



Molecular identification of bacteria associated with the coral reef ecosystem of Gulf of Mannar Marine Biosphere Reserve using 16S rRNA sequences

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With an objective of identifying the bacterial diversity associated with coral reef ecosystem of Gulf of Mannar Marine Biosphere Reserve, bacteria were isolated from the coral tissues. Isolates exhibiting different morphological features were selected for molecular identification. Nine isolates exhibited differences in morphological features and these bacteria were subjected to molecular identification. 16S rRNA genes were successfully amplified from the genome of the selected isolates using Universal Eubacterial 16S rRNA primers. The resulted amplification products were subjected to Amplified ribosomal DNA restriction analysis (ARDRA) using *Hinf*I to identify different polymorphic groups among amplicons. The ARDRA analysis revealed five different polymorphic groups. Based on the results of ARDRA analysis, 16S rDNA amplicons of the five representative strains were subjected to automated DNA sequencing. Partial 16S rDNA sequences obtained were compared directly with sequences in the NCBI database using BLAST as well as with the sequences available with Ribosomal Database Project (RDP). The Sequence analysis revealed that four isolates belong to the *Bacillus.sp* and the fifth one being *Enterobacter cloacae*.

Introduction

Corals have been known to harbor many different species of bacteria in their gastric cavity and on their surface, where their carbohydrate rich mucus is utilized as a medium for microbial growth. Previous studies also show that there is a dynamic microbiota living on the surface and within the tissue of the corals and in the surrounding reef waters⁹. Corals harbor nitrogen fixing bacteria, which suggest that there is a symbiotic relationship between the bacteria and the coral colony, where the coral obtains fixed nitrogen from the bacterium and in turn feeds and protects the associated bacteria^{3,10}. Numerous natural products from marine invertebrates show striking structural similarities to known metabolites of microbial origin, suggesting that microorganisms (bacteria, microalgae) are at least involved in their biosynthesis or are in fact the true sources of these respective metabolites⁸.

To gain thorough knowledge on coral associated bacterial diversity and coral bacterial interactions it is important to identify and characterize the microbes present on and in the corals. Traditionally, bacterial identification has been conducted using a variety of physical and biochemical tests that allow the grouping of microbial isolates into genera and species.

The disadvantages of phenotypic identification are over reliance on a small number of subjectively chosen properties, which might lead to placing the bacteria in the wrong genus and the properties looked for are themselves inadequate for identification. Moreover phenotypic identification is time consuming, laborious and also affected by cultural conditions. Molecular identification by 16S rDNA sequence is a valuable tool for identifying and characterizing bacterial diversity and it overcomes many of the shortcomings mentioned above. In this paper we describe molecular identification of coral associated bacteria by 16S rRNA gene sequencing.

Materials And Methods

Description of Study Area

The Gulf of Mannar Marine Biosphere Reserve

The Gulf of Mannar Biosphere Reserve is situated in the southern part of the peninsular India which extends from Adams Bridge to Cape Comorin. There are 21 islands forming the 'archipelago' in this Gulf, lying between Rameswaram and Tuticorin. These islands are surrounded by coral reefs in a discontinuous manner for a distance of about 140km¹¹. The reefs are mostly fringing type arising from the shallow sea floor and the depth around here is around 6 meters. This area is remarkable for its richness and a variety of flora and fauna and it also sustains a good fishery.

Sampling Station

Hare Island

Hare Island (9^o 12' lat. and 79^o 5'E long.) spreads over an area of 120 ha. This is the largest island in the Gulf of Mannar. Extensive coral reefs are found in the south west end of the Hare Island including mangrove and other succulent plants⁷.

Sample Collection

Coral tissues were collected from the Gulf of Mannar Biosphere Reserve (Hare Islands).The coral tissues 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 sea water to remove any loosely attached bacteria⁹. The coral sample was placed in a 250 ml of conical flask containing 50 ml of sterile sea water and agitated to isolate the bacteria associated on the surface and within the coral tissue. The coral samples were serially diluted and plated on Artificial Sea Water Nutrient Agar and incubated at 28°C for 24 to 48 hours. Most of the colonies exhibited differences in the morphological features such as shape, colour and growth rate. Nine different isolates exhibiting different morphological features were selected and subjected to molecular cataloging.

Molecular identification of coral associated bacteria

Genomic DNA Extraction

The selected colonies were inoculated in Artificial Sea water Nutrient broth and incubated overnight at 28°C. The culture was spun at 7000 rpm for 3 min. The pellet was resuspended in 400 µl of SucroseTE. Lysozyme was added to a final concentration of 8 mg/ml and the solution was incubated for 1h at 37°C. To the tube, 100 µl of 0.5M EDTA (pH 8.0), 60 µl of 10% SDS and 3 µl of proteinase K were added and incubated at 55 °C overnight. Extracted with equal volume of phenol: chloroform (1:1), centrifuged (10000 rpm; 10 min) and the supernatant was transferred to a sterile tube. The supernatant was extracted twice with phenol: chloroform and once with chloroform: isoamylalcohol (24:1) and ethanol precipitated. The DNA pellet was resuspended in sterile distilled water and stored at 4°C for immediate use and at - 20°C for long-term storage.

PCR Amplification of 16S rDNA

Bacterial 16S rDNA was amplified from the extracted genomic DNA by using the following universal eubacterial 16S rRNA primers: forward primer 5' AGAGTTTGATCCTGGCTCAG 3' and reverse primer 5' ACGGCTACCT TGTTACGACTT 3'¹². Polymerase chain reaction was performed with a 50-µl reaction mixture containing 2 µl (10 ng) of DNA as the template, each primer at a concentration of 0.5 µM, 1.5 mM MgCl₂, and each deoxynucleotide triphosphate at a concentration of 50 µM, as well as 1 U of *Taq* polymerase and buffer used as recommended by the manufacturer (MBI Fermentas). After the initial denaturation for 4.5 min at 95°C, there were 40 cycles consisting of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min and then a final extension step consisting of 5 min at 72°C; Mastercycler Personal (eppendorf, Germany) was used. The amplification of 16S rDNA was confirmed by running the amplification product in 1% agarose gel electrophoresis in 1X TAE.

Amplified ribosomal DNA restriction analysis (ARDRA)

To examine the ARDRA profile, 10µl of the PCR product was digested with *Hinf*I restriction enzyme at 37 °C for 3hrs. Digested DNA were analysed by submarine horizontal electrophoresis in 2% agarose gel. The 100 bp ladder (New England BioLabs Inc.,) was used as DNA marker.

Purification of 16S rDNA amplicon

The amplified 16S rDNA products were run in 1% preparative agarose gel electrophoresis .The amplified 1,500-bp product was purified from the gel slice using GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences) according to manufacturer's instruction.

Sequencing of 16S rRNA genes

Sequencing reaction was carried out using ABI PRISM 310 Genetic Analyzer (PE applied Biosystems). For sequencing reaction Big Dye Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Perkin-Elmer) was employed.

Sequence analysis

Sequence analysis was performed with sequences in the NCBI database using BLAST as well as with the sequences available with Ribosomal Database Project (RDP)⁴.

Phylogenetic analysis

Sequences were aligned by using the Clustalw program and phylogenetic tree was constructed using software from the PHYLIP package².

Results

Sample collection and Coral species identification

Isolation of bacteria from coral tissues

Coral samples were collected from Hare islands, Gulf of Mannar Marine Biosphere Reserve. These coral samples were identified as *Acropora latistella*, *Acropora digitifera* and *Acropora spicifera*. Bacteria were isolated from coral tissues. To begin with, nine different isolates exhibiting different morphological features were selected and subjected to molecular cataloging. Morphological features like shape, color and size of the colonies were considered.

Amplification of 16S rRNA gene

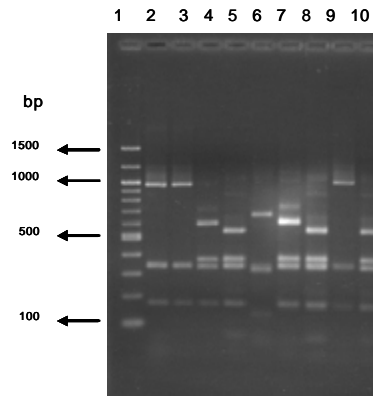
16S rRNA genes were successfully amplified from genome of all nine coral associated bacteria using universal eubacterial 16S rRNA primers: forward primer 5' AGAGTTTGATC CTGGCTCAG 3' and reverse primer 5' ACGGCTACCTTGTTACGACTT 3'.

ARDRA analysis

In order to differentiate similar isolates, all 9 isolates were examined by Amplified Ribosomal DNA restriction Analysis. The 16S rDNAs were amplified by PCR using universal primers and the amplified product was cleaved with the restriction enzyme *Hinf*I. The ARDRA profile obtained with *Hinf*I revealed the presence of five different polymorphic groups among the nine isolates (Fig1.) ARDRA profile revealed that Lanes 2, 3 and 9 had similar band patterns, belonging to one group. Lanes 5, 8 and 10 were found to constitute the second polymorphic group. Lane 4, 6 and 7 possessed a unique ARDRA pattern. From each group, one isolate was selected for 16S rRNA gene sequencing.

Sequencing of 16S rDNA gene of coral associated bacteria

Based on the result of ARDRA profiles, 16S rDNA amplicons of the five representative strains were selected for sequencing. Sequencing reaction was carried out using ABI PRISM 310 Genetic Analyzer (PE applied Biosystems). For sequencing reaction Big Dye Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Perkin-Elmer) was employed. Partial sequences of about 300-400 bases from both ends were obtained from the 1.5kb amplicon (16S rRNA gene sequences corresponding to approximately positions 8 - 1494 of *E. coli* numbering). Sequences obtained were matched with previously published bacterial 16S rDNA sequences available in NCBI using BLAST and the Ribosomal Database Project (RDP). Sequence analysis results revealed that among the five different polymorphic groups, four sequences (HCTB -1,3,4 and 6) matched with *Bacillus sp* and the remaining one sequence matched with *Enterobacter cloacae* (HCTB -2).



Lane1: 100bp DNA Ladder (NEB)
Lane: 2 -10: ARDRA profile of nine coral associated bacteria

Figure 1: Amplified ribosomal DNA restriction analysis of coral associated bacteria

Phylogenetic Analysis

The sequences were aligned using clustalw and a phylogenetic tree was constructed.

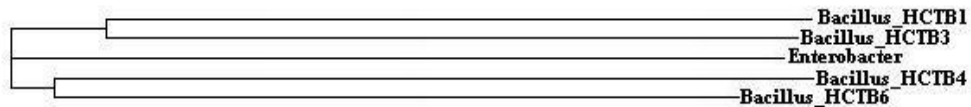


Figure2: Phylogenetic analysis of coral associated bacteria

Discussion

Coral reefs have proven to be a chemical repository for many bioactive compounds. Many of these compounds show structural similarity with compounds from bacteria of terrestrial origin⁸. To ensure that these bioactive compounds are of bacterial origin and to tap this valuable source, bacterial identification is of utmost importance. Molecular identification of coral bacteria might prove valuable in identification of these bacteria. In this study, we have identified coral associated bacteria by amplifying the 16S rRNA gene from the genome of coral associated bacteria.

The five separate restriction fragment length polymorphism groups obtained by Amplified ribosomal DNA restriction analysis reveal that there are different bacteria population associated within the coral reef. Sequencing of the five unique amplicons and comparison with the available databases revealed the presence of both gram positive (*Bacillus sp*, N=4) and gram negative bacteria (*Enterobacter cloacae*, N=1). This indicates the fact that both Gram positive and Gram negative bacteria are associated with the coral tissue and can coexist in that environment. The phylogenetic tree also indicates the presence of four distinct strains in the Bacillus group. Tree constructed using the full length sequence will throw more light on the nature of diversity.

Presence of *Bacillus sp* in the coral tissues promises much useful enzymes/bioactive compounds for exploration. Many bioactive compounds such as bacilysin, subtilisin, subtilancin, surfactin and bacilysin have already been derived from *Bacillus subtilis* which can be used as antibacterial and antifungal agents^{5, 6}. Moreover enzymes can also be tapped from these marine bacteria and employed in high salt environment where enzymes from terrestrial bacteria are rendered inactive/ineffective. *Enterobacter cloacae* could be used as a potential biocontrol agent against plant pathogen.

Typing of the remaining bacterial isolates of the coral reef tissue available in hand and samples to be collected from that region is expected to reveal the possible rich diversity associated with the coral reef ecosystem of the Gulf of Mannar Marine Biosphere Reserve. PCR amplification, ARDRA analysis and sequencing lead to the proper identification and the true bacterial genera present in coral reef ecosystem. This in a way overcomes the disadvantages of conventional phenetic system which is time consuming and might also result in classifying the Genera in the wrong group. This study once again emphasizes the importance of employing molecular techniques for assessing bacterial diversity and to gain knowledge about the microbial world beneath the sea.

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