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Molecular characterization of the nitrifying bacterial consortia employed for the activation of bioreactors used in brackish and marine aquaculture systems

V.J. Rejish Kumar [a], Vrinda Sukumaran [a], Cini Achuthan [a], Valsamma Joseph [a], Rosamma Philip [b], I.S. Bright Singh [a,*]

[a] National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin 682016, India
[b] Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Fine Arts Avenue, Cochin 682016, India

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ABSTRACT

The addition of commercial nitrifying bacterial products has resulted in significant improvement of nitrification efficiency in recirculating aquaculture systems (RAS). We developed two nitrifying bacterial consortia (NBC) from marine and brackish water as start up cultures for immobilizing commercialized nitrifying bioreactors for RAS. In the present study, the community compositions of the NBC were analyzed by universal 16S rRNA gene and bacterial amoA gene sequencing and fluorescence in situ hybridization (FISH). This study demonstrated that both the consortia involved autotrophic nitrifiers, denitrifiers as well as heterotrophs. Abundant taxa of the brackish water heterotrophic bacterial isolates were *Paenibacillus* and *Beijerinckia* spp. whereas in the marine consortia they were *Flavobacterium*, *Cytophaga* and *Gramella* species. The bacterial amoA clones were clustered together with high similarity to *Nitrosomonas* sp. and uncultured beta Proteobacteria. FISH analysis detected ammonia oxidizers belonging to β subclass of proteobacteria and *Nitrosospira* sp. in both the consortia, and *Nitrosococcus mobilis* lineage only in the brackish water consortium and the halophilic *Nitrosomonas* sp. only in the marine consortium. However, nitrite oxidizers, *Nitrobacter* sp. and phylum *Nitrospira* were detected in both the consortia. The metabolites from nitrifiers might have been used by heterotrophs as carbon and energy sources making the consortia a stable biofilm.

1. Introduction

The aquaculture industry is driven towards more intensification under recirculation due to the compelling factors such as limited space, water conservation, and restrictions in water discharge and easiness in the management of diseases. RAS reduces the water demand and discharges by recycling water and increases the food conversion efficiency resulting in less waste generation from feed (Losordo et al., 1998). In saltwater systems, RAS plays an important role in the production of healthy and properly sized fingerlings for stocking in net pens or ponds (Fielder and Allan, 1997). The most prominent characteristic of any RAS is a nitrifying biofilter to prevent accumulation of metabolites like ammonia and nitrite; which at high levels undermine the commercial production as their toxic impacts are manifested through impaired growth or chronic diseases (Tomasso, 1994; Cheng et al., 2004; Sovobodova et al., 2005). Fixed film biofilters are commonly used for total ammonia nitrogen (TAN) removal in RAS, where attached growth as biofilm offers several advantages such as handling convenience, increased process stability to shock loading and prevention of the bacterial population from being washed off (Fitch et al., 1998; Seo et al., 2001; Shnel et al., 2002).

Nitrification is the biological oxidation of ammonia to nitrate via nitrite by two groups of chemolithotrophic bacteria, ammonia oxidizers and nitrite oxidizers; both having a low specific growth rates (Prosser, 1989; Bock et al., 1991). Recent discoveries of anammox (Strous et al., 1999) and ammonia oxidizing archaea (Koenneke et al., 2005) have augmented the depth and breadth of ammonia oxidizing microorganisms. Nitrification in biofilters often relies on natural colonization of the nitrifying bacteria in the production systems. However, this natural method can take a relatively long time (4–8 weeks) to establish a healthy and viable population of both ammonia and nitrite-oxidizing bacteria (Manthe and

* Corresponding author. Tel./fax: +91 484 2381120.
E-mail addresses: bsingh@md3.vsnl.net.in, isbsingh@gmail.com (I.S. Bright Singh).

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Malone, 1987; Masser et al., 1999). Moreover, the nitrifying bacterial population is much sensitive to chemical and physical stresses (Malone and Pfeiffer, 2006; Emparanza, 2009). Therefore, a viable start up culture is vital for enhanced performance of RAS, which can overcome the initial lag and can also be used for quick reactivation of the system whenever some inhibition occurs. Kuhn et al. (2010) observed a significant improvement in the nitrification efficiency of recirculating systems by the addition of commercial nitrifying bacterial products. Accordingly, for the application in tropical recirculating systems, we developed nitrifying bacterial consortia (NBC) from the marine and brackish water by enrichment method (Achuthan et al., 2006) and mass cultured them in indigenously designed fermentors (Kumar et al., 2009a). Since then, these consortia are being applied as start up cultures for the activation of commercial packed bed bioreactor (PBBR) (Kumar et al., 2009b) and stringed bed suspended bioreactor (SBSBR) (Kumar et al., 2009c) (http://www.nitrifying-bioreactor.com, Patent no. 241648). The NBC were successfully demonstrated for activation and performance of the bioreactors in larval rearing systems of *Penaeus monodon* and *Macrobrachium rosenbergii* (Kumar et al., 2009b, c), in the rearing of adult *P. monodon* (Kumar et al., 2010) and also in *P. monodon* maturation systems (Kumar et al., 2011). These consortia were also successfully employed for bioaugmenting nitrification in *P. monodon* grow out systems by immobilizing on wood particles (Manju et al., 2009).

Enumeration, characterization and identification of ammonia- and nitrite-oxidizing bacteria in environmental samples by traditional microscopical and microbiological methods are difficult, because of the limited species specific morphological variety, slow growth rate (Watson et al., 1989), and their low growth yields (Gay and Corman, 1984; Wood, 1986). The use of molecular techniques now enables us to circumvent these limitations. The availability of molecular tools such as 16S ribosomal RNA (rRNA) sequence analyses have made it possible to explore slow growing or uncultivated bacterial species in different environments (Heal et al., 1998; O'Donnell and Gorres, 1999). In the present study, community composition of the NBC enriched from both the marine and brackish water systems have been analyzed using universal 16S rRNA gene and bacterial amoA gene sequencing and by FISH analyses.

2. Materials and methods

2.1. Nitrifying bacterial consortia: culturing and storage

NBC were cultured in 2 l fermentor (Bioflo 2000, NewBrunswick Scientific, USA) using simple seawater based medium (salinity adjusted to 15 g l^{-1} for brackish and 30 g l^{-1} for marine consortia) supplemented with 10 mg l^{-1} substrate $(NH_4)_2$ SO_4 and 2 mg l^{-1} KH_2PO_4 at an optimum temperature of 28 °C and pH of 8.0. After attaining log phase, the cultures were harvested and maintained at 4 °C with periodic addition of the substrate and adjustment of pH (using 1% Na_2CO_3) to the optimum.

2.2. DNA extraction, amplification of the 16S rRNA and amoA genes

DNA from each of the consortia was extracted following Burrell et al. (1998). Aliquots of 2 ml of the active consortium each were centrifuged at 12000 g for 5 min and the pellets were resuspended in 500 µl saline − EDTA (150 mM NaCl, 100 mM EDTA, pH 8.0). After the addition of 100 µl of freshly prepared 100 mg ml^{-1} lysozyme the mixtures were incubated at 37 °C for 1.5 h and then subjected to four cycles of freeze-thaw sequentially at −20 and 65 °C. Thereafter, 100 µl 25% (w/v) sodium dodecyl sulfate and 50 µl 2% (w/v) proteinase K each were added to the mixtures and incubated at 60 °C for 1.5 h. This was followed by phenol-chloroform extraction, and the residual RNA was removed by adding 3 µl of 10 mg l^{-1} RNAse and incubated at 37 °C for 1 h. The DNA at 100 ng concentration was maintained at −20 °C for further use.

Bacterial and archaeal 16S rRNA genes were amplified using primers 16S1 (5′-GAGTTTGATCCTGGCTCA-3′)/16S2 (5′-ACGGC-TAC CTTGTTACGACTT-3′) (Lane, 1991) and Arch21F (5′-TCCGGTTG ATCCYGCCGGA-3′)/Arch958R (5′-YCCGGCGTTGAMTCCAAT T-3′) (DeLong, 1992) respectively as per the conditions described by the authors.

For the amplification of bacterial amoA gene, primers *amo* A-1F (5′-GGGGTTTCTACTGGTGGT- 3′)/*amo*A 1R (5′-CCCCTCKGS AAAGCCTTCTTC-3′) (Rotthauwe et al., 1997) were used. The reactions were performed in a solution containing 1× PCR buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton ×100, 20 mM Tris−HCl, pH 8.8), 20 nmol of each dNTPs, 30 pmol each primer, 1 µl of template DNA (100 ng) and 2.5 U of Taq DNA polymerase (New England Biolabs). The Taq polymerase was added after the first denaturation step. The reaction cycle followed, 5 min at 94 °C; pause at 80 °C to add Taq polymerase; then 42 cycles consisting of 90 s at 56.8 °C, 90 s at 72 °C, and 60 s at 94 °C and a final elongation of 72 °C at 7 min. Aliquots (10 µl) of the PCR products were visualized in 1% agarose gels by standard electrophoresis procedures.

2.3. Cloning and screening

Fresh PCR products of 16S rRNA and *amo*A genes were cloned into the pGEM-T Easy vector (Promega). The ligation mix (10 µl) consisted of 5 µl ligation buffer (2×), 0.5 µl vector (50 ng μl^{-1}), 3 µl of PCR product and 1 µl of T4 DNA ligase (3 U μl^{-1}). This was incubated at 4 °C overnight. The entire ligated mix was used to transform *Escherichia coli* JM109 competent cells prepared using calcium chloride method. The ligation mix was added to 10 ml glass tube previously placed on ice to which 50 µl of competent cells were added and incubated on ice for 20 min. A heat shock at 42 °C was given for 90 s, immediately the tubes were placed on ice for 2 min and then 600 µl of SOC medium was added and incubated for 2 h at 37 °C with shaking at 250 rpm. The transformation mixture (100–200 µl) was spread on Luria-Bertani (LB) agar plates supplemented with ampicillin (100 µg ml^{-1}), IPTG (100 mM) and X-Gal (80 µg ml^{-1}). After overnight incubation at 37 °C the positive clones were selected using the blue/white screening. The white colonies were selected and streaked to purity on LB-Amp + X-gal + IPTG plates and incubated at 37 °C overnight. To confirm the insert, colony PCR of the white colonies were carried out using the vector primers T7 (5′TAATACGACTCACTATAGGG3′) and SP6 (5′GATTTAGGTGACACTATAG3′). White colonies (template) picked from the transformed plate were dispensed into the PCR reaction mix (25 µl) containing 2.5 µl 10× PCR buffer, 2.5 µl 2.5 mM dNTPs, 1 µl 10 pmol μl^{-1} of T7 and SP6 primers, 0.5 U Taq polymerase, and the remaining volume was made up with MilliQ water. The thermal cycling conditions were as follows: 95 °C for 5 min; 35 cycles of 94 °C for 15 s, 57 °C for 20 s, 72 °C for 60 s and a final extension of 72 °C for 10 min.

Plasmids from the positive clones were extracted using the 'GenElute HP' plasmid miniprep kit (Sigma). Plasmids were further screened by restriction digestion using *Eco*R1 enzyme (New England Biolabs) to release the insert. The reaction mix (20 µl) consisted of 2 µl 10× buffer, 5 µl plasmid DNA, 0.5 µl *Eco*R1 enzyme (20,000 U ml^{-1}) and the rest was made up using sterile water (MilliQ). The reaction mix was incubated at 37 °C for 1 h and the enzyme was heat inactivated at 65 °C for 20 min.

2.4. Phylogenetic analyses

Screened clones were sequenced using ABI Prism 3700 Big Dye sequencer at Microsynth AG, Switzerland. After removing