Prokaryotic Diversity in Oxygen Depleted Waters of the Bay of Bengal Inferred Using Culture-Dependent and -Independent Methods

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Received: 7 December 2018 / Accepted: 4 February 2019 / Published online: 11 February 2019
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Abstract There are regions in the world oceans where oxygen saturation is at its lowest, evident at depths between shelf to upper bathyal zone. These regions are known as Oxygen Minimum Zones (OMZs), which reportedly support phylogenetically diverse microbes. In this study, we aimed to characterize prokaryotic diversity in the water samples collected from 43, 200 and 1000 m depth of the Bay of Bengal Time Series location (BoBTS—18.0027°N, 89.0174°E) in the OMZ region. Illumina sequencing generated 3,921,854 reads of 16S rRNA gene amplicons, which corresponded to 5778 operational taxonomic units. The distribution of bacteria at class level varied with depth and oxygen concentration. α -Proteobacteria was found in abundance in 43 m and 1000 m depth water samples. γ -Proteobacteria was prominently detected in oxygen-depleted depths of 200 m and 1000 m. AB16 (Marine Group A, originally SAR406) was restricted at dissolved oxygen concentration of 1.5 μ M at 200 m. Archaeal members were observed in low abundance (2%), with a high occurrence of phylum Euryarchaeota at 43 m, while Crenarchaeota was detected only at 200 m depth.

Select bacterial cultures were screened for their ability to reduce nitrate in vitro, to obtain insights into their possible role in the nitrogen cycle. A total of 156 bacterial isolates clustered majorly with *Alcanivorax*, *Bacillus*, *Erythrobacter*, *Halomonas*, *Idiomarina* and *Marinobacter*. Among them, 11 bacterial genera showed positive nitrate reduction in the Griess test. A large percentage (63.55%) of 16S rRNA gene amplicons corresponded to unidentified OTUs at genus or higher taxonomic levels, suggesting a greater undiscovered prokaryotic diversity in this oxygen depleted region.

Keywords Bacteria · High throughput sequencing · Nitrate reducers · North Indian Ocean

Introduction

Microbial communities play critical roles in decomposition, mineralisation and recycling of organic and inorganic compounds [1]. Bacteria are known to utilise a broad range of electron donors (organic and inorganic) as their energy sources, as well as alternative electron acceptors for respiration in the absence of molecular oxygen. The metabolic versatility enables bacteria to survive and thrive in the oxygen-depleted environment [2]. Areas in the ocean that experience high primary productivity and sluggish ventilation of sub-surface waters show depleted dissolved oxygen (DO) below 20 μ M at intermittent depths due to consumption of DO through respiration. These regions in the ocean are described as oxygen minimum zones (OMZs). The most extensive permanent OMZs observed are in the Eastern Tropical North Pacific (ETNP), Eastern Tropical South Pacific (ETSP), Arabian Sea (AS) and Bay of Bengal (BB) in the northern Indian Ocean [3].

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12088-019-00786-1>) contains supplementary material, which is available to authorized users.

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The Bay of Bengal in the north-eastern Indian Ocean experiences intense depletion of dissolved oxygen ($\leq 5 \mu\text{M}$) at intermediate depths ($\sim 100/150$ to 1000 m) and prevents large-scale denitrification from occurring [4] unlike Arabian Sea-OMZ. The variation of oxygen intensity in the BB-OMZ is attributed to lack of detritus transport and immense freshwater inputs from major rivers of the western boundaries [5].

In OMZ waters, microbial community varies in their abundance, composition, and metabolic activities as a result of steep gradients in nutrient and energy availability. Low DO in the OMZ influences the microbial community in playing an active role in the cycling of carbon, nitrogen, sulphur and trace gases [6]. Low oxygen concentrations in the OMZs create conditions for facultative bacteria to use nitrate and nitrite as terminal electron acceptors in respiration, a process that is termed as denitrification [7]. The N_2 production due to denitrification in BB-OMZ accounts for about 2.5% of the global water column production in the OMZ regions [4]. Culture-independent analyses of the total community DNA can detect a considerable fraction of uncultivable organisms in addition to those that can be cultured [8]. Culture-independent methods are complemented by cultivation method for making inferences on the physiological and metabolic properties of the organisms. In the present study, we report the 16S rRNA gene diversity inferred through culture-independent approach and isolated the cultivable bacteria, which showed to have nitrate reducing ability from the open ocean oxygen depleted waters of the Bay of Bengal.

Materials and Methods

Site Description and Sampling

Water samples were collected from the Bay of Bengal Time Series location (BoBTS—Latitude: 18.002728°N Longitude: 89.01749°E , water depth: 2230 m) of the Bay of Bengal, during the cruises onboard RV Sindhu Sadhana (SSD005 and SSD020) in the years 2014 and 2016, respectively. This site is located in the north-eastern Bay of Bengal and is an existing OMZ accompanied with low but significant loss of nitrogen [4]. Depths were chosen based on the oxygen gradient of the water column using Conductivity-Temperature-Depth (CTD) rosette system fitted with Niskin bottle sampler (Seabird Electronics, Washington, USA) mounted with an oxygen sensor. Samples once retrieved were processed immediately in the onboard laboratory.

Culture-Independent Bacterial Studies

Five-litre water samples from depths of 43 m , 200 m and 1000 m were collected during the cruise SSD005 at BoBTS, using Niskin bottles from CTD rosette system. The water collected was filtered through $0.22 \mu\text{m}$ sterivex filters (Millipore, USA) with a peristaltic pump. The filters were filled with DNA storage buffer (50 mM Tris pH 8.3, 40 mM EDTA and 0.75 M sucrose), sealed and stored at -20°C until DNA was extracted in the laboratory. The total genomic DNA was extracted from the sterivex filter using the Power Water DNA Isolation kit protocol (MO BIO Laboratories, Carlsbad, USA). The purity of the DNA was estimated using a Nanodrop spectrophotometer (Thermo, US). A total of $\sim 1000 \text{ ng}$ DNA was vacuum dried in a vacuum concentrator (Eppendorf, Germany) and outsourced to obtain sequenced amplicon of V3 (150×2) paired-end regions of the bacterial 16S rRNA gene using 16S rDNA cyclic-array sequencing (Illumina NextSeq) instrument. The Illumina paired raw reads were quality checked using Fast QC tool, while QIIME pipeline was used to ensure quality and clean sequences were clustered into operational taxonomic units (OTUs) at $\geq 97\%$ sequence similarity against the curated chimera free 16S rRNA database (Greengenes5 v 13.8). QIIME program was further used to calculate taxonomic richness (OTUs, Chao-1 and Shannon diversity) [9]. R studio V3.5.1. was used to construct the plots using the Phyloseq and ggplot2 package. The raw Illumina paired reads generated during the current study were deposited in NCBI's Sequence Read Archive (SRA) under the accession SRP 152551 associated with BioProject PRJNA79567 (SAMN09623311-13).

Isolation of Cultivable Bacteria

Bacteria were isolated using fourteen different organic and inorganic media. Excluding Zobell marine agar (ZMA) and NO_3 agar (HiMedia), remaining 12 media were modified for bacterial isolation (Table S1). A total volume of $100\text{--}200 \text{ mL}$ water was filtered through $0.22 \mu\text{m}$ nitrocellulose membrane and placed onto the respective media. Culture media, including controls, were incubated at room temperature ($25\text{--}28^\circ\text{C}$), and bacterial isolates were obtained across 3–15 days. Bacterial isolates were sub-cultured to obtain pure cultures, and genomic DNA was extracted using the ZR Bacterial DNA MicroPrep Kit (Zymo Research, CA). Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was performed using universal primers 27F ($5'\text{-AGAGTTTGATCMTGGCT-CAG-}3'$) and 1492R ($5'\text{-TACGGYTACCTTGTTAC GACTT-}3'$) [10]. PCR programme followed: initial denaturation at 95°C for 5 min, followed by 95°C for 1 min, 54°C for 45 s, and 72°C for 1 min for 35 cycles

Table 1 Culture-independent depth wise diversity indices at Bay of Bengal time series (BoBTS)

Depths (m)	OTU picked/observed species	H (Shannon diversity)	Simpsons	Chao 1
43	2547	6.1964	0.964	3669.714
200	3431	6.221	0.95	4697.534
1000	4103	7.011	0.9686	55,519.35

with a final 10 min extension at 72 °C. The amplified PCR products were purified using PCR Clean-Up System (Promega Corporation, US) and sequenced using Genetic Analyzer 3130 × 1 (ABI) by the Big Dye Terminator v 3.1 (Chain terminator) chemistry. NCBI GenBank database was used for similarity search of all the sequences using BLASTn, which was later confirmed using EzBioCloud database. The sequences were aligned using MUSCLE embedded in MEGA X. Evolutionary analyses were conducted in MEGA with reference sequences retrieved from EzBioCloud database. The partial 16S rRNA gene sequences of the bacterial isolates in this study have been deposited in NCBI GenBank (Table S2).

Qualitative Analysis of Nitrate Reducers

The bacterial isolates were tested for their nitrate-reducing capabilities by growing them in nitrate broth (HiMedia, India). The test was performed as per the Griess method, which is based on the activity of the enzyme nitrate reductase. The protocol and interpretation followed was as mentioned in Mulla et al. [7].

Results and Discussion

Prokaryotic Diversity at BoBTS

The DO concentrations measured during SSD005 cruise sampling, varied along the vertical depths showing low oxygen layers between 200 m and 1000 m, whereas at 43 m and near bottom 2000 m depths were well oxygenated (Fig. S1a). Quality filtration of the raw sequencing 16S rRNA gene amplicons led to a total number of 3,921,854 reads for the 3 samples, corresponding to 5778 OTUs with varied abundance in different samples. The highest number of observed OTUs was found in the 1000 m depth and 200 m, whereas a lower richness of prokaryotic OTUs was observed at the surface depth of 43 m (Fig. S1b). As seen in our studies, Stevens and Ullao [11] have also reported higher bacterial in the oxygen-deficient waters attributing it to the presence of various terminal electron acceptors, as compared to oxygenated surface or deep oxycline depths where oxygen is the primary electron acceptor. The Shannon and Chao 1 index was highest at 1000 m, while 43 m showed lower Shannon

and Chao 1 index indicating higher rare species at 1000 m (Table 1). Thus signifying more diverse bacterial OTUs in the low oxygen concentration depths. The rarefaction curves (Fig. S2) of the three sampling depths showing the annotated species richness depicting more intensive sampling could yield only a few additional species.

Prokaryotic Community Structure at BoBTS

The OTUs identified grouped into 47 prokaryotic phyla, with most sequences affiliated to Actinobacteria, Cyanobacteria, Proteobacteria and SAR406 (Fig. 1a). The presence of Proteobacteria increased along the oxygen gradient (43 m—50.27%; 200 m—51.07%; 1000 m—67.01%), followed by Cyanobacteria in the oxygenated surface waters (43 m—25.03%), and SAR406 in the less oxygenated waters (200 m—36.04% and 1000 m—13.7%). At class level (Fig. S3a) the bar graph revealed the dominance of α -Proteobacteria at 43 m and 1000 m depths. Class AB16 also known as SAR406, renamed as Marinimicrobium [12], was present at 200 m where the oxygen concentration was the lowest (1.5 μ M), while γ -Proteobacteria was distributed at all depths sampled. Synechococcophycideae class was present in high concentrations in the 43 m sample among the phototrophs. At 43 m non-specific 16S rRNA gene amplification of algal and plant chloroplast was observed in high concentrations, which is likely due to the homology between the 16S rRNA genes in bacteria and chloroplast. However, the amplification of chloroplast 16S rRNA gene could be omitted with use of improved primer sets [13].

The bacterial community at 43 m was dominated by the members of Acidimicrobiales, Chlorophyta, Oceanospirillales, Rickettsiales and Synechococcales. While the Arctic96B-7, Flavobacteriales and Rhodobacterales were found in small proportions. Most of the sequences at 200 m were clustered into Arctic96B-7, Oceanospirillales, Rickettsiales, and Thiotrichales. Acidimicrobiales, Alteromonadales, Desulfobacterales, Flavobacteriales, Rhodobacterales, Sva0853, Thiohalorhabdaceae and ZA3648c contributed comparatively minor members in the bacterial diversity. The Arctic96B-7, Rickettsiales and Sphingomonadales dominated at 1000 m water depth. The dominance of the Arctic96B-7 (class AB16) at 200 m depth, in comparison to 1000 m exhibits depth-dependent changes in abundance along the oxygen gradient [14].

Fig. 1 Bacterial diversity at the phylum level obtained from BoBTS location with each method used. **a** The number of nucleotide sequence read in each phylum for Illumina Nextseq culture-

independent method, < 0.01 were grouped as others. **b** The total isolates obtained from culture media

Members of the Arctic96B-7 are suggested to be involved in the reduction or oxidation of reduced sulphur compounds in the oxygen-depleted waters [15]. Interestingly,

the Sphingomonadales was found to be dominant at lower depths of BoBTS. Though the order has been previously reported from the upper euphotic zone [16], we are

reporting its occurrence in the OMZ of the Bay of Bengal for the first time. The bacterial clads Thiotrichales and Desulfobacterales were observed at 200 m and 1000 m in the oxygen-depleted waters at BoBTS. Thiotrichales and Desulfobacterales are reported to be linked to the sulphur cycle and play an active role in the cryptic sulphur cycle in the OMZ [17].

Among the 465 assigned genera of the 3 sampled depths, the relative abundance of top 27 bacterial genera (Fig. S3b) showed a high relative content of *Prochlorococcus* and *Candidatus Portiera* at 43 m depth. *Prochlorococcus* is most abundant in oligotrophic waters over *Synechococcus* which prefers well lit, nutrient rich waters [18]. *SAGH499*, *Candidatus Portiera*, *SargSea-WGS* and *Nitrospina* were represented with a relatively high abundance in the 200 m sample. *Erythrobacter*, *SAGH499*, *SargSea-WGS*, *Marinobacter* and *Nitrospina* were identified as dominant genera in the 1000 m depth. *Candidatus Portiera* was one of the most abundant OTU in our dataset which is a genus belonging to an endosymbiont of the whitefly. The presence of this taxon is likely an error in Greengenes database [19]. However, this genus is a part of the order Oceanospirillales, which are common marine γ -Proteobacteria having the ability to degrade hydrocarbons [20]. The presence of anaerobic *Anaerospira* was identified at oxygen minimum depths only (200 m and 1000 m). However a high percentage of unclassified OTUs (43 m—73%, 200 m—81%, 1000 m—64%) at genus level was present in all depths which was filtered out during analysis to resolve the most abundant classified genera. The presence of such high percentage of unclassified genera (63.55%) can harbour some of the novel clades in this region.

A few sequences were obtained of the archaeal 16S rRNA gene, which clustered into two phyla Euryarchaeota and Crenarchaeota. Euryarchaeota (Marine group II) was dominant at 43 m (1.2%) in comparison to 200 m (0.3%) and 1000 m (0.3%). At class level Thermoplasmata was dominant at all samples highest at 43 m, a similar pattern was found in the OMZ of the Eastern Tropical South Pacific. The occurrence of Thermoplasmata in surface waters is linked to the higher availability of labile and terrigenous derived organic matter at surface depths [15]. Very few sequences clustered into MGB and Halobacteria during this study. Marine Group III (MG III) was identified only at low oxygen concentration depths of 200 and 1000 m.

Cultured Bacteria and Their Nitrate Reducing Ability

Considering the limitations of culture media routinely used in bacterial isolation [21], a wide range of culture media

(Table S1) was employed in this study to isolate bacteria from water samples collected during successive cruises (SSD005 and SSD020) (Table S3) to improve recovery of cultivable bacteria. In total, 156 bacterial isolates were recovered during 2 cruises at BoBTS along the sampled depths and varying oxygen concentrations. Ten isolates were obtained from ZMA and NO_3 agar (SSD005) and 146 from TMSM, MBM, RM, SOM1, SOM2, TDM, TTM, Thiopar, Thioox, Thiobac and DMA- NO_3 (SSD020). Most suitable media that gave us maximum isolates were Thiobac and RM (Table S3). Based on the 16S rRNA gene sequence analysis, the bacterial isolates clustered within four phyla, i.e. Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria (Fig. 1b). Culture-based techniques, show bias towards the “Big Four” bacterial phyla, namely, Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria [22], which were successively recovered during our isolation. The phylogenetic tree of the bacterial isolates clustered into six major clades, α -Proteobacteria, β -Proteobacteria, γ -Proteobacteria, Actinobacteria, Bacilli and Flavobacteriia (Fig. S4). A total of 18 genera were isolated at BoBTS (Fig. 2), a few of the most abundant genera identified from the culture independent method were isolated using cultivation media viz., *Alcanivorax*, *Bacillus*, *Erythrobacter*, *Halomonas*, *Idiomarina*, *Marinobacter*. The nitrate reduction performed using Griess test showed positive to genera *Achromobacter*, *Alcanivorax*, *Bacillus*, *Erythrobacter*, *Halomonas*, *Idiomarina*, *Marinobacter*, *Nitrateductor*, *Pseudomonas*, *Rheinheimera* and *Zunongwangia* (Table S4). These species have been tentatively identified as *Alcanivorax dieselolei*, *Erythrobacter citreus*, *Idiomarina fontislapidosi*, *Idiomarina marina*, *Marinobacter alkaliphilus*, *Marinobacter hydrocarbonoclasticus*, *Nitrateductor aquibiodomus*, *Nitrateductor kimnyeongensis* and *Pseudomonas stutzeri*. Earlier reports show, *Marinobacter hydrocarbonoclasticus* and *Pseudomonas stutzeri* to be classified as aerobic denitrifiers under microaerobic conditions in the presence of nitrate [23, 24]. Studies have also reported the presence of denitrification genes in *Halomonas* spp.: *narH*, *nirS*, and *nosZ*. While, a few of the genera isolated during this study were also found to perform denitrification in the Arabian Sea OMZ viz., *Alcanivorax*, *Bacillus*, *Halomonas* and *Marinobacter* [7]. Among the β -Proteobacteria; *Achromobacter* provides an interesting insights into bacterial ecology in oxygen-deficient waters of the Bay of Bengal. Recently, Menezes et al. [21] showed the isolation of *Achromobacter* sp. from the OMZ of the Bay of Bengal and confirmed its sulphur-oxidising ability by in vitro qualitative oxidation of thiosulfate to sulfate. Most of the isolates in the present study that reduced nitrate, belonged to α -Proteobacteria and γ -Proteobacteria, which could serve as candidates for denitrification in these regions under favorable conditions.

Fig. 2 The relative abundance of cultivable bacterial isolates at the genus level from BoBTS

Recent reports suggest that despite the lack of evidence for anoxia in BB-OMZ waters, this area supports active denitrification as indicated by functional gene abundances of nitrate reductase and nitrite oxidoreductase genes [4].

Conclusion

In the present study, the culture independent approach was used to examine bacterial and archaeal diversity, attempt was made to isolate cultivable bacteria by amending the culture media with varying components of nitrogen and sulphur source, which could support the growth of bacteria existing in low oxygen concentrations by acting as alternate electron donors and acceptor. The diversity study at BoBTS location revealed the dominance of α -Proteobacteria, and γ -Proteobacteria, while a few of the dominant genera were capable of reducing nitrate in Griess test. Most genera sampled during this study remain unclassified implying the presence of possible novel clades present in the OMZ waters of the Bay of Bengal. With the occurrence and potential of a few genera to reduce nitrate we can hypothesize that they play an active role in the BB-OMZ nitrogen cycle. Extended investigation is however, required to confirm the active role of these isolates in the sulphur and nitrogen cycles with the use of biomarkers in the OMZ.

Acknowledgements The authors are thankful to Director, CSIR-NIO, and Head, Biological Oceanography Division for all the infrastructure and facilities. We are grateful to the Chief Scientists, Captains and Crew of the cruises SSD005 and SSD020. We thank Dr

Damodar M. Shenoy from the Chemical Oceanography Division and anonymous reviewers for their valuable suggestions to improve the manuscript. The cruises were part of the SIBER program (GAP2425) funded by the Ministry of Earth Sciences (MoES), Government of India. CSIR India is acknowledged for funding the research through the PSC0108 (INDIAS IDEA) project. This publication has CSIR-NIO contribution number 6351.

Author Contributions SD and BDS planned the work and was executed by GF and LM. GF was involved in sampling. RM has carried out the sequencing of the cultured isolates using in-house DNA sequencer. The results were analysed by SD, BDS, GF and LM. The manuscript was written and reviewed by SD, BDS, GF and LM.

Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

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