the RFLP pattern in clone library. Bootstrap values above 50 (100 iterations) are shown at each node. Scale bar represents the nucleotide substitution percentage. The 16S rDNA sequences from the γ-Proteobacterial AOB, *Nitrosococcus oceani* and *Nitrosococcus halophilus*, are used as outgroups.

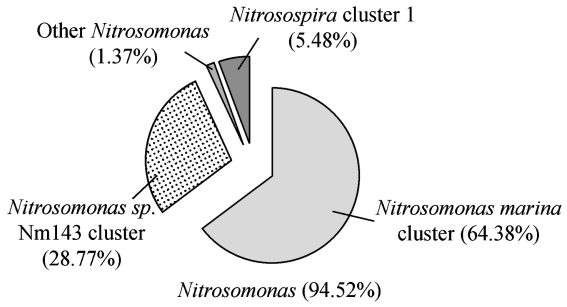

Fig. 4. Pie chart showing the community composition of β-Proteobacterial AOB from the prawn farm sediment. The percentage in parentheses indicates the abundance of clones of the respective AOB cluster in the pooled 16S rRNA gene library.

found to be widely distributed in seawater, freshwater, sand dune [2] and in sediment of estuary [26]. Nm143 cluster can also be distributed widely in different estuarine or marine habitats. The sampling sites included polluted and non-polluted fish farm sediments [14], estuarine [27] and even anoxic sediments [25]. A common feature among the sites investigated is salinity values above 10 ppt. Similarly, in the present study, the salinity in the prawn farm pond was 24–28 ppt. Interestingly, however, members of this Nm143 lineage were not yet detected in the open sea [9]. Members of *Nitrosospira* were subdivided into four clusters [2]. *Nitrosospira* cluster 1 is composed entirely of environmentally retrieved sequences [25] and is distinct from the other three clusters. It mainly contained marine clones while the others (clusters 2, 3 and 4) contained soil clones [14]. This is in agreement with our result that only *Nitrosospira* cluster 1 was detected in the mariculture environment.

McCaig [11] investigated the community structure of AOB in marine fish farm sediments and found that *Nitrosospira* cluster 1 was the most abundant group and occupied 53% to 83% of the total hybridization signal in each sample while *Nitrosomonas marina* cluster comprised less than 20%

in each sample. On the contrary, the present study showed that *Nitrosomonas marina* cluster was dominant and occupied more than 60% of the clone library while *Nitrosospira* cluster 1 was just a minor component (occupied 5.48%, Fig. 4) in prawn farm sediment. Furthermore, *Nitrosomonas* cluster 5 detected in the fish farm sediments was absent in the present study. These disagreements indicated that, despite in the same mariculture environment, the species and relative abundance of AOB were different in fish farm sediment and in prawn farm sediment. The reason for AOB variations in different mariculture environments is not fully understood but is most likely due to variations in environmental factors, such as the physical and chemical characteristics in the sediments, especially the concentrations of different forms of nitrogen as well as organic substances. Future research including a broad range of environmental parameters – physical, chemical, and biological may help to better understand the differences of AOB community structure in different environments.

AOB are generally members of the β subdivision of the class *Proteobacteria*, but it also includes a small number of members belonging to γ-Proteobacteria. Recently, some *Planctomycetales*-affiliated bacteria are proved to be capable of anaerobic ammonia oxidation (anammox) and these anammox-related *Planctomycetales* are globally distributed [28]. Furthermore, more and more evidence indicated that *Crenarchaeota* (one of the kingdoms of *Archaea*) contains the ammonia monooxygenase subunit A (*amo*A) gene and have the ability of ammonia oxidation [28]. These newly discovered AOB play an unneglected role in the nitrogen cycle. In addition, the chemolithotrophic nitrite-oxidizing bacteria (NOB) are responsible for the second step of nitrification. However, in this case, only β-Proteobacteria AOB are considered, so it is unlikely that the inspected AOB can fully reflect the community structure of ammonia oxidizer in prawn farm sediment. Clearly studies considering other ammonia oxidizer and NOB will both be required in the future to better understand the role of these bacteria in nitrogen cycle of prawn farm environments.

In conclusion, our study demonstrated that the community structure of β-Proteobacterial AOB in the prawn farm sediment were mainly composed of *Nitrosomonas* marina cluster, Nm143 cluster and *Nitrosospira* cluster 1. The *N. marina* cluster was predominant, occupying more than 60% of the clone library. Nm143 cluster was abundant while *Nitrosospira* cluster 1 was present just as a minor component. Comparison of AOB diversity in the prawn farm sediment and that in the fish farm sediment showed that the community structure of β-proteobacterial AOB in the two environments was different, indicating that the ecological niches of AOB are associated with environmental characteristics. The results revealed here would expand our understanding of the genetic diversity of AOB and would be significant for further functional studies of these microbes in mariculture environments.

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