**Streptomyces** associated with marine sponges collected from Andaman Sea and its antibacterial activity

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**ABSTRACT**

**Aims:** Sponges harbour diverse prokaryotic and eukaryotic microbes. However, diversity of sponge-derived *Actinomycetes* is of high interest because of its bioactive compounds. In the present study, diversity of *Streptomyces* associated with marine sponges collected from Pongibalu (south Andaman) was investigated.

**Methodology and results:** Sponges samples were collected by underwater SCUBA diver and Kuster’s agar media was used for isolation of actinobacteria. Colony morphology and 16s rRNA were studied for identification of isolates and phylogenetic tree was constructed using MEGA.6. A total of ten *Streptomyces* species were identified based on 16S rRNA gene sequencing from three sponge species (*Hemisterella* spp. *Tentorium* spp. and *Tethyopsis* spp.). The organic extracts of these ten isolates revealed bioactivity against tow Gram positive and eight Gram negative pathogenic bacteria.

**Conclusion, significance and impact of study:** This study suggests prospects and potentials of the diverse population of *Streptomyces* with bioactivity in marine sponges. It would be a potential source in the pharmaceutical industries. As well as actinobacteria associated with sponge may prevent the sponge from pathogenic bacteria, fungi, virus and other microflora by secretion of secondary metabolites on surface and inside. To understand the sponge and actinobacteria association and its bioactivity, a profound study need to be conducted.

**Keywords:** Marine sponges, *Streptomyces*, diversity, bioactivity, Andaman Islands

**INTRODUCTION**

Sponges (*Porifera*) are the ancient multicellular phylum with a fossil record dating back to Precambrian times (Hentschel et al., 2012). Sponges populate tropical reefs in great abundance (Schmitt et al., 2012), and are commonly known to harbour diverse microbes (Hentschel et al., 2006; Wang et al., 2006). Studies show these are phylogenetically diverse, yet highly sponge-specific, prokaryotic microbial groups. Actinomycetes have been cultivated from the marine environment including sea water (Zhang et al., 2012), marine snow, and marine sediments (Becerril-Espinosa et al., 2013). So far, at least 32 bacterial phyla and candidate phyla were described from marine sponges by both cultivation-dependent and cultivation-independent techniques; with the most common phyla being *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Cyanobacteria*, *Gemmatusimonadetes*, *Nitrospira*, *Planctomycetes*, *Proteobacteria* (Alpha, Delta, Gamma subclases) and *Spirochaetes* (Hentschel et al., 2012; Schmitt et al., 2012). *Actinomycetes* have also been cultivated from different marine invertebrates (Abdelmoohsen et al., 2010; Sun et al., 2012; Wyche et al., 2012), with the majority being isolated from sponges (Zhang et al., 2006a; Xi et al., 2012). Marine *actinomycetes* produced a multitude of novel lead compounds with biotechnological and pharmaceutical applications.

The genus *Streptomyces* of the family *Streptomycetaceae* contains the largest number of species among the genera of the *Actinomycetales* (Goodfellow et al., 1992). Because of the importance of the *Streptomyces* as a source of novel bioactive compounds, more than 3000 *Streptomyces* species have been proposed. Biological activities such as antibacterial, antifungal, antiparasitic, antimalarial, immunomodulatory, anti-inflammatory, antioxidant, and anticancer activities were reported from sponge-
associated actinomycetes (Bull and Stach, 2007; Pimentel-Elardo et al., 2010; Abdelmohsen et al., 2012; Blunt et al., 2013). Actinomycetes account for approximately half of the natural products [MarinLit database 2013 (John Blunt, Marin Lit, University of Canterbury, New Zealand)] (Lam, 2006; Thomas et al., 2010). A total of 411 natural products from marine Actinomycetes were reported in the Marin Lit database in 2013 (J. Blunt, Marin Lit, University of Canterbury, New Zealand) of which 22% were obtained from marine sponge-associated Actinomycetes (Abdelmohsen et al., 2014). At present, the finding of rare or novel marine Actinomycetes has become a major focus in the search for the next generation of pharmaceutical agents (Bull et al., 2000). Andaman and Nicobar islands have a rich source for flora and fauna diversity, a total of 88 species of sponges was reported from Andaman and Nicobar islands. But very few reports are available pertaining to sponge associated bacteria especially actinobacteria from Andaman and Nicobar Islands. In this study, Streptomyces were isolated from marine sponge species collected from the Andaman Islands. The obtained Streptomyces were phylogenetically characterized based on 16S rRNA gene sequencing and their biological activities against pathogenic bacteria are reported.

MATERIALS AND METHODS

Sponge collection

Three sponge species (Hemiasterella sp., Tentorium sp. and Tethyopsis sp.) were collected by SCUBA diving at depths of 5-10 m in Pongibalu, south Andaman Island. Sponges were transferred to plastic bags containing sterile sea water and transported to the laboratory. Sponge specimens were rinsed in sterilized sea water and ddH₂O, cut into pieces of 1 cm³ and thoroughly homogenized in a sterile mortar with 10 volumes of sterile sea water. The supernatant was diluted in 10-fold series and subsequently plated out on agar plates.

Streptomyces isolation and identification

Kusters agar and actinomycetes agar was supplemented with 30 μg/mL of the fungicide cyclohexamide and 20 μg/mL nalidixic acid to facilitate the isolation of actinobacteria and deter the growth of sponge associated fungi and gram negative bacteria on the plates. Aliquots of 100 μL were transferred from each sponge homogenate dilution to the two selective media in triplicate. The plates were incubated at 28 °C for 3-4 weeks until actinomycetes were observed growing on the plates. Individual colonies were isolated and plated on new agar plates with the same media that was used for their initial growth. Following this procedure, actinomycetes were isolated and identified through sequencing of the 16S rRNA gene.

Isolation of DNA and PCR amplification

Streptomyces isolates were cultured in Kuster’s agar. The genomic DNA was extracted following the procedure described by Ausubel et al. Polymerase chain reaction (PCR) was performed using 16S rRNA primers Forward 5'- AGA GTT TGA TCM TGG CTC AG -3', Reverse 5'-ACG GCT ACC TTG TTA CGA CT -3' following the standard protocol (Lane et al., 1985). The amplified products were sequenced using DNA analyzer 3730 (Applied Biosystems, CA, USA). The partial 16S rRNA gene sequences and sequences of closely related strains in the NCBI database were retrieved and assembled using MEGA 5 software (Tamura et al., 2011) and the unknown sequences were identified using the CLUSTALW alignment tool of MEGA 5.

Test bacterial strains for bioactivity

Gram positive bacteria (Bacillus spp. MTCC-3133 and Staphylococcus aureus MTCC-3160) and Gram negative bacteria (Klebsiella pneumoniae sub MTCC-3040, Salmonella infantis MTCC-1167, Lactococcus lactis sub spp. 1-440, Escherichia coli (Electro aggregative) ICMR, Vibrio cholerae 01 Inaba - ICMR, Pseudomonas spp. (KU), Proteus spp. MTCC- 425, Citrobacter spp. (KU) and Shigella flexneri.

Screening for bioactivity

The Streptomyces spp. were inoculated into a 5 L Halfman flask containing 2.5 L production media broth (dextrose- 20 g, soya bean- 20 g, soluble starch-5 g, peptone- 5 g, (NH₄)₂SO₄- 2.5 g, MgSO₄-7H₂O- 0.25 g, K₂HPO₄- 0.02 g, NaCl- 4 g, CaCO₃, 2 g, seawater- 500 mL, ddH₂O- 500 mL and pH 7±0.2), and incubated at 28±2 °C on a rotary shaker at 250 rpm for seven days. The fermented broth was centrifuged at 10000 rpm at 4 °C for 20 min and the supernatant was filtered using 0.45 μm filter (Millipore). An equal volume (1:1) of the organic solvent (ethyl acetate) was added to the cell free culture filtrates and mixed on a shaker for 12 h, the solvent was separated and the extract was evaporated using a rotary evaporator and the crude powder was collected as antimicrobial compound. The crude powder was mixed with DMSO and used to test the activity against tested pathogens as well as DMSO alone was maintained as a control.

RESULTS

Isolation and phylogenetic identification

A total of 37 actinobacteria were isolated from three sponges in two used media, among them 16, 12 and 9 were observed in Hemiasterella sp., Tentorium sp. and Tethyopsis sp. respectively. Off 37 only ten isolates were found to be distinguished on the colony morphology and aerial and substrate spore color. Among the ten 4, 3 and 3 were obtained from Hemiasterella sp., Tentorium sp. and
Tethyopsis sp. respectively. These Streptomyces were observed under light microscope (400×) for its confirmation before 16S rRNA analysis. The microscopic observation proved distinguished morphology among them. Distinguished Streptomyces were depicted with Gene bank accession number in Table 1.

DNA based molecular methods have been used for species differentiation and the identification of Streptomyces. Significance of phylogenetic studies based on 16S rDNA sequences enhanced the knowledge on the systematics of actinobacteria (Yokota, 1997). Sequences of 16S rDNA have provided actinobacteriologists with a phylogenetic tree that allowed the investigations on the evolution of actinobacteria and also provides the basis for identification. In the present study, variations in the 16S rDNA sequences of ten sponge associated different Streptomyces spp. were observed. The phylogenetic analysis of partial 16S rDNA sequence showed maximum similarity with Streptomyces and no similarity among the sponge isolated Streptomyces genus. The same was analysed with various genus of actinobacteria and other Gram positive and negative bacteria, it was distinguished from the Gram negative and positive bacteria when it shows maximum similarity with Streptomyces and moderately with other actinobacterial species (Figure 1). It shows different species of Streptomyces associated with marine sponges.

**Antibacterial activity of ethyl acetate extract of Streptomyces**

All the morphologically distinguished strains were tested against two Gram positive and eight Gram negative pathogenic bacteria. The organic solvent extract of the Streptomyces spp. showed activity against most of the tested pathogens. Among the isolates, Streptomyces sp.: DOSMB SP10 showed maximum activity in range of 14.33mm to 40mm against all the test pathogens except *S. infantis*. SP3, SP5, SO7, SP9, SP11, SP20, SP23, SP24 also showed activity against most of the tested pathogens (Table 2). Almost all isolate showed activity against both Gram positive and negative bacteria.

**Table 1:** Identification of the sponge associated bacterial isolates by alignment with 16S rRNA gene sequences of published bacterial species in the NCBI database.

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Host sponge spp.</th>
<th>Sequence length (bp)</th>
<th>Closest published database accession</th>
<th>Proposed identity</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOSMB-SP9</td>
<td>Hemiasterella spp.</td>
<td>565</td>
<td>KF477115</td>
<td>Streptomyces xiamenensis</td>
<td>KJ933452</td>
</tr>
<tr>
<td>DOSMB-SP7</td>
<td>Hemiasterella spp.</td>
<td>772</td>
<td>NR109175</td>
<td>Streptomyces abyssalis</td>
<td>KJ933451</td>
</tr>
<tr>
<td>DOSMB-SP6</td>
<td>Hemiasterella spp.</td>
<td>753</td>
<td>KT163794</td>
<td>Streptomyces thermocarboxydus</td>
<td>KJ933450</td>
</tr>
<tr>
<td>DOSMB-SP24</td>
<td>Hemiasterella spp.</td>
<td>694</td>
<td>NR044534</td>
<td>Streptomyces nansensis</td>
<td>KJ933449</td>
</tr>
<tr>
<td>DOSMB-SP5</td>
<td>Tentorium spp.</td>
<td>534</td>
<td>EU158356</td>
<td>Streptomyces spp.</td>
<td>KJ933448</td>
</tr>
<tr>
<td>DOSMB-SP10</td>
<td>Tentorium spp.</td>
<td>766</td>
<td>AB498661</td>
<td>Streptomyces spp.</td>
<td>KJ933447</td>
</tr>
<tr>
<td>DOSMB-SP20</td>
<td>Tentorium spp.</td>
<td>694</td>
<td>KF444511</td>
<td>Streptomyces plicatus</td>
<td>KJ933446</td>
</tr>
<tr>
<td>DOSMB-SP23</td>
<td>Tethyopsis spp.</td>
<td>653</td>
<td>HQ238361</td>
<td>Streptomyces sindenensis</td>
<td>KJ933445</td>
</tr>
<tr>
<td>DOSMB-SP11</td>
<td>Tethyopsis spp.</td>
<td>723</td>
<td>KM215732</td>
<td>Streptomyces cyaneus</td>
<td>KJ933444</td>
</tr>
<tr>
<td>DOSMB-SP3</td>
<td>Tethyopsis spp.</td>
<td>863</td>
<td>KJ002642</td>
<td>Streptomyces gancidicus</td>
<td>KJ933435</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Marine ecosystems consist of taxonomically and biologically diverse macro- and microorganisms which exhibit unique physiological and structural features enabling them to survive under the extremes of pressure, salinity and temperature. Many marine organisms are further endowed with the ability to produce novel molecules with interesting therapeutic applications not observed in their terrestrial counterparts (Lane, 2008; Li and Vederas, 2009; Rateb and Ebel, 2011).

Andaman and Nicobar Islands are the one of the most biodiverse environment in Indian Ocean. However, few studies have been carried out so far to investigate actinobacterial communities from sponges of Andaman Islands. *Actinomycetes* are known for their unprecedented ability to produce novel lead compounds of clinical and pharmaceutical importance. The most powerful approach to solve the taxonomic problems of actinobacteria is the study of nucleic acids. Comparison of nucleic acids yields considerable information on the true relatedness. Molecular systematic which includes both classification and identification, has its origin in the early nucleic acid hybridization studies has achieved a new status following the identification of nucleic acid sequences through sequencing techniques (Brenner *et al.*, 2001).
Figure 1: The inferred phylogenetic tree showing the diversity of 10 sponge associated *actinomycetes* isolates, including 31 reference strains. Evolutionary distances were determined with pairwise dissimilarities of the 16S rRNA gene sequences and the dendrogram was generated using the neighbor-joining algorithm (MEGA5). Closed sphere closed triangle, closed square indicate isolated from *Hemiasterella* spp., *Tentorium* spp. and *Tethyopsis* spp. respectively.
Table 2: Antimicrobial activity of organic solvent extract of *Streptomyces* spp.

<table>
<thead>
<tr>
<th>Test pathogens</th>
<th><em>Streptomyces</em> spp. Mean values are presented (Zone of inhibition mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SP3</td>
</tr>
<tr>
<td>Gram positive</td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>17.33±</td>
</tr>
<tr>
<td></td>
<td>±0.19</td>
</tr>
<tr>
<td><em>Bacillus</em> spp.</td>
<td>21.66±</td>
</tr>
<tr>
<td></td>
<td>±0.19</td>
</tr>
<tr>
<td>Gram negative</td>
<td></td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>17.66±</td>
</tr>
<tr>
<td></td>
<td>±0.19</td>
</tr>
<tr>
<td><em>S. infantis</em></td>
<td>15.33±</td>
</tr>
<tr>
<td></td>
<td>±0.19</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>14.66±</td>
</tr>
<tr>
<td></td>
<td>±1.9</td>
</tr>
<tr>
<td><em>V. cholerae</em></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>±1.9</td>
</tr>
<tr>
<td><em>S. flexneri</em></td>
<td>16.66±</td>
</tr>
<tr>
<td></td>
<td>±0.19</td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td>13.66±</td>
</tr>
<tr>
<td>spp.</td>
<td>±0.19</td>
</tr>
<tr>
<td><em>Proteus</em> spp.</td>
<td>8.66±</td>
</tr>
<tr>
<td></td>
<td>±0.19</td>
</tr>
<tr>
<td><em>Citrobacter</em></td>
<td>11.33±</td>
</tr>
<tr>
<td>spp.</td>
<td>±0.19</td>
</tr>
<tr>
<td><em>Bacillus</em> spp.</td>
<td>21.66±</td>
</tr>
<tr>
<td></td>
<td>±0.19</td>
</tr>
</tbody>
</table>

A total of 10400 actinomycete 16S rRNA gene sequences were thus far obtained by isolation from marine sources. This compares to about 36000 16S rRNA gene sequences from terrestrial Actinomycetes, which, however, have a much longer history of exploration. Actinomycetes were found in association with different marine invertebrates such as sponges, tunicates and fish (Bull et al., 2005; Abdelmohsen et al., 2010; Sun et al., 2012; Wyche et al., 2012) but the majority was isolated from sponges (Hentschel et al., 2003; Zhang et al., 2006a; Selvin et al., 2010).

It has long been known that actinomycetes can be cultured from marine sources (Grein and Meyers, 1958) yet it was not clear whether these typically soil-derived bacteria should be considered as terrestrial “contaminants” or as true components of the marine ecosystem (Weyland, 1969). Early evidence for the existence of indigenous marine actinomycete populations came from the description of the first marine species, *Rhodococcus marinonascenc* in 1984 (Helmke and Weyland, 1984), the metabolic activity of some *Streptomyces* strains in marine sediments (Moran et al., 2012).
1995) and the isolation of obligate marine strains (Jensen et al., 1991).

In the present study, we have isolated ten phylogenetically distinguished Streptomyces species from three sponges of Andaman Islands with antibacterial properties. Similarly, Abdelmohsen et al. (2014) has reported the diverse actinobacteria associated with marine sponges of Red Sea. During the investigation of actinobacterial diversity from intertidal marine sponges, a novel actinobacterium, Actinoalloteichus hymeniacidonis was isolated from *Hymeniadon perleve* (Zhang et al., 2006b). Similarly, novel species such as *Streptomyces halclionae*, *S. marinus*, and *S. tatayamensis* were isolated from the marine sponge, *Haliclona* sp. (Khan et al., 2011). However, very few reports were available for actinobacteria associated with *Hemiasterella* spp. and *Tentorium* spp. and *Tethyopsis* sp.

Abdelmohsen et al., (2014) and others had also been reported bioactivity of sponge associated actinomycetes against variety of Gram positive and negative bacteria, fungi, human parasites and virus. Understandable in the present study all the ten Streptomyces isolates showed activity against least of eight out of ten test pathogen used. Pimentel-Elardo et al. (2011) obtained tetracyclins 1-4 with antibacterial potential from marine sponge *Axinella polypoides* associated *Streptomyces axinellae* Pol001T. Similarly, lobophorin C and D with cancer activity was purified from *Streptomyces carnosus* strain AZS17 associated with marine sponge *Hymeniadon* sp. (Wei et al., 2011). Mayamycin with cytotoxicity and antibacterial activity was isolated from *Streptomyces* sp. strain HB202 associated with marine sponge *Halichondria panacea* (Schnemann et al., 2010). Gao et al. (2010) obtained *Streptomyces* sp. DA18 with antimicrobial activity from marine sponge. Likewise *Salinispora* sp. strain M403 with antibacterial activity was isolated from marine sponge *Pseudoceratina clavata* (Kim et al., 2006). *Brevibacterium* sp. KMD 003 with antibacterial potential was isolated from marine sponge *Callyspongia* sp. (Simmons et al., 2011). As well as Karupiah et al. (2015) reported that sponge-associated actinobacteria are the largest source of natural *Callyspongia* sp. (Simmons et al., 2011) products, which account for about 56% followed by mollusk and mangrove-derived actinobacteria. In comparison to actinomycetes from other environmental sources, the sponge associated actinomycetes are remarkably rich, with higher chances of finding new chemotypes and fewer problems with rediscovery (Abdelmohsen et al., 2014).

**CONCLUSION**

In conclusion, this study on streptomycetes associated with three marine sponges from the Andaman Islands has revealed the future prospect of finding diverse microbiota in this unexplored ecosystem. Present and previous studies explored the diversity of actinobacteria with potential bioactivity in the marine fauna (Sponge). However, to understand the biodiversity of marine flora and fauna associated actinobacteria with special reference to industrial application has to be explored for novel secondary metabolites.

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