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Genetic and physiological characterization of denitrifying bacteria from brackishwater shrimp culture ponds of India

Nagarajan Dineshkumar, Chinnasamy Saravanakumar, Muthuraman Vasanth, Moturi Muralidhar, Shankar Vinayakrao Alavandi*

Aquatic Animal Health and Environment Division (AAHED), Central Institute of Brackishwater Aquaculture (Indian Council of Agricultural Research), #75 Santhome High Road, R A Puram, Chennai 600 028, India

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ABSTRACT

Denitrifying bacteria in brackishwater shrimp culture pond sediments were isolated and characterized from a total of eight samples. Out of the 264 isolates obtained, only 108 (40.96%) were positive for nitrate reduction and only 14 (12.96%) were positive for both nitrate and nitrite reduction. These fourteen isolates have been characterized based on complete reduction of nitrate to gas, presence of denitrification genes (*nirS*, *nirK* and *nosZ*), reverse transcriptase PCR for *nirS* gene and quantification of nitrous oxide after blocking the nitrous oxide reductase with acetylene. Out of the 14 isolates, nine were identified as *Marinobacter* spp., and the others belonged to *Shewanella* sp., *Aquamicrobium* sp., *Marinimicrobium* sp., *Microbulbifer* sp. and *Janibacter* sp. None of the denitrification genes could be detected in two of the isolates (CDN1 and 12) that reduced both nitrate and nitrite. RT-PCR analysis of all the *Marinobacter* isolates showed that *nirS* gene expression is better under anoxic than under oxic conditions indicating their ability to adapt to varying dissolved oxygen concentration. Results revealed that all the *Marinobacter* isolates are capable of denitrification under oxic, oxygen tolerant and anoxic conditions and indicated that *nirS* gene containing bacteria of the order *Alteromonadales* are one of the predominant denitrifying bacteria in brackishwater shrimp culture ponds of India. The study highlights the significance of such brackishwater ecosystems as study sites for future investigations on distribution and diversity of denitrifying bacteria and their role in the nitrogen cycle.

1. Introduction

Brackishwater aquaculture practices involve growing shrimp in high densities using feeds rich in nitrogen. The biogeochemistry of nitrogen in shrimp culture ponds is dominated by biological transformations of organic and inorganic nitrogen (Hargreaves, 1998). Toxic forms of nitrogen viz. ammonia and nitrite are converted to nitrate and nitrogen gas through nitrification and denitrification processes. Denitrification and anammox are the key microbial processes responsible for the removal of fixed nitrogen from wastewater through the production of dinitrogen (Castine et al., 2012).

Denitrification is a respiratory process in which nitrate and nitrite are converted into gaseous nitrogen intermediates viz. nitric oxide (NO), nitrous oxide (N_2O) and finally nitrogen (N_2) through the action of series of enzymes viz. nitrate reductase (*narG* and *napA* genes), nitrite reductase (*nirS* and *nirK* genes), nitric oxide reductase (*norB* gene) and nitrous oxide reductase (*nosZ* gene) (Philippot, 2002). Denitrification is said to be ubiquitous in most aquatic sediments (Canfield et al., 2005) and is considered an important process of ecological significance since it permanently removes nitrogen from a system that would otherwise be available for primary production. In aquaculture ponds, denitrification takes place in the sediments, due to the presence of anoxic conditions and through degradation of organic matter (Hargreaves, 1998; Gross et al., 2000). Although denitrification has been intensively studied at the process level, less is known on the microbial species composition, distribution, and functional dynamics.

Denitrifying bacteria (DNB) are one of the important groups of bacteria involved in the nitrogen (N) cycle. They are genetically and metabolically diverse with members from almost all phylogenetic groups (Zumft, 1992). Most denitrifiers are aerobic heterotrophic organisms that are more frequent within α and ß subclasses of Proteobacteria and Archaea with a large number from the genera

* Corresponding author. Tel.: +91 44 24618817; fax: +91 44 24610311.
E-mail addresses: svalavandi@yahoo.com, alavandi@ciba.res.in (S.V. Alavandi).

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Pseudomonas and *Bacillus* (Zumft, 1992, 1997). Though denitrification is reported to be the dominant N_2 production pathway through isotope tracer studies in tropical aquaculture settlement ponds (Castine et al., 2012), information on the group of bacteria involved in denitrification in aquaculture ecosystem remains scanty (Abraham et al., 2004). The fact that only a few studies (Song et al., 2011; Cao et al., 2012) on denitrifying bacteria in shrimp culture ponds have been carried out, emphasizes the need for more such studies for better understanding of the bacterial communities involved in denitrification. Hence the present study was conducted to gain a better understanding of the heterotrophic bacterial communities involved in denitrification in shrimp culture ponds. We identified denitrifying bacteria based on their activity *in vitro,* presence of denitrification genes, detection of nitrous oxide and 16S rRNA gene sequence analysis.

2. Materials and methods

2.1. Sampling

Sediment samples ($n = 8$) used for this study were collected during August 2007–September 2009 from brackishwater shrimp culture ponds located in Tamil Nadu and Andhra Pradesh on the East coast and Kerala and Gujarat on the west coast of India. Seven of these farms practiced semi-intensive shrimp culture, while one farm practiced traditional shrimp culture. The culture period in these farms varied from 27 days to 110 days and the salinity ranged from 20 to 25 ppt. Composite core samples of sediments was collected using sterile PVC corers as described by Abraham et al. (2004), in sterile sample containers and were transported to the laboratory in ice box, refrigerated on arrival and processed within 24 h.

2.2. Isolation and characterization

Sediment samples from shrimp culture ponds were serially diluted and plated on Nitrate agar medium (Himedia, India) supplemented with 1.5% NaCl. They were then incubated at 32 °C for 24–48 h. Isolates were randomly selected, purified on nitrate agar and tested for their ability to reduce nitrate and nitrite by inoculating them in 10 ml of sterile nitrate broth (Himedia, India) and BTB-free Giltay nitrite (GN) medium (Matsuzaka et al., 2003) respectively. Both the media were supplemented with 1.5% NaCl and final pH was set to 7.0 ± 0.2. Cultures were incubated in a shaker (120 rpm) at 32 °C for 3 days. Nitrate reduction was tested by adding nitrite reagent (Nitrite Test kit 1.14658.0001, Merck, Germany) to 5 ml of culture broth. Formation of red colour indicated the presence of nitrite, the end product of nitrate reduction. If no nitrite could be detected, the samples were tested for nitrate by addition of zinc dust. Formation of red colour indicated that nitrate is not reduced to nitrite. Isolates that were found positive for nitrate and nitrite reduction were subjected to denitrification tests. Denitrification activity was tested as described by Matsuzaka et al. (2003) using modified GN medium supplemented with 1.5% NaCl and the final pH was adjusted to 7.0 ± 0.2. Each isolate was inoculated into test tubes containing 10 ml of sterile modified GN medium and an inverted Durham tube. Cultures were incubated in a shaker (120 rpm) at 32 °C for 3 days. The ability to carry out denitrification under oxygen tolerant condition was indicated by a change in color of the GN medium from green to blue and the formation of air bubble in the Durham tube.

Isolates that were found positive for both nitrate and nitrite reduction were inoculated into sterile nitrate broth and BTB-free GN medium in Schott bottles and incubated in an orbital shaker (120 rpm) at 32 °C for 12 h. Log phase culture (1 ml, $OD_{600nm} = 0.1$) of the respective isolates in marine broth (Himedia, India) was used to inoculate each bottle. A volume of 5 ml of the culture broth from both media was tested for nitrite using nitrite reagent. Dissolved oxygen (DO) in the culture broth was monitored using a D.O probe (Thermo, Singapore) during 0, 6 and 12 h. Optical density was measured using a UV–Visible spectrophotometer (SmartSpec, Biorad, USA).

2.3. Presence of denitrification genes

All the selected isolates were subjected to PCR for nitrite reductase (*nirS* and *nirK*) and nitrous oxide reductase (*nosZ*) genes. Isolates CDN1-14 were screened using primers nirS1F – nirS6R and nirK1F - nirK5R (Table 2) for *nirS* and *nirK* genes respectively (Braker et al., 1998) following touchdown PCR protocol as described in Nogales et al. (2002). Isolates that were negative for nirS and nirK genes were subjected to an additional round of screening using primers cd3aF – R3cd and F1aCu – R3Cu (Table 2) as described by Throback et al. (2004) and Hallin and Lindgren (1999) respectively.

nosZ gene was screened using three primer combinations viz. Nos661F – Nos1773R, Nos661F – Nos1527R and Nos1527F –

Table 1
Denitrification properties of the isolates from shrimp culture pond sediments.

S.No	Isolate code	Sampling station	Organism	Aerobic			Anaerobic			Presence of denitrification genes			RT-PCR for *nirS* gene		N_2O conc. (ppm) at 24 h after blocking with acetylene
				NO_3 red.	NO_2 red.	Gas Pdn.	NO_3 red.	NO_2 red.	Gas Pdn.	*nirS*	*nirK*	*nosZ*	Aerobic	Anaerobic	
1.	CDN1	Mamallapuram,TN	*Aquamicrobium* sp.	+	+	–	–	–	–	–	–	–	–	–	ND
2.	CDN2	Marakanam,TN	*Marinobacter* sp	+	+	+	+	+	+	+	–	+	+	+	3.42×10^2
3.	CDN3	Nellore, AP	*Marinobacter* sp.	+	+	+	+	+	+	+	–	+	+	+	7.78×10^1
4.	CDN4	Bapatla, AP	*Marinobacter* sp	+	+	+	+	+	+	+	–	+	+	+	9.41×10^2
5.	CDN5	Nagapattinam, TN	*Marinobacter* sp.	+	+	+	+	+	+	+	–	+	+	+	5.97×10^2
6.	CDN6	Marakanam,TN	*Marinobacter* sp.	+	+	+	+	+	+	+	–	+	+	+	57.2×10^{-1}
7.	CDN7	Cuddalore, TN	*Marinobacter* sp.	+	+	+	+	+	+	+	–	+	+	+	91.7×10^{-1}
8.	CDN8	Cuddalore, TN	*Marinobacter* sp.	+	+	+	+	+	+	+	–	+	+	+	1.94×10^3
9.	CDN9	Cochin, Kerala	*Marinimicrobium* sp.	+	+	–	–	–	–	–	+	+	ND	ND	ND
10.	CDN10	Valsad, GJ	*Marinobacter* sp.	+	+	+	+	+	+	+	–	+	+	+	4.22×10^2
11.	CDN11	Valsad, GJ	*Marinobacter* sp.	+	+	+	–	–	–	+	–	+	+	+	8.28×10^{-1}
12.	CDN12	Marakanam,TN	*Microbulbifer* sp.	+	+	–	–	–	–	–	–	–	+	–	ND
13.	CDN13	Mamallapuram, TN	*Janibacter* sp.	+	+	–	–	–	–	–	–	+	ND	ND	ND
14.	CDN14	Valsad, GJ	*Shewanella* sp.	+[a]	–	–	+	+	+	+	–	+	–	+	2.38×10^4

ND-not determined.
[a] Poor growth.