IMPACT: International Journal of Research in Applied, Natural and Social Sciences (IMPACT: IJRANSS)
ISSN (P): 2347-4580; ISSN (E): 2321-8851
Vol. 6, Issue 5, May 2018, 91-98

MICROBIAL DIVERSITY ASSOCIATED WITH CORALS OF JOLLY BOUY, ANDAMAN AND NICOBAR

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Received: 02 May 2018 **Accepted:** 06 May 2018 **Published:** 18 May 2018

ABSTRACT

The present study was carried out in Jolly Bouy Island of Andaman and Nicobar. The selected location is very famous for water corals. Hence, we made an attempt to identify the bacteria associated with corals of Jolly Bouy. The corals collected are Acropora robusta, Favites abtida, Favites complanata, Favites flexuosa, Favites pentagona, Favites russelli, Goniastrea rotiformis, Montastrea colemani, Pavona venosa, Porites solida, Psammacora obtusangula. Molecular characteristics of microbes isolated from corals were also done using universal bacterial primers, corresponding to E. coli positions 27f (5'-AGRGTTTGATCMTGGCTCAG-3') and 1525r (5'-AAGGAGGTGWTCCARCC-3'). Amplification was performed in Veriti Thermal Cycler (Applied Biosystems, USA) and 1X GT PCR master mix (TaKaRa). The 16S r DNA of the bacterial species was sequenced and the sequences of isolates were searched against the NCBI and EzTaxon database. The sequence analysis revealed 100% homology to Ruegeria arenilitoris G-M8 (JQ807219), Vibrio neocaledonicus NC470 (JQ934828), Vibrio Azureus NBRC 104587 (BATL01000140), 99.87% homology to Vibrio Azureus NBRC 104587 (BATL01000140) and 99.62% homology to Vibrio alginolyticus NBRC 15630 (CP006718). The identified strains formed an evolutionarily distinct lineage. Earlier studies had shown the identified bacterial species were the main pathogens responsible for coral bleaching. These pathogenic infections might be one of the reasons for the decrease in the coral population. Further studies are needed to evaluate the same.

KEYWORDS: *Jolly Bouy Island, Vibrio Neocaledonicus, Vibrio Azureus, Vibrio Alginolyticus*

INTRODUCTION

Coral reefs are the complex organogenic framework of calcium carbonate, which forms a rocky eminence on the sea floor and customarily grow upwards to the low-tide limit (Fairbridge, 1969). Corals harbor many different species of bacteria in and on their surface. Corals provide three habitats for bacteria they are lipid-rich surface mucus layer, tissues and skeleton each has the distinct bacterial population (Bourne and Munn, 2005; Koren and Rosenberg 2006). The coral mucus is dominated by alpha-proteobacteria, including Roseobacter (Munn, 2004), Spongiobacter (Bourne *et al.*, 2009) and phototrophs (Bourne and Munn, 2005) whilst tissue is dominated by gamma-proteobacteria. Hermatypic corals require the ability to regulate symbiont populations to maintain their health and protect them from pathogens in their surrounding (Kelman *et al.,* 2006). Coral bacteria can be either pathogenic or non-pathogenic. The pathogenic bacteria can initiate coral disease and that can change coral characteristics. Most common coral disease are Black band disease (Antonius 1985, Carlton and Richardson, 1995), White plague type II (Smith *et al.*, 1996; Richardson *et al.*, 1998), tissue necrosis (Hodgson 1990; Ben Haim and Rosenberg, 2002). On another hand many coral-associated

bacteria have antimicrobial properties and act as antagonists against the pathogens (Nissimov *et al.*, 2009; Harder *et al.*, 2002; Kelmen *et al.*, 2009). Bacteria act as an alternative source of nutrition under the condition of nutrients are scared and some bacteria have the ability to fix nitrogen or carbon for consumption (Shashar *et al.*, 1994; Ducklow and Mitchell, 1979). To gain the knowledge of coral-associated bacterial diversity, it is very important to identify and characterize the bacteria present in and on the corals. In this study, we describe the molecular identification of coral-associated bacteria by 16S rRNA gene sequencing.

MATERIALS AND METHOD

The coral samples were collected from Jolly buoy N 11^{o} 30.368' E 92^{o} 36.933' (Andaman Island). The corals were placed in a sterile plastic bag and sealed airtight. The samples were transported to the laboratory within two hours by keeping the samples in ice. In the laboratory, the coral sample was washed with sterile seawater to remove any loosely attached bacteria. The coral sample was placed in a 250 ml of the conical flask containing 50 ml of sterilesea water and agitated to isolate the bacteria associated with the surface and within the coral tissue. The coral samples were serially diluted and plated on Zobell marine agar (Zobell, 1941) and incubated at 28^{o}C for 24 to 48 hours. Most of the colonies exhibited differences in the morphological features such as shape, color and growth rate were selected and subjected to molecular cataloging.

DNA Extraction from Bacterial Isolates

Pure bacterial isolates from coral samples were subcultured at 30°C for 48 h. Cell suspensions were lysed using lysozyme and Proteinase K at 37 and 55°C, respectively for 1 hr. Genomic DNA was isolated by the standard phenol/chloroform/isoamyl alcohol (25:24:1) extraction and isopropanol precipitation method (Sambrook *et al.*, 1989) for polymerase chain reaction (PCR) amplification. The integrity of the extracted DNA was checked by 0.8% horizontal agarose gel electrophoresis in TAE buffer (40 mm is, 20 mm acetate, 2 mm EDTA) and visualized by ethidium bromide staining (Figure 1).

Figure 1: Genomic DNA Extracted from the Isolated Bacteria

PCR Amplification and Sequencing of the 16S rRNA Gene

The 16S rRNA genes were amplified using universal bacterial primers, corresponding to E. coli positions 27f (5′-AGRGTTTGATCMTGGCTCAG-3′) and 1525r (5′-AAGGAGGTGWTCCARCC-3′). Amplification was performed in

Veriti Thermal Cycler (Applied Bios stems, USA) in 25 µl reaction volume containing, 20–50 ng of DNA template and 1X GT PCR master mix (TaKaRa). The final volume of the PCR mixture was adjusted by nuclease free PCR grade water. A reagent blank containing all components of the reaction mixture except template DNA was included in every PCR procedure. The thermal cycling includes an initial denaturation at 95°C for 5min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min.

PCR products were analyzed by electrophoresis and purified using the QIA quick PCR purification kit (Qiagen, USA) according to the manufacturer's suggested protocol(Figure 2)Sequencing reaction of purified PCR product was performed using Big Dye Terminator v3.1 cycle sequencing kit according to manufacturer's instructions (Applied Biosystems) with primer 907r (5′-CCGTCAATTCCTTTGAGTTT-3′)and the sequences were determined using an ABI-3500 genetic analyzer (Applied Biosystems Inc., Foster City, USA). The 16S rDNA sequences were checked manually using CHROMASPRO version 1.5 software (www.technelysium.com.au/ChromasPro.html). The identification of phylogenetic neighbors was initially carried out using BLAST (Altschul *et al*., 1997) in the Gen Bank database (URL, HTTP ://www.ncbi.nlm.nih.gov), the reference 16S rRNA gene sequences of related type strains were obtained from EzTaxon server (See URL, http://www.eztaxon.org/; Chun *et al.*, 2007).

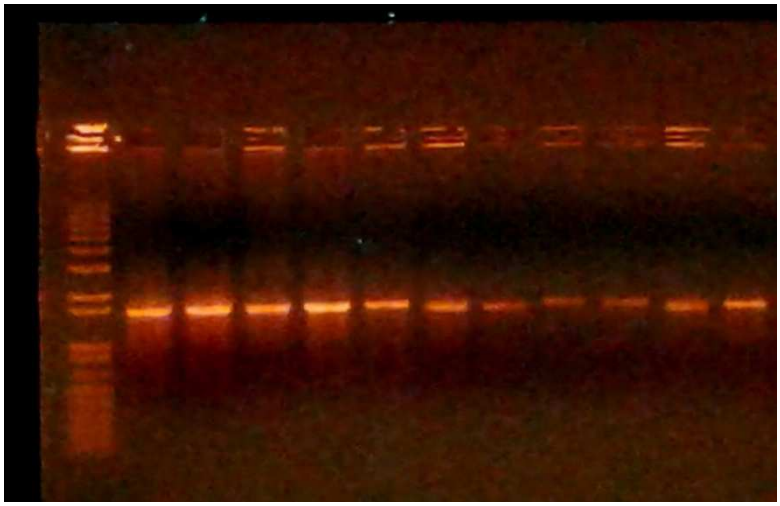

Figure 2: Purified 16S rDNA (1500bp) PCR Product of Bacterial Isolates. DNA Ladder is 1 Kb plus (Invitrogen)

Phylogenetic Analysis of Isolates

The 16S rDNA sequences of bacteria isolated from corals were searched against the NCBI and EzTaxon database containing the type strains with validly published prokaryotic names (http://www.ezbiocloud.net/eztaxon; Kim *et al.*, 2012) using BLASTN (Altschul *et al.*, 1997) program. The type strain sequences with highest identity values were selected and downloaded from NCBI. ClustalOmegaat EMBL-EBI web services (Mc William *et al.*, 2013) was used to carry out the multiple sequence alignment and corrected manually using DAMBE version 5.3.46 (Xia 2013). Evolutionary genetic distances were computed according to the algorithm of Kimura 2-parameter (Kimura 1980) and the tree topologies were inferred with Neighbor-Joining (Saitou &Nei 1987) method using MEGA program version 6.05 (Tsmura *et al.*, 2013).

RESULTS AND DISCUSSIONS

The coral samples were collected from Jolly buoy, Andaman Island was identified as *Acropora robusta, Favites abtida, Favites complanata, Favites flexuosa, Favites pentagona, Favites russelli, Goniastrea rotiformis, Montastrea colemani, Pavona venosa, Porites solida, Psammacora obtusangula.* The coral samples were identified with the help of taxonomic manuals on corals and published literature on coral taxonomy (Veron& Smith, 2000; Pillai, 1967 a, b, c, d, e, f; Pillai& Jasmine, 1989; Venkataraman *et al*., 2003).

The bacterial isolates from these corals are subjected to 16S rDNA sequences and showed homology to the genus *Vibrio alginolyticus* NBRC 15630 (CP006718), *Vibrio neocaledonicus*NC470 (JQ934828), *Vibrio Azureus* NBRC 104587 (BATL01000140) and *Ruegeria arenilitoris* G-M8 (JQ807219), in NCBI and EzTaxon database. The elucidated phylogenetic relationship (Figure 3) withstands the 16S rDNA sequence identity results (Table 1) and showed that strains formed an evolutionarily distinct lineage within the cluster comprising closely related type strains.

Table 1: List of Isolates Identified Based on the Taxonomic Assessment of 16S rDNA Sequences to the EzTaxon Type Strain Database

S. No.	Representative Isolate (Accession No.)	Closest Type Strain (Accession no.)	Sequence Similarity (%)
	Alphaproteobacteria		
1	B5c (*Acroporarobusta*..)	*Ruegeria arenilitoris* G-M8 (JQ807219)	100
	Gammaproteobacteria		
2	B1b (*Favitesabtida*)	*Vibrio azureus*NBRC 104587 (BATL01000140)	99.87
3	B1e (*Favitescomplanata*)	*Vibrio neocaledonicus*NC470 (JQ934828)	100
4	B2a (*Favitesflexuosa*)	*Vibrio alginolyticus* NBRC 15630 (CP006718)	99.62
5	B2b (*Favitespentagona*)	*Vibrio alginolyticus*NBRC 15630 (CP006718)	99.62
6	B2c (*Favitesrusselli*)	*Vibrio neocaledonicus*NC470 (JQ934828)	100
7	B3a (*Goniastrearotiformis*)	*Vibrio neocaledonicus*NC470 (JQ934828)	100
8	J1a (*Montastreacolemani*)	*Vibrio alginolyticus*NBRC 15630 (CP006718)	99.62
9	J1c (*Pavonavenosa*)	*Vibrio alginolyticus*NBRC 15630 (CP006718)	99.62
10	B5b (*Poritessolida*)	*Vibrio alginolyticus*NBRC 15630 (CP006718)	99.62
11	B4b (*Psammacoraobtusangula*)	*Vibrio azureus*NBRC 104587 (BATL01000140)	100

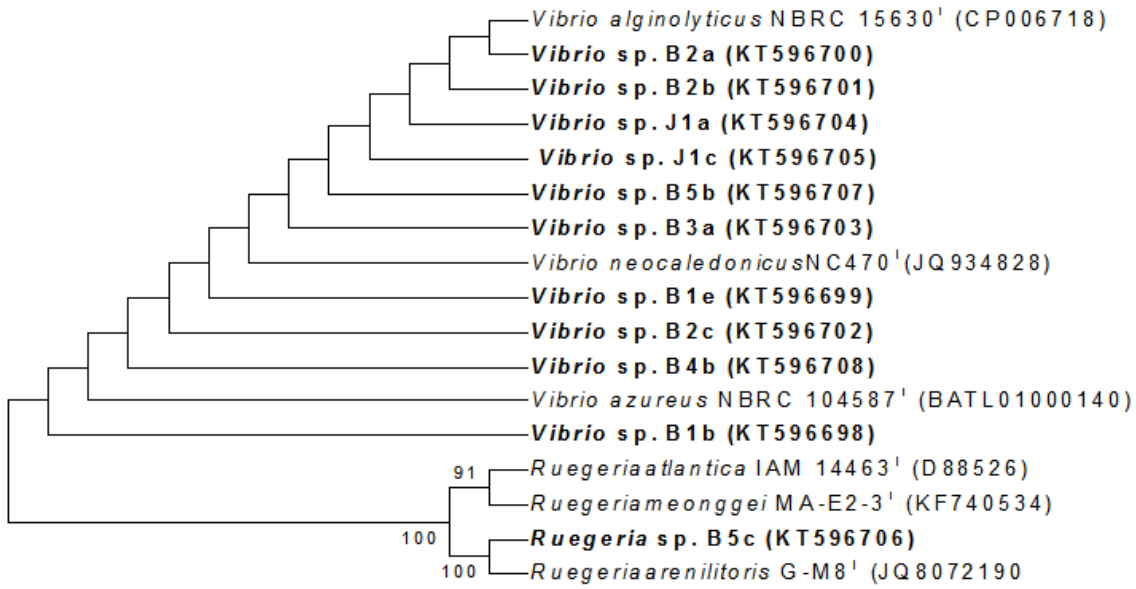

Figure 3: Neighbor-Joining Tree Based on Aligned 16S rDNA Sequences Showing the Evolutionary Position of Isolated Bacteria (Bold Facet) among the Closest Type Strains and Members of Other Related Genera. The Gen Bank Accession Numbers are given in Parentheses

Identification of bacteria by 16S ribosomal RNA gene (16S rRNA) sequences has been used extensively for molecular taxonomic studies as an attractive method. The rRNA genes are highly conserved DNA in all cells. (Boyle *et al*., 1999). The 16S rRNA gene is now most commonly used for bacterial taxonomic purposes. (Woo *et al*., 2008). Using 16S rRNA sequencing, bacterial identification is more robust, reproducible, and accurate.

In order to understand better the nature of microbial communities associated with coral, it is effective to use cloning and sequencing of 16S rRNA genes to describe the complete microbial community composition. Sabdono *et al.*, 2005 had investigated the general insights into the diversity of the bacterial community associated with the coral *Galaxea fascicularis* by the 16S rDNA sequencing. The findings had concluded that the microbial diversity on *Galaxea fascicularis* was very high.

The microorganisms cultured from the collected coral species had 16SrRNA gene sequences that were closely related to bacteria previously isolated and cultured from a variety of marine environments. Studies by Rohwer *et al*., 2001; Raina *et al*., 2009 reveals that *Pseudoalteromonas sp* and *Vibrio sp* are commonly recovered when culturing bacteria from corals indicating their ubiquity in the marine environment and symbiotic relationship with marine organisms.

Earlier studies by Barneah *et al*., 2007; Arotsker *et al*., 2009 had focused on the specific microbial group of *Vibrio sp* and 16S rRNA sequencing studies. These studies reveal that *Vibrio sp* play a vital role in BBD (Black Band Disease). According to Thompson *et al.*, 2004 *Vibrios* are known pathogens with characteristic pathogenic and virulence properties.

In our study the 16S rRNA sequencing assists to resolve the exact taxonomic position of coral bacteria and provided more detailed information on their phylogenetic position among their closest relatives. Future investigations should take into account the multifactorial nature of coral an etiology study and combine a number of molecular, physiological and histopathological tools when assessing corals and their relationship with microbes.

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