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

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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.

Table 2
Primers used for screening nirS, nirK, nosZ and 16S rRNA genes in this study.

Gene	Primer	Sequence (5′_ to 3′_)	Reference
nirS	nirS1F	CCTAYTGGCCGCCRCART	Braker et al. (1998)
	nirS6R	CGTTGAACTTRCCGGT	
	cd3aF	GTS AAC GTS AAG GAR ACS GG	Michotey et al. (2000)
	R3cd	GAS TTC GGR TGS GTC TTG A	Throback et al. (2004)
nosZ	Nos661F	CGGCTGGGGGCTGACCAA	Scala and Kerkhof (1999)
	Nos1773R	ATRTCGATCARCTGBTCGTT	
	Nos1527F	CGCTGTTCHTCGACAGYCA	
	Nos1527R	CTGRCTGTCGADGAACAG	
nirK	nirK1F	GGMATGGTKCCSTGGCA	Braker et al. (1998)
	nirK5R	GCCTCGATCAGRTTRTGG	
	F1aCu	ATCATGGTSCTGCCGCG	Hallin and Lindgren (1999)
	R3Cu	GCCTCGATCAGRTTGTGGTT	
16S rRNA	fD1	AGAGTTTGATCCTGGCTCA (positions 7–26 in *Escherichia coli*)	Weisburg et al., 1991
	rP2	ACGGCTACCTTGTTACGACTT (positions 1513–1494 in *Escherichia coli*)	

Nos1773R (Table 2) as described by Scala and Kerkhof (1999) with an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 95 °C for 0.5 min, 56 °C for 1 min, 72 °C for 1.5 min and a final extension at 72 °C for 10 min. Amplification was performed in an iCycler (Bio-Rad, USA) using 25 μl 1 × Taq Master Mix Red (Ampliqon, Denmark) containing 50 ng of each primer and 25–50 ng of template. A 5-μl aliquot of each amplification product was electrophoresed on a 1% agarose gel in 0.5X TBE buffer at 50 V for 45 min, stained with ethidium bromide, and the PCR products were visualized using gel documentation system (Bio-Rad, USA).

2.4. Reverse transcriptase (RT)-PCR for nirS gene

All the isolates were grown in test tubes containing 10 ml of nitrate broth supplemented with 1.5% NaCl with inverted Durham tubes. One set of the inoculated tubes was incubated in an orbital shaker (120 rpm) (Gallenkamp, Germany) at 32 °C and the other set was incubated under anaerobic conditions in a 3.5 L Anaerobic System Mark III (Himedia, India). Anaerobic conditions were maintained using Anaerogas Pack 3.5 L (Himedia, India) and absence of oxygen was confirmed using the Anaero Indicator tablets (Himedia, India). From these aerobic and anaerobic cultures, 1.0 ml was withdrawn at 12 and 24 h and total RNA was extracted using Ez-10 Total RNA Minipreps Super kit (Biobasic, Canada) according to the manufacturer's instructions. The total RNA was treated with DNAse I (NEB, USA) to remove DNA contamination and purified by a phenol-chloroform extraction. The purified total RNA aliquot was then subjected to PCR with primers nirS1F and nirS6R using 1 × Taq Master Mix Red (without reverse transcriptase) to check for DNA contamination. First strand cDNA was synthesized using iScript cDNA synthesis kit (BIO-RAD, USA) using random primers according to the manufacturer's instructions. The first strand cDNA thus synthesized, was used as the template for PCR amplification of *nirS* gene using primers (Braker et al., 1998) following touchdown PCR protocol as described in Nogales et al. (2002).

2.5. Identification and phylogenetic analysis

All the isolates were subjected to 16S *rRNA* gene sequence analysis for identification. Genomic DNA was extracted from 1 ml of overnight culture in Zobell marine broth using DNA express kit (Himedia, India) as per the manufacturer's instructions. Nearly full length sequences of 16S rRNA gene were amplified using universal primers fD1 and rP2 (Weisburg et al., 1991) (Table 2). Amplification was performed in 50 μl 1 × Taq Master Mix Red (Ampliqon, Denmark) with 25–50 ng of template DNA, 50 ng of each of the primers using an iCycler (BioRad, USA) with an initial denaturation at 94 °C for 4 min followed by 30 cycles of 94 °C for 1 min, 57 °C for 1 min, 72 °C for 1.5 min and a final extension at 72 °C for 7 min. Amplified products were electrophoresed on a 1.5% agarose gel in 0.5 × TBE buffer at 50 V for 45 min, stained with ethidium bromide and visualized using a Gel Documentation system (BioRad, USA) and purified using HiYield PCR Clean-up kit (RBC, Taiwan) and sequenced using automated DNA sequencer (*1st Base*, Malaysia).

The 16S ribosomal RNA gene sequences of the isolates thus obtained were checked for anomalies using Pintail program (Ashelford et al., 2005). They were aligned using SILVA Incremental Aligner (SINA version 1.2.11) (Pruesse et al., 2012) to the sequences retrieved from the Ribosomal Database Project (Cole et al., 2009). Phylogeny was inferred using neighbor-joining algorithm in the MEGA5 software package (Tamura et al., 2011) with 1000 bootstrap replications after complete deletion of gaps and missing data.

2.6. Nitrous oxide (N_2O) measurements

All the isolates were inoculated into sterile 20 ml glass vials with 10 ml of sterile nitrate broth containing 10 mM KNO_3 (Himedia, India) supplemented with 1.5% NaCl and 0.85% sodium citrate. The vials were then closed with sterile butyl septum and sealed using aluminium crimp. Headspace was flushed with high purity nitrogen for 3 min and replaced with acetylene to inhibit reduction of N_2O to dinitrogen as described by Falk et al. (2010). The vials were incubated at 32 °C in an orbital shaker at 120 rpm for 24 h and subjected to gas chromatography (7890A system, Agilent Technologies, USA) using head space analysis (G1888 network headspace sampler, Agilent Technologies, Italy). N_2O was separated using 1/8 inch stainless steel HayeSep Q column (Agilent Technologies, USA) and detected by micro-electron capture detector (μ-ECD) using 5% methane in Argon as make up gas at a flow rate of 2 ml min^{-1}. Column oven and μ-ECD detector were maintained at 60 °C and 300 °C respectively. The calibration gas mixture of 1 ppm N_2O in nitrogen with analytical accuracy of ±2%, traceable to NIST weights were obtained from Agilent Technologies, USA and nitrous oxide was quantified using gas standard analysis.

2.7. Nucleotide sequence accession numbers

The 16S rRNA gene sequences of isolates CDN1-CDN14 were deposited in NCBI Genbank with the following accession numbers HQ671195 – HQ671204, HQ693231 – HQ693234.

3. Results

3.1. Isolation and characterization of DNB

A total of 264 isolates were obtained from the 8 samples processed. Out of these isolates, 108 (40.9%) were found positive for nitrate reduction and only 14 (12.96%) were positive for both nitrate and nitrite reduction. These 14 isolates were subjected to physiological and molecular characterization. All the 14 isolates were characterized for denitrification properties (nitrate and nitrite reduction to gas) under oxic and anoxic conditions and screened for the presence of *nirS*, *nirK* and *nosZ* genes using PCR. Though all the isolates (CDN1-14) were positive for both nitrate and nitrite reduction, only 11 were (CDN2-8, 10, 11 and 14) positive for gas production (air bubbles in Durham tube). While other isolates produced gas under both oxic and anoxic conditions, CDN14 was

found to produce gas only under anoxic conditions. In CDN2-8, 10, 11 and 14, no gas was observed in the initial experiments under anoxic conditions, however inoculating the tubes with double the size of the inoculum resulted in gas production. In isolates CDN2-8, 10 and 11, nitrate and nitrite reduction was comparatively more rapid under oxic conditions than anoxic conditions except CDN14 that was found efficient only under anoxic conditions. In nitrate broth, CDN14 exhibited slow growth under oxic conditions with negligible nitrite production only inside the Durham tube indicating preference for oxygen-limited conditions.

Isolates CDN2-8, 10 and 11, completely reduced nitrate to gas in less than 12 h under oxic conditions and in 24 h under anoxic conditions. CDN 14 took 24 h for complete reduction of nitrate to gas under anoxic conditions. For all the *Marinobacter* isolates, DO was found to decrease (7.5 mg L^{-1} to almost 0 mg L^{-1}) during complete reduction of nitrate to gas in 12 h with concomitant increase in the biomass (Fig. 1). Experiments in nitrate broth showed that CDN2-8, 10, 11 and 14 are capable of complete reduction of nitrate to gas but isolates CDN1, 9, 12 and 13 could only reduce nitrate to nitrite but not gas. Further reduction of nitrite did not occur even after 120 h of incubation but addition of C source (citrate or pyruvate) resulted in complete reduction of nitrite in less than 3 days. Though all the 14 isolates reduced nitrate and nitrite, only CDN2-8, 10 and 11 were positive for denitrification properties under oxygen-tolerant conditions (i,e. media turned green to blue with gas production in the Durham tube). CDN14 exhibited denitrification activity only under anoxic conditions. Isolates that effected no change in the colour of the media even after 120 h of incubation were considered negative for denitrification.

3.2. Presence of denitrification genes and RT-PCR for nirS gene

PCR analysis (Table 1) showed that *nirS* gene could be detected in all the isolates except CDN1, 9, 12 and 13. Of all the isolates, only CDN9 was found to possess *nirK* gene. All the isolates were positive for *nosZ* gene, except CDN1 and 12. Though the isolates CDN1 and 12 were found to reduce both nitrate and nitrite, none of the denitrification genes (*nirS, nirK* and *nosZ*) could be detected even after screening with additional primer combinations. Isolates that were positive for *nirS* gene were subjected to reverse transcriptase PCR to check *nirS* gene expression. Isolates CDN2-8, 10 and 11 were found positive for *nirS* gene expression (Fig. 2) under both oxic and anoxic conditions except CDN14 that was found to express *nirS* gene only under anoxic conditions.

3.3. Identification and phylogenetic analysis

Isolates CDN1-CDN14 were identified based on the similarity of the 16S rRNA gene sequences. Out of the 14 isolates, 10 (CDN2-8, 10 and 11) were identified as *Marinobacter* spp. with 97–99% similarity to the sequences available in the NCBI database. Isolates CDN1, 9, 12 and 13 were identified as *Aquamicrobium* sp., *Marinimicrobium* sp., *Janibacter* sp. and *Shewanella* sp. respectively. The 16S *rRNA* gene sequence based phylogenetic tree (Fig. 3) constructed with other validly described denitrifying and nitrate reducing bacteria formed 6 distinct clusters comprising *Marinobacter* spp., *Microbulbifer spp., Shewanella* spp., *Marinimicrobium* spp., *Aquamicrobium* spp., and *Janibacter* spp.. The tree showed that all the isolates belonged to the phyla Proteobacteria except *Janibacter* sp. that belonged to the phylum Actinobacteria. While all the other *Marinobacter* isolates formed a monophyletic cluster with *Marinobacter hydrocarbonoclasticus* (T), CDN11 formed a similar cluster with *Marinobacter lipolyticus* (T). Also the tree showed that CDN 12 (*Microbulbifer* sp.) formed a separate lineage within the *Microbulbifer* group. All other isolates CDN1, 9, 13 and 14 formed monophyletic clusters with their respective type strains.

3.4. N_2O measurements

All the *Marinobacter* isolates were subjected to N_2O measurements after blocking nitrous oxide reductase with acetylene to confirm denitrification (Table 1). N_2O produced by the isolates ranged between 8.28×10^{-1} to 2.38×10^{4} ppm. While a few of the isolates produced higher amounts of N_2O, five isolates produced lower amount of N_2O between 8.28×10^{-1} and 7.78×10^{1} ppm.

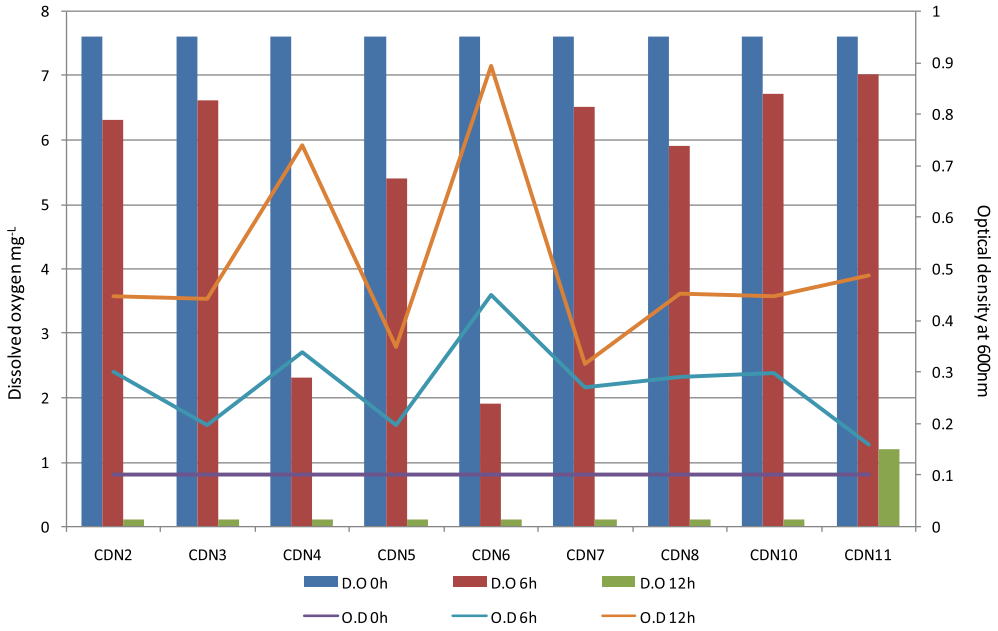
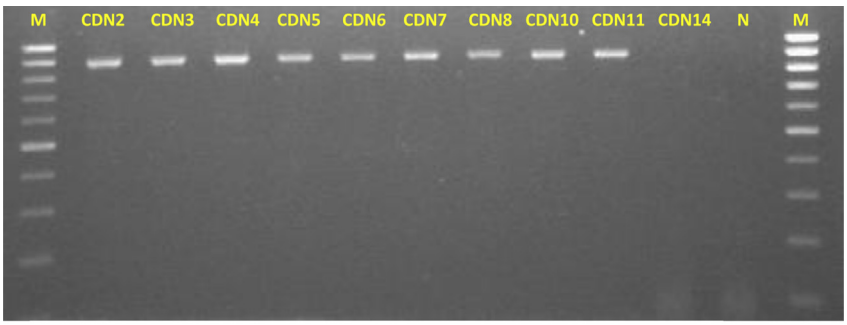
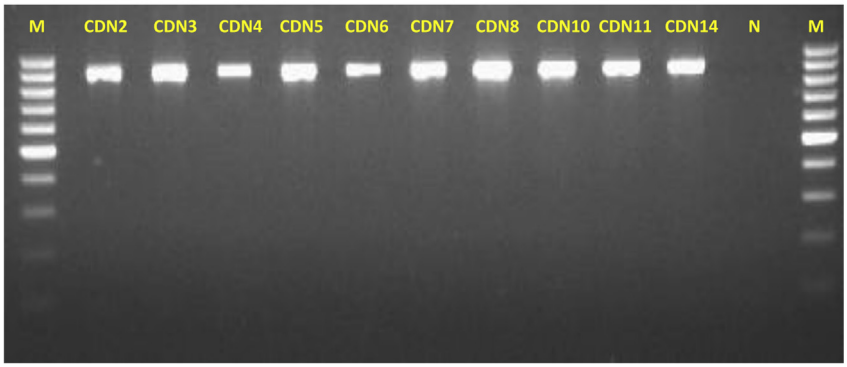


Fig. 1. Biomass and dissolved oxygen levels in broth culture of *Marinobacter* isolates during denitrification. Dissolved oxygen levels (Left axis) and biomass (Optical density units-Right axis) during denitrification under oxic conditions (shake culture) by *Marinobacter* spp. Data shown is an average of experiments conducted in triplicates.



a. *nirS* gene expression under oxic conditions



b. *nirS* gene expression under anoxic conditions

Fig. 2. Reverse transcriptase PCR for *nirS* gene. Fig. 2a *nirS* gene expression under oxic conditions. Fig. 2b *nirS* gene expression under anoxic conditions M-100 bp DNA ladder N-negative control Isolates CDN2-8, 10, 11 expressed *nirS* gene under oxic and anoxic conditions CDN14 expressed *nirS* gene only under anoxic conditions.

4. Discussion

With nitrite reduction being considered the key step in denitrification to distinguish true denitrifiers from nitrate reducers (Braker et al., 2012), only 12.96% of the isolates from this study were found to reduce both nitrate and nitrite. Out of the 14 DNB isolated and characterized for denitrification properties from brackishwater shrimp culture ponds, 9 isolates with considerable difference in their ability to denitrify were identified as *Marinobacter* spp.. While most of the isolates belonged to the order *Alteromonadales*, a few belonged to the order *Rhizobiales* and *Actinomycetales*. It should be noted that members of the family *Alteromonadaceae* and other *Alteromonas*-like Proteobacteria usually do not denitrify (Ivanova et al., 2004) except a few (Jean et al., 2006; Lin and Shieh, 2006). While *Marinobacter* spp. isolated from different ecosystems are reported to carry out denitrification (Gauthier et al., 1992; Gorshkova et al., 2003; Shivaji et al., 2005; Yoshie et al., 2006) some are reported to degrade hydrocarbons under denitrifying conditions (Gauthier et al., 1992; Lu et al., 2011). Though the occurrence of *Marinobacter hydrocarbonoclasticus* in shrimp culture ponds has been reported using a culture independent approach (Krishnani, 2010) based on the *nosZ* gene sequence similarity, efforts to isolate and characterize such denitrifying bacteria remains limited. Our study provides the proof of experimental verification of denitrification in *Marinobacter* spp..

In the pond environment, dissolved oxygen level in pond water is one of the most important limiting factors for shrimp growth and survival. In semi-intensive shrimp culture ponds, shrimps account for 10–15% ($\sim$ 0.01–0.16 mg O_2 L^{-1} h^{-1}) of the total pond respiration (Fast and Boyd, 1992), with the bacterial decomposition of organic matter, consuming significant part of the dissolved oxygen (Avnimelech and Ritvo, 2003), phytoplanktons were also considered a major consumer of DO in ponds (Garcia and Brune, 1991). Such conditions favour denitrification that offers a unique advantage over nitrification in that it can completely remove nitrogen from the system.

In our experiments with *Marinobacter* spp. under oxic conditions, the bacterial biomass was found to increase creating oxygen-limited conditions thereby inducing denitrification. However, higher biomass was recorded under oxic than anoxic conditions (Fig. 1) owing to the fact that aerobic respiration is a high energy yielding process. Being a complete denitrifier and facultative aerobe (Sanford et al., 2012), *Marinobacter* isolates from this study were found to exhibit denitrification properties under oxic, anoxic and oxygen tolerant conditions. Though, RT-PCR revealed that nirS gene expression is better under anoxic than under oxic conditions, it indicated that they are highly adaptable to varying dissolved oxygen concentration. Under such conditions, use of *Marinobacter* for pond water quality management would be an advantage over other bacteria for active cycling of nitrate and nitrite through denitrification. Such isolates could also be exploited for formulation of microbial products or can be used in biofilters for *in situ* or *ex situ* water quality management in hatchery and pond environment.

Rapid denitrification in *Marinobacter* isolates were observed as indicated by nitrous oxide buildup in the headspace in 24 h compared to the longer incubation period (48 h) required for *Shewanella* sp. In isolates CDN1, 9, 12 and 13, experiments conducted in nitrate broth supplemented with citrate or pyruvate showed that complete reduction of nitrate occurred in less than 3 days. Also in these isolates, nitrite reduction was found to be affected in the absence of carbon source but not by the availability of oxygen showing that they are capable of denitrification under oxygen tolerant conditions. The requirement of carbon source for isolates CDN1, 9, 12 and 13 to reduce nitrite to gas suggests that these

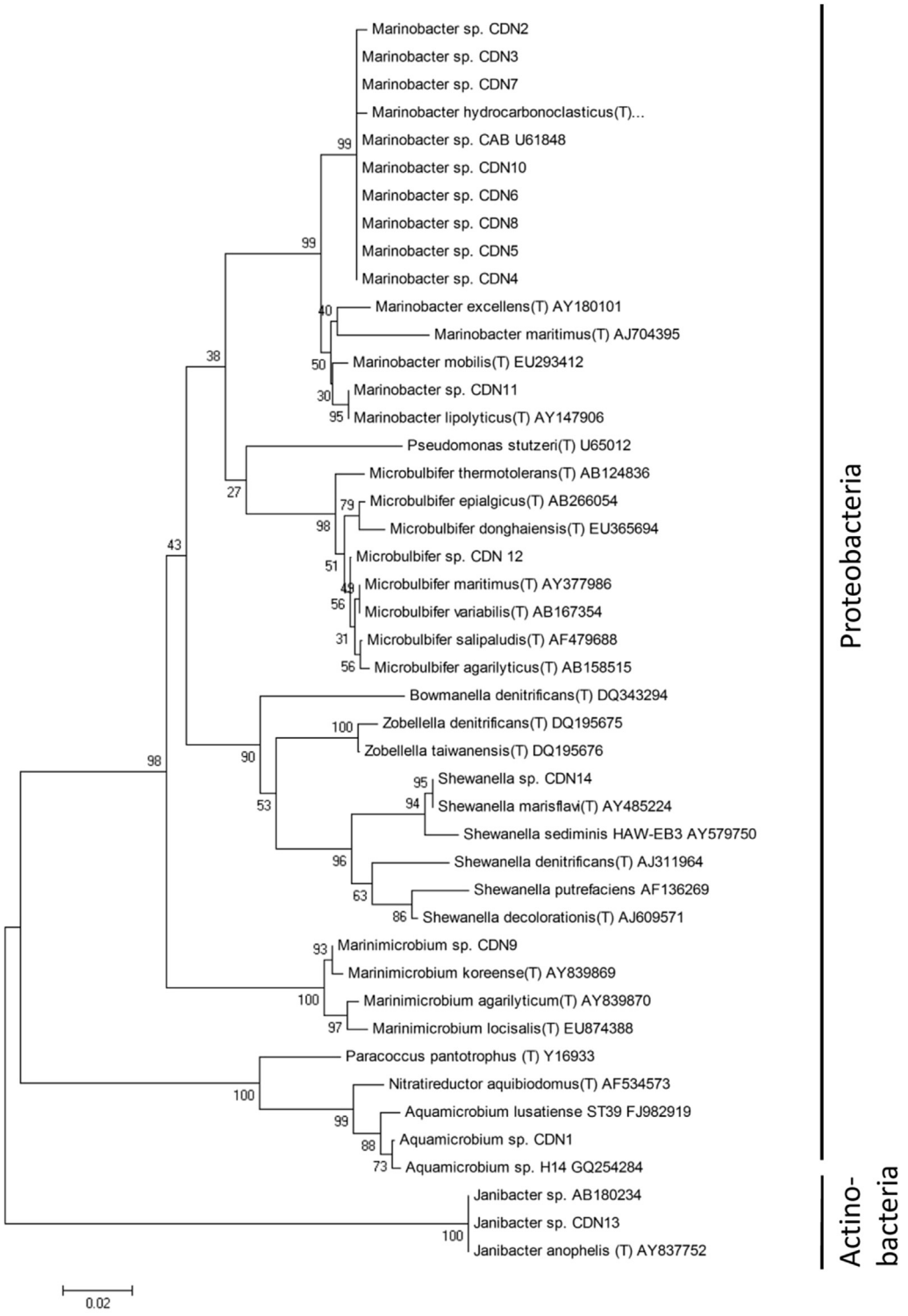


Fig. 3. Phylogenetic tree of denitrifying bacteria from shrimp culture ponds.

isolates could probably be mixotrophs. Though stoichiometric conversion of NO_3^- or NO_2^- to N_2O and N_2 could not be established, the isolates CDN2-8, 10, 11 and 14 characterized for their denitrification properties met the criteria for respiratory denitrifiers proposed by Mahne and Tiedje (1995).

Isolate CDN 14 identified as *Shewanella* sp. was found capable of denitrification under anoxic conditions and nitrate reduction under oxic conditions similar to the respiratory denitrifier *Shewanella putrefaciens* (Krause and Nealson, 1997). *Shewanella* spp. is a complete denitrifier (Sanford et al., 2012) and several strains of *Shewanella* viz. *Shewanella denitrificans*, *Shewanella sediminis* and *Shewanella decolorationis* are capable of denitrification (Brettar et al., 2001, 2002; Xu et al., 2005; Zhao et al., 2005). So far *Aquamicrobium* sp., *Marinimicrobium* sp., *Microbulbifer* sp. and *Janibacter* sp. identified in the present study have not been reported to reduce both nitrate and nitrite.

While most of the *Microbulbifer* strains are reported to reduce nitrate (Wang et al., 2009; Zhang et al., 2012) and a few can degrade phenol under denitrifying conditions (Sueoka et al., 2009) but the isolate CDN12 was found to reduce both nitrate and nitrite though it was found negative for *nirS* and *nosZ* genes. Phylogenetic analysis suggests that the isolate CDN12 could probably form a novel species within the genus *Microbulbifer*.

Though *Pseudomonas* species has been widely reported to carry out denitrification under oxic conditions (Su et al., 2001; Takaya et al., 2003; Chen et al., 2006; Kim et al., 2008; Miyahara et al., 2010), none of the isolates recovered in this study belonged to the genus *Pseudomonas* supporting previous reports that *Pseudomonads* does not seem to be an important group in pelagic marine environment (Fuhrman et al., 1993; Giovannoni and Rappe, 2000; Brettar et al., 2001). Also, this could be possible that the media employed in this study may not be suitable for isolation of denitrifying bacteria from diverse phyla and hence it is not surprising that many isolates of *Marinobacter* species were obtained. However, analysis of three heterotrophic nitrifying isolates of *Pseudomonas* spp. that were isolated from shrimp culture ponds and maintained in our laboratory revealed that they all harbored *nirS* and *nosZ* genes but no gas production could be observed (data not shown). Though the commonly reported denitrifiers could not be isolated in this study, the methodology employed in this study was found suitable for isolating aerobic and oxygen-tolerant denitrifying bacteria.

Isolates that were found positive for both nitrate and nitrite reduction with gas production in Durham tubes possessed *nirS* and *nosZ* genes and have been confirmed as denitrifiers based on RT-PCR for *nirS* gene and acetylene blocking assay. This indicates that these tests can be used to select presumptive denitrifying isolates for further study. Despite being positive for nitrate and nitrite reduction, none of the denitrification genes could be detected in CDN 1, indicating that it may possess genes that even the commonly used primers could not detect (Throback et al., 2004; Henry et al., 2006). Also, the isolate (CDN9) that was found to possess a *nirK* type nitrite reductase gene is from a traditional shrimp culture pond that unlike other intensive culture ponds did not receive any commercial microbial formulations for enhancing water quality. More studies focused on traditional shrimp culture ponds would reveal the autochthonous micro flora that are involved in denitrification.

Commercially available microbial products for water quality management in shrimp culture ponds are mainly composed of organic matter degrading bacteria and nitrifying bacteria (Antony and Philip, 2006) and less attention is given for the use of denitrifying bacteria. It should be noted that the microbial cultures used in marine denitrification reactors are commonly derived and adapted from freshwater-activated sludge (Borges et al., 2008). Hence, further studies in this regard may offer efficient indigenous strains for use in brackishwater aquaculture systems.

In conclusion, we provide the first report on the isolation and thorough characterization of denitrifying bacteria from shrimp culture pond sediments. The genotypic characteristics of some of the denitrifying isolates recovered in the present study show that there exist a diverse group of heterotrophic bacteria with genes yet to be characterized, that are involved in denitrification. Moreover studies on the diversity and physiology of such denitrifying bacteria may lead to identification of novel bacteria and help in understanding the ecological significance of denitrifying bacteria in shrimp culture pond sediments. Our results demonstrate the ability of the *Marinobacter* isolates to carry out denitrification under oxic, oxygen tolerant and anoxic conditions. Most of the denitrifying isolates harbored *nirS* gene and belonged to the order *Alteromonadales*. The study has showed that simple experiments can be used to check denitrification properties along with RT-PCR to confirm *nirS* gene expression. Though the observed diversity in this study might be an underestimate of the existing denitrifier communities, large scale analysis of such ecosystems would help resolve the same. Without characterizing the distribution and abundance of these isolates, their role in ecosystem functioning remains unknown and hence future research through application of culture-independent molecular techniques will focus on finding their *in situ* importance and whether they really represent the denitrifying members in shrimp culture ponds.

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References

Abraham, T.J., Ghosh, S., Nagesh, T.S., Sasmal, D., 2004. Distribution of bacteria involved in nitrogen and sulphur cycles in shrimp culture systems of West Bengal, India. Aquaculture 239, 275–288.

Antony, S.P., Philip, R., 2006. Bioremediation in shrimp culture systems. Naga WorldFish Cent. Q. 29, 62–66.

Ashelford, K.E., Chuzhanova, N.A., Fry, J.C., Jones, A.J., Weightman, A.J., 2005. At least 1 in 20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. Appl. Environ. Microbiol. 71, 7724–7736.

Avnimelech, Y., Ritvo, G., 2003. Shrimp and fish pond soils: processes and management. Aquaculture 220, 549–567.

Borges, M.T., Sousa, A., De Marco, P., Matos, A., Honigova, P., Castro, P.M.L., 2008. Aerobic and anoxic growth and nitrate removal capacity of a marine denitrifying bacterium isolated from a recirculation aquaculture system. Microb. Ecol. 55, 107–118.

Braker, G., Dorsch, P., Bakken, L.R., 2012. Genetic characterization of denitrifier communities with contrasting intrinsic functional traits. FEMS Microbiol. Ecol. 79, 542–554.

Braker, G., Fesefeldt, A., Witzel, K.P., 1998. Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. Appl. Environ. Microbiol. 64, 3769–3775.

Brettar, I., Christen, R., Hofle, M.G., 2002. *Shewanella denitrificans* sp. nov., a vigorously denitrifying bacterium isolated from the oxic-anoxic interface of the Gotland Deep in the central Baltic Sea. Int. J. Syst. Evol. Microbiol. 52, 2211–2217.

Brettar, I., Moore, E.R.B., Hofle, M.G., 2001. Phylogeny and abundance of novel denitrifying bacteria isolated from the water column of the central Baltic Sea. Microb. Ecol. 42, 295–305.

Canfield, D.E., Kristensen, E., Thamdrup, B., 2005. Aquatic Geomicrobiology. Academic Press.

Cao, H., Wang, H., He, S., Ou, R., Hou, S., Yang, X., 2012. Isolation and characterization of a denitrifying *Acinetobacter baumannii* H1 using NO_2-N as nitrogen source from shrimp farming ponds. Afr. J. Microbiol. Res. 6, 2258–2264.

Castine, S.A., Erler, D.V., Trott, L.A., Paul, N.A., De Nys, R., Eyre, B.D., 2012. Denitrification and anammox in tropical aquaculture settlement ponds: an isotope tracer approach for Evaluating N_2 production. PloS one 7, e42810.

Chen, F., Xia, Q., Ju, L.K., 2006. Competition between oxygen and nitrate respirations in continuous culture of *Pseudomonas aeruginosa* performing aerobic denitrification. Biotechnol. Bioeng. 93, 1069–1078.

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., et al., 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. 37, D141–D145.

Falk, S., Liu, B., Braker, G., 2010. Isolation, genetic and functional characterization of novel soil *nirK*-type denitrifiers. Syst. Appl. Microbiol. 33, 337–347.

Fast, A., Boyd, C., 1992. Water circulation, aeration and other management practices. In: Fast, A., Lester, J. (Eds.), Marine Shrimp Culture: Principles and Practices. Elsevier Science Publishers, Amsterdam, pp. 457–495.

Fuhrman, J.A., McCallum, K., Davis, A.A., 1993. Phylogenetic diversity of subsurface marine microbial communities from the Atlantic and Pacific Oceans. Appl. Environ. Microbiol. 59, 1294–1302.

Garcia, A., Brune, D.E., 1991. Transport limitation of oxygen in shrimp culture ponds. Aquac. Eng. 10, 269–279.

Gauthier, M.J., Lafay, B.n.d., Christen, R., Fernandez, L., Acquaviva, M., Bonin, P., Bertrand, J.C., 1992. *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new, extremely halotolerant, hydrocarbon-degrading marine bacterium. Int. J. Syst. Evol. Microbiol. 42, 568–576.

Giovannoni, S., Rappe, M.S., 2000. Evolution, diversity and molecular ecology of marine prokaryotes. In: Kirchman, D.L. (Ed.), Microbial Ecology of the Oceans. Wiley, New York, pp. 47–84.
Gorshkova, N.M., Ivanova, E.P., Sergeev, A.F., Zhukova, N.V., Alexeeva, Y., Wright, J.P., Nicolau, D.V., Mikhailov, V.V., Christen, R., 2003. *Marinobacter excellens* sp. nov., isolated from sediments of the Sea of Japan. Int. J. Syst. Evol. Microbiol. 53, 2073–2078.
Gross, A., Boyd, C.E., Wood, C.W., 2000. Nitrogen transformations and balance in channel catfish ponds. Aquac. Eng. 24, 1–14.
Hallin, S., Lindgren, P.-E., 1999. PCR detection of genes encoding nitrite reductase in denitrifying bacteria. Appl. Environ. Microbiol. 65, 1652–1657.
Hargreaves, J.A., 1998. Nitrogen biogeochemistry of aquaculture ponds. Aquaculture 166, 181–212.
Henry, S., Bru, D., Stres, B., Hallet, S., Philippot, L., 2006. Quantitative detection of the *nosZ* gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, *narG*, *nirK*, and *nosZ* genes in soils. Appl. Environ. Microbiol. 72, 5181–5189.
Ivanova, E.P., Flavier, S.b., Christen, R., 2004. Phylogenetic relationships among marine Alteromonas-like proteobacteria: emended description of the family *Alteromonadaceae* and proposal of *Pseudoalteromonadaceae* fam. nov., *Colwelliaceae* fam. nov., *Shewanellaceae* fam. nov., *Moritellaceae* fam. nov., *Ferrimonadaceae* fam. nov., *Idiomarinaceae* fam. nov. and *Psychromonadaceae* fam. nov. Int. J. Syst. Evol. Microbiol. 54, 1773–1788.
Jean, W.D., Chen, J.S., Lin, Y.T., Shieh, W.Y., 2006. *Bowmanella denitrificans* gen. nov., sp. nov., a denitrifying bacterium isolated from seawater from An-Ping Harbour, Taiwan. Int. J. Syst. Evol. Microbiol. 56, 2463–2467.
Kim, M., Jeong, S.Y., Yoon, S.J., Cho, S.J., Kim, Y.H., Kim, M.J., Ryu, E.Y., Lee, S.J., 2008. Aerobic denitrification of *Pseudomonas putida* AD-21 at different C/N Ratios. J. Biosci. Bioeng. 106, 498–502.
Krause, B., Nealson, K.H., 1997. Physiology and enzymology involved in denitrification by *Shewanella putrefaciens*. Appl. Environ. Microbiol. 63, 2613–2618.
Krishnani, K.K., 2010. Detection and diversity of nitrifying and denitrifying functional genes in coastal aquaculture. Aquaculture 302, 57–70.
Lin, Y.T., Shieh, W.Y., 2006. *Zobellella denitrificans* gen. nov., sp. nov. and *Zobellella taiwanensis* sp. nov., denitrifying bacteria capable of fermentative metabolism. Int. J. Syst. Evol. Microbiol. 56, 1209–1215.
Lu, X., Zhang, T., Han-Ping Fang, H., Leung, K.M.Y., Zhang, G., 2011. Biodegradation of naphthalene by enriched marine denitrifying bacteria. Int. Biodeterior. Biodegradation 65, 204–211.
Mahne, I., Tiedje, J.M., 1995. Criteria and methodology for identifying respiratory denitrifiers. Appl. Environ. Microbiol. 61, 1110–1115.
Matsuzaka, E., Nomura, N., Nakajima-Kambe, T., Okada, N., Nakahara, T., 2003. A simple screening procedure for heterotrophic nitrifying bacteria with oxygen-tolerant denitrification activity. J. Biosci. Bioeng. 95, 409–411.
Michotey, V., Méjean, V., Bonin, P., 2000. Comparison of methods for quantification of cytochrome cd 1-denitrifying bacteria in environmental marine samples. Appl. Environ. Microbiol. 66, 1564–1571.
Miyahara, M., Kim, S.W., Fushinobu, S., Takaki, K., Yamada, T., Watanabe, A., Miyauchi, K., Endo, G., Wakagi, T., Shoun, H., 2010. Potential of aerobic denitrification by *Pseudomonas stutzeri* TR2 to reduce nitrous oxide emissions from wastewater treatment plants. Appl. Environ. Microbiol. 76, 4619–4625.
Nogales, B., Timmis, K.N., Nedwell, D.B., Osborn, A.M., 2002. Detection and diversity of expressed denitrification genes in estuarine sediments after reverse transcription-PCR amplification from mRNA. Appl. Environ. Microbiol. 68, 5017–5025.
Philippot, L., 2002. Denitrifying genes in bacterial and archaeal genomes. Biochimi. Biophys. Acta (BBA)-Gene Struct. Expr. 1577, 355–376.
Pruesse, E., Peplies, J.R., Glockner, F.O., 2012. SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. Bioinformatics 28, 1823–1829.
Sanford, R.A., Wagner, D.D., Wu, Q., Chee-Sanford, J.C., Thomas, S.H., Cruz-Garcia, C., Rodriguez, G., Massol-Deya, A., et al., 2012. Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. Proc. Natl. Acad. Sci. USA 109, 19709–19714.
Scala, D.J., Kerkhof, L.J., 1999. Diversity of nitrous oxide reductase (*nosZ*) genes in continental shelf sediments. Appl. Environ. Microbiol. 65, 1681–1687.
Shivaji, S., Gupta, P., Chaturvedi, P., Suresh, K., Delille, D., 2005. *Marinobacter maritimus* sp. nov., a psychrotolerant strain isolated from sea water off the subantarctic Kerguelen islands. Int. J. Syst. Evol. Microbiol. 55, 1453–1456.
Song, Z.F., An, J., Fu, G.H., Yang, X.L., 2011. Isolation and characterization of an aerobic denitrifying *Bacillus* sp. YX-6 from shrimp culture ponds. Aquaculture 319, 188–193.
Su, J.J., Liu, B.Y., Chang, Y.C., 2001. Identifying an interfering factor on chemical oxygen demand (COD) determination in piggery wastewater and eliminating the factor by an indigenous *Pseudomonas stutzeri* strain. Lett. Appl. Microbiol. 33, 440–444.
Sueoka, K., Satoh, H., Onuki, M., Mino, T., 2009. Microorganisms involved in anaerobic phenol degradation in the treatment of synthetic coke-oven wastewater detected by RNA stable-isotope probing. FEMS Microbiol. Lett. 291, 169–174.
Takaya, N., Catalan-Sakairi, M.A.B., Sakaguchi, Y., Kato, I., Zhou, Z., Shoun, H., 2003. Aerobic denitrifying bacteria that produce low levels of nitrous oxide. Appl. Environ. Microbiol. 69, 3152–3157.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.
Throback, I.N., Enwall, K., Jarvis, A., Hallin, S., 2004. Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiol. Ecol. 49, 401–417.
Wang, C.S., Wang, Y., Xu, X.W., Zhang, D.S., Wu, Y.H., Wu, M., 2009. *Microbulbifer donghaiensis* sp. nov., isolated from marine sediment of the East China Sea. Int. J. Syst. Evol. Microbiol. 59, 545–549.
Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697–703.
Xu, M., Guo, J., Cen, Y., Zhong, X., Cao, W., Sun, G., 2005. *Shewanella decolorationis* sp. nov., a dye-decolorizing bacterium isolated from activated sludge of a wastewater treatment plant. Int. J. Syst. Evol. Microbiol. 55, 363–368.
Yoshie, S., Ogawa, T., Makino, H., Hirosawa, H., Tsuneda, S., Hirata, A., 2006. Characteristics of bacteria showing high denitrification activity in saline wastewater. Lett. Appl. Microbiol. 42, 277–283.
Zhang, D.S., Huo, Y.Y., Xu, X.W., Wu, Y.H., Wang, C.S., Xu, X.F., Wu, M., 2012. *Microbulbifer marinus* sp. nov. and *Microbulbifer yueqingensis* sp. nov., isolated from marine sediment. Int. J. Syst. Evol. Microbiol. 62, 505–510.
Zhao, J.S., Manno, D., Beaulieu, C., Paquet, L., Hawari, J., 2005. *Shewanella sediminis* sp. nov., a novel Na^+-requiring and hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine-degrading bacterium from marine sediment. Int. J. Syst. Evol. Microbiol. 55, 1511–1520.
Zumft, W.G., 1992. The denitrifying prokaryotes. The Prokaryotes. In: Balows, A., Truper, H.G., Dworkin, M., Harder, W., Schleifer, K.-H. (Eds.), The Prokaryotes. A handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications, second ed., 1. Springer-Verlag, New York, N.Y, pp. 554–582. vol. 1.
Zumft, W.G., 1997. Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. 61, 533–616.