Vertical Distribution of Bacterial Communities in the Indian Ocean as Revealed by Analyses of 16S rRNA and *nasA* Genes

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Abstract Bacteria play an important role in the marine biogeochemical cycles. However, research on the bacterial community structure of the Indian Ocean is scarce, particularly within the vertical dimension. In this study, we investigated the bacterial diversity of the pelagic, mesopelagic and bathypelagic zones of the southwestern Indian Ocean (50.46°E, 37.71°S). The clone libraries constructed by 16S rRNA gene sequence revealed that most phylotypes retrieved from the Indian Ocean were highly divergent from those retrieved from other oceans. Vertical differences were observed based on the analysis of natural bacterial community populations derived from the 16S rRNA gene sequences. Based on the analysis of the *nasA* gene sequences from GenBank database, a pair of general primers was developed and used to amplify the bacterial nitrate-assimilating populations. Environmental factors play an important role in mediating the bacterial communities in the Indian Ocean revealed by canonical correlation analysis.

Keywords 16S rRNA gene sequence · Nitrate assimilation · Assimilatory nitrate reductase gene *nasA* · Indian Ocean

Introduction

Bacteria are an important component of the marine ecosystem. The vertical distribution of bacteria has been described in the Atlantic and Pacific oceans [1–3]. Highly diverse and novel patterns among picoeukaryotes have been observed in the Indian Ocean based on molecular surveys [4]. However, limited data is available regarding microbial diversity in the Indian Ocean. Functional communities play a significant role in oceanic biogeochemical cycles [5]. Denitrifiers in the coastal environments of the Indian Ocean have been completely investigated using molecular methods [6, 7] and were found to be particularly important in the oceanic oxygen minimum zone (OMZ) [5, 8]. Inorganic nitrogen, mainly nitrate, is discharged to the coastal water and triggers a series of environmental events. The uptake of nitrate by marine bacteria has important implications for the global nitrogen and carbon balance [9, 10]. Nitrogen-related functional microbial communities can potentially consume a substantial fraction of dissolved inorganic nitrogen [11]. A recent study using isotope labeling and metagenomics determined that bacterial nitrate reduction is closely related to the carbon:nitrogen ratio [12]. Bacterial nitrate uptake potentially impacts estimates of new production and carbon export to the deep ocean [13].

Nitrate is an important nitrogen source for a diverse group of heterotrophic bacteria [10, 14]. The concentration of nitrate was high in the deep ocean. The spacial distribution of nitrate-assimilating bacteria (NAB) has been investigated using the *nasA* gene in various oceanic surface waters [15–17]. However, these studies were not emphasized on the difference of NAB in the vertical dimension. A study showed bacterial nitrate assimilation comprises a greater proportion (up to 93 %) at the bottom of the Mid-

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Atlantic Bight [18]. However, information regarding NAB diversity in the twilight and aphotic zones is scarce, thereby limiting our understanding of nitrogen cycles in the water column.

The *nasA* gene primers were believed to be biased in the previous studies [15, 16]. In order to better understand the NAB communities, it is extremely urgent to improve the *nasA* gene primers allowing amplification of *nasA* gene fragments from diverse prokaryotes. We analyzed the *nasA* nucleotide sequences available in the GenBank database. The conserved regions were found and a new pair of primers was designed, they were used to amplify the *nasA* gene fragments of environmental samples. To confirm the amplicates obtained were indeed *nasA* gene fragments, direct sequencing of the PCR fragments were carried out. The NAB populations revealed by the new primers were distinct, the primer designed in present study could be significantly important supplement to the previous studies. To our knowledge, this is the first study to survey the *nasA*-containing bacteria in the water column of Indian Ocean based on primer improvement.

The aim of the current study was to identify the bacterial diversity along the vertical profile of the Indian Ocean. We found that the bacterial communities in the Indian Ocean are unique based on phylogenetic analyses of 16S rRNA and *nasA* gene sequences. The result indicated that diverse bacterial communities are significant to the biochemical transformation in the water column of Indian Ocean.

Materials and Methods

Sample Collection

Seawater samples were collected from depths ranging from 5 to 1600 m at station YD (50.46°E, 37.71°S) in the Indian Ocean using Niskin bottles during the 21st global cruise in 2009. Seawater totaling 2–3 L was filtered through 0.22 µm pore size polycarbonate filters (Millipore Co., Bedford, MA) under a pressure <0.03 MPa. Filtered samples were immediately frozen and stored at –80 °C until commencement of DNA extraction procedures. The physicochemical parameters of seawater (water depth, temperature, and salinity) were measured in situ using a conductivity, temperature, depth (CTD) system. Nitrate concentrations were measured with the nutrient AutoAnalyzer AA3 (Bran and Luebbe AA3, Germany).

DNA Extraction, Amplification of *nasA* and 16S rRNA Gene and Sequencing

DNA was extracted using the UltraClean Soil DNA kit (MoBio, San Diego, CA, USA) according to the protocol of

the manufacturer. A total of five depth samples (100, 200, 500, 1000, and 1600 m) from the Indian Ocean were used to construct clone libraries. The universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTACGACTT-3') were used to amplify community DNA. The nitrate assimilation gene *nasA* was amplified using the primers *nas964* and *nasA1735* [15] and newly designed primer set: *nasF30* (5'-TAYTGYGGBGTNGGNTG-3') and *nasR1080* (5'-CCCATNGCRTTNGGYTG-3') in this study, with the following cycling conditions: 95 °C for 4 min, followed by 30 cycles, 95 °C for 50 s, 30 s at 53 °C, 50 s at 72 °C, with a 7 min final extension. The PCR products were gel-purified and ligated into pMD18-T vectors, then transformed into *Escherichia coli* DH5a competent cells (TaKaRa, Dalian, China). Colony PCR with M13 primers for the vector was used to test positive clones. Sixty positive clones containing *nasA* genes and one hundred positive clones containing 16S rRNA genes were randomly selected for sequencing in each clone library (Invitrogen, Shanghai, China).

Phylogenetic and Statistical Analysis

The sequence alignments, rarefaction analysis, phylogenetic trees and canonical correspondence analysis (CCA) were using the previous method [17].

Nucleotide Sequence Accession Numbers

The sequences obtained in the current study have been deposited in the GenBank database under the accession numbers JX441384–JX441599 and JQ628107–JQ628379 and JX423855–JX423972.

Results

Bacterial Community Structure in the Vertical Dimension of the Indian Ocean as Revealed by 16S rRNA Gene Sequences

A total of 445 reliable sequences from five clone libraries (90 from 100 m, 87 from 200 m, 87 from 500 m, 92 from 1000 m, and 89 from 1600 m) were used for diversity analysis. All the sequences were assigned to 215 operational taxonomic units (OTUs) based on a 97 % sequence identity cutoff and affiliated with the bacterial lineages described below (Fig. 1).

The highest diversity of phylotypes was found within the *Alphaproteobacteria* cluster (175 sequences, 86 OTU), dominated by SAR11 cluster (85 sequences), assigned to 30 OTU. SAR11 sequences were found

Fig. 1 Maximum likelihood phylogenetic tree by 16S rRNA gene sequences. Nodes supported by bootstrap values >50 % are indicated by circles. The taxonomic sequence groups were separated by different color ranges and showed on the right (color figure online)

throughout the water column and were abundant in the mesopelagic zone. In addition, *Rhodobacterales* (17 sequences, 8 OTU) and unidentified Alpha I (21 sequences, 19 OTU) were relatively abundant within the *Alphaproteobacteria*. The *Rhodobacterales* cluster was mainly distributed in the euphotic zone, accounting for 71 % of the total sequences of *Rhodobacterales*. The remaining sequences were grouped into *Sphingomonadales* (12 sequences, 7 OTU), *Rhizobiales* (10 sequences, 8 OTU), and unidentified *Alphaproteobacteria* (30 sequences, 14 OTU).

One hundred fifty-eight sequences (63 OTU) were clustered into the *Gammaproteobacteria*. The dominant group (43 sequences) was affiliated with the

Alteromonadales cluster and classified to 10 OTU and was mainly distributed in the euphotic zone (100 m depth). In addition, two clusters were related to the unidentified gamma-affiliated sequences, namely unclassified gamma I (40 sequences, 8 OTU) and the unclassified gamma II (34 sequences, 16 OTU), suggesting that many bacterial groups exist in the Indian Ocean that have not been identified previously. The remaining clone sequences were grouped into the *Legionellales* (11 sequences, 9 OTU), *Xanthomonadales* (10 sequences, 3 OTU), *Chromatiales* (5 sequences, 3 OTU), *Alcanivorax* (four sequences, 4 OTU), *Vibrio* (3 sequences, 3 OTU), *Marinobacter* (2 sequences, 2 OTU), *Halomonas* (1 sequence), and unclassified *Gammaproteobacteria* (four sequences, 4 OTU).

Forty-one sequences (16 OTU) were clustered into the *Burkholderiales*, which belonged to *Betaproteobacteria*. There were nine clones (8 OTU) grouped into the *Deltaproteobacteria*. Clones numbering 18 and classified into 9 OTUs were affiliated with the *Flavobacteriales*. The remaining sequences belonged to the *Actinobacteria* (7 sequences, 6 OTU), the *Cyanobacteria* (8 sequences, 4 OTU), the *Planctomycetales* (6 sequences, 5 OTU), *Verrucomicrobiales* (6 sequences, 4 OTU), the *Acidobacteria* (8 sequences, 7 OTU), and the unidentified bacteria (9 sequences, 7 OTU).

Canonical correlation analysis (CCA) was performed to identify the environmental factors shaping the bacterial community structure in the vertical dimension (Fig. 2a). All the environmental factors analyzed, including nitrate concentration, salinity, and temperature, demonstrated that multiple factors ($p > 0.05$) influence the vertical distribution of natural bacterial communities in the Indian Ocean.

Diversity of NAB in the Vertical Dimension of the Indian Ocean

In total, 278 *nasA* sequences were retrieved from the five clone libraries representing the vertical dimension of the Indian Ocean. Our results showed that the dominant NAB belonged to *Gammaproteobacteria* (60 % to the total sequences). The two most abundant groups were *Vibrio* (67 sequences, 9 OTU) and the *Marinobacter* group (40 sequences, 3 OTU) (Fig. 3), with the former dominating the euphotic zone and the latter dominating the deep depths of the Indian Ocean. The remaining *Gammaproteobacterial* sequences were allocated to the *Alcanivorax*, *Acinetobacter*, *Oceanospirillum*, *Alteromonas* and the

Psychrobacter. Of the total sequences, 17 % were affiliated to *Alphaproteobacteria*, dominated by the *Roseobacter*-like group (25 sequences, 2 OTU). The remaining sequences were grouped into unidentified *Alphaproteobacteria* and the *Hirschia* group. An unidentified bacteria group deserves to be mentioned, with 43 sequences assigned to 2 OTUs, and may represent a novel NAB ecotype of the Indian Ocean.

To determine the environmental factors mediating the *nasA*-containing bacterial communities occupying the vertical dimension, CCA analysis was performed (Fig. 2b). The results showed that salinity ($p = 0.033$) is the most significant environmental factor among all assessed environmental factors including depth, temperature, and nitrate concentration.

Improvement of *nasA* Gene Primers and Experimental Test

We analyzed the DNA and inferred amino acid sequences encoded by *nasA* genes in the GenBank database and found the present primers used to amplify *nasA* sequences might underestimate the *nasA* gene diversity. In present study we found two conserved regions suitable for designing universal primers covering almost all the *proteobacterial* phylogenies (Table 1). A pair of new primers (*nasF30*, *nasR1080*) was designed for further study on bacterial nitrate assimilation. In order to confirm the effective of the primers, we amplified 1000 bp fragments in the environmental samples. 118 sequences at the 500 and 1600 m depths of the Indian Ocean obtained using the new primers. We attempted to amplify the *nasA* fragment from surface water but failed, indicating that NAB may be low

Fig. 2 Canonical correspondence analysis ordination plots of the relationship between the environmental parameters and bacterial communities in the vertical dimension of the Indian Ocean (**a** Bacterial communities with environmental factors; **b** *nasA*-containing bacteria with environmental factors)

Fig. 3 Phylogenetic analysis of *nasA*-containing bacteria in the vertical dimension of the Indian Ocean based on the neighbor-joining methods of mega4.0 software. The bootstrap values exceeding 50 %

are shown at the nodes. Clone number of every operational taxonomic unit representing five clone libraries is shown in the *square bracket*

abundance in the surface water. The most abundant NAB bacteria were related to *Gammaproteobacteria* (69 clones), most were related to unidentified gamma-affiliated sequences, indicating the existence of novel genotype of *nasA* genes. 7 sequences were affiliated into *Alphaproteobacteria*. The remaining sequences were grouped into *Bacteroidetes* (2 clones) and *Betaproteobacteria* (1 clone).

Discussion

Vertical Distribution of the Bacterial Community Structure in the Indian Ocean

In the current study, the vertical distribution pattern of the bacterial community was revealed by the 16S rRNA gene

sequence analysis in the Indian Ocean. The diversity analysis reflected changes in bacterial composition along the depth profile (Fig. 4). Sequences in the SAR11 cluster were abundant in the bacterial community and dominant in the 500 and 1000 m depth. *Betaproteobacteria* was distributed at all depths, but was dominant in the 200 m depth. Unclassified *Alphaproteobacteria* was abundant in the deeper oceanic waters (500–1600 m). These results suggested that specific depth-related bacterial genotypes exist in the Indian Ocean. Light quality, temperature, and organic matter show dramatic changes within the ocean depth profile and may influence microbial distribution. Specific bacteria characteristic of different depths may play important ecological roles.

Statistical analysis of the five clone libraries showed that the diversity within the 1600 m depth clone library

Table 1 Conserved-site analysis by alignment of partial nucleic acid sequences of *nasA* genes

Genus	GenBank no.	Sequence sites								
		22–44 bp	964–980 bp	1735–1751 bp	1933–1952 bp					
<i>Klebsiella</i>	4755081	TGCCCTAT	TGCGGGTTG GCTG	CAACCTA	ACGCCATGGG	GATCAA	TGGCACACCA	T	TTGTGCC	GATGCACTGG
	16329170	TGCCCTTAC	TGCGGTGTGG GCTG	CAACCAA	ACGCCATGGG	GGCCAC	TGGCATACTC	A	TTGTGCC	GATGCACTGG
<i>Synococcus</i>	71905642	TGCTGCTAT	TGTGGGTGCG GTTG	CAGCCGA	ATGCCATGGG	GATCAT	TGGCACGGCA	T	GGATGCC	GATGCACTGG
	89052491	TGCCCTAT	TGCGGGGTGCG GCTG	CAACCCA	ACGCCATGGG	GACCAA	TGGCACACGA	T	TGCAACC	AATGCACTGG
<i>Alteromonas</i>	196154825	TGCCCTTAT	TGCGGCGTGG GTTG	CAGCCGA	ATGCCATGGG	GATCAA	TGGCATACCA	T	TCAATCC	TATCCATGG
	296271545	TGTGGCTAT	TGTGGTGTG GTTG	CAACCAA	ATGCCATGGG	GATCAA	TGGCATAGTG	G	TTATACC	AGT
<i>Arcobacter</i>	110279108	TGCTCGTAT	TGCGGTGTG GTTG	CAACCGA	ATGCCATGGG	GATCAA	TGGCATACCA	T	TTTTACC	CATGCACTGG
<i>Cytophaga</i>	411529184	TGCTCTTAT	TGCGGAGTTG GATG	CAACCCA	ATGCCATGGG	GATCAG	TGGCATACCA	T	TTCTTCC	CATGCACTGG

was highest while diversity in the 100 m depth was the lowest (Table 2). The high diversity within the 1600 m depth was mainly due to abundant specific unclassified clones, which were detected only in this library, suggesting that many species or ecotypes were responsible for the high diversity in the bathypelagic ocean. The rarefaction curves reflected that the 100 m depth diversity was lowest, characterized by high dominance (0.12), with the dominant group being *Alteromonadales* occupying 39 % of the total clones in this library. The result was different with that in the North Pacific Ocean, which showed that *Alteromonadales* was abundant within the 3000 m depth zone [2]. Other studies have shown that the organisms within *Alteromonadales* are prevalent in various oceanic environments [19].

Vertical Distribution of NAB in the Indian Ocean

In the present study, we studied the *nasA*-harboring bacterial diversity in a vertical profile of the Indian Ocean. The results showed that NAB communities are more diverse than ever before realized. We found that *Marinobacter* were dominant in the deep water of the Indian Ocean, with the highest proportion being 42 % of the total sequences. Previous studies have found that the *Marinobacter* group is of significance for nitrate assimilation and is positively correlated to nitrate concentration [9]. *Marinobacter* strains have been applied to successfully treat reservoir souring [20]. *Marinobacter* associated with *Alteromonadales* were abundant in the water column, indicating an important role in nitrate assimilation in the Indian Ocean. *Alteromonadales* is distributed over a wide range of oceanic environments, from surface to deep ocean [21]. The *Roseobacter*-like group affiliated to *Alphaproteobacteria* was the second most abundant NAB in the Indian Ocean. The genome sequences of many *Roseobacter* clade strains contain *nasA* genes [22]. The *Roseobacter* cluster constituted 10–15 % of total bacteria at the DNA level and 12–25 % of total bacteria on the rRNA level during a phytoplankton bloom [23]. Our results suggest that *Alteromonadales* and *Rhodobacterales* may be of particular significance within nitrate assimilation and may play an important role in the oceanic nitrogen cycle.

Based on the clone library analysis, we found that the diversity of *nasA* genes was lower in surface water and higher in the deep water of the Indian Ocean (Table 2). The PCR product of *nasA* genes from the 5 m depth zone was not sufficient to obtain gel purification, despite repeated amplification and PCR optimization. The diversity index indicated that the 100 m depth zone showed the lowest diversity of the five depth samples along a depth profile ranging from 100 to 1600 m in Indian Ocean. The

Fig. 4 The bacterial community composition in different depths of Indian Ocean (color figure online)

highest diversity of *nasA* gene was found at the 500 m depth. The OTU number was highest (20 OTU, 54 sequence), reflecting the complexity of the NAB community composition in the mesopelagic zones. In the current study, we obtained many unique deep-water sequences (from 500 m to 1600 m) which remain unidentified. These unique depth-related sequences were dispersed through the *nasA* phylogeny with extended branch lengths and may represent novel clades. The low similarity values in nucleotides and amino acids clearly demonstrated an unexpectedly high diversity of the *nasA* genes in the deep ocean. The results show that the community structure of *nasA* genes is different from that of the other marine environments, and that the NAB community is more diverse in the mesopelagic oceans. Therefore, we speculate that nitrate concentration may be a critical factor driving depth-related differences of NAB communities in the vertical dimension.

Primer Discussion on the *nasA* Gene

In previous studies, the results showed most of the *nasA*-containing bacteria were assigned to *Gammaproteobacteria* using primer sets *nas964* and *nasA1735* [15, 17]. In order to clarify the fact that the primer bias exists or not in the experiments, a work to improve the primers specific to *nasA* genes need to be carried out. The pool of the *nasA* nucleotide sequences available in the GenBank is expanding. We made a chance to improve the *nasA* gene primers. Based on these gene analysis, we found the outermost primers (*nas22*, *nas1933*) used in previous

studies [15] could not match many of the bacterial *nasA* genes. That means nest-PCR method cannot amplified many *nasA* genotypes and underestimated the NAB diversity information. The two conserved regions of *nasA* genes were suitable to primer designation new primers (*nasF30*, *nasR1080*). Our results showed gamma-like *proteobacterial nasA* genes were abundant in the Indian Ocean using the newly designed primers, the results were consistent with the previous studies [15–17]. We speculated that *Gammaproteobacteria* were significantly important in nitrate assimilation. We also obtained unrecognized NAB groups based on the primers designed in this study. We suggest that they could be supplemental primers and applicable to study the nitrate-assimilating bacteria in various environments.

In conclusion, vertical differences in bacterial community were observed based on the phylogenetic analysis of the depth profile at division and subdivision levels. The sequences retrieved in the current study showed high similarity to uncultured bacteria, where novel populations were recognized. Our results certified that the NAB communities within *Gammaproteobacteria* are important functional groups, and their wide distributions may play an important role in the nitrogen cycle of the Indian Ocean. The CCA analysis indicated that the distribution of the natural bacterial communities along the vertical dimension is affected by the combined effect of multiple assessed factors, whereas the distribution of the NAB communities is mainly influenced by salinity. The abundant unclassified sequences found in the bathypelagic ocean showed that novel bacterial populations exist,

Table 2 The environmental parameters and clone libraries based on 16S rRNA and *nasA* genes in the Indian Ocean

Depth (m)	Longitude/latitude	Sampling date	Total depth (m)	Temperature (°C)	Salinity (‰)	NO ₃ ⁻ (μmol/L)	Total clones (16SrRNA/nasA)	Coverage (%) (16SrRNA/nasA)	Taxa (16SrRNA/nasA)	Dominance (16SrRNA/nasA)	Shannon (16SrRNA/nasA)	Simpson (16SrRNA/nasA)
100	50.46E/37.71 N	2/2010	2211	18.8	35.6	18.8	90/56	58.9/87.5	47/14	0.12/0.26	3.14/1.90	0.88/0.74
200				17.0	35.6	23.1	87/57	59.8/82.5	46/17	0.06/0.20	3.38/2.12	0.94/0.80
500				12.0	34.8	27.9	87/54	50.6/81.5	56/20	0.03/0.10	3.78/2.61	0.97/0.90
1000				3.0	34.5	29.5	92/57	52.2/91.2	56/14	0.04/0.13	3.70/2.30	0.96/0.87
1600				2.1	34.7	31.6	89/54	42.7/80.0	62/19	0.03/0.12	3.86/2.47	0.97/0.88

suggesting further research is required to elucidate the roles of bacteria to facilitate the biogeochemical cycle in the Indian Ocean.

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