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Original Article

Molecular analysis of sponge associated bacteria in Gulf of Mannar Coast and their antibacterial activity against fish pathogens

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Abstract

Marine bacteria are a rich source of potentially useful antibacterial substance producer. However, much of the microbial diversity in marine ecosystems with its potential for uncovering new antimicrobial compounds remains to be discovered. One hundred and nine bacterial strains associated with fourteen species of sponges were isolated from the Gulf of Mannar Coast, India. The double agar overlay methods was used to screen for antibiotic production by these strains against five fish pathogens, viz. *Vibrio fischeri, Vibrio vulnificus, Vibrio harveyi, Aeromonas hydrophila* and *Aeromonas sobria*. Fifteen percent of the bacterial strains were found to be antibiotic producers and their activities ranged from broad spectral to species specific. A strain coded SAR11 was found to be highly potent against the target strains. Phylogenetic analysis based on comparative analysis of sequenced 16S rRNA of the active strains indicated a preponderance of bacteria belonging to *Bacillus* and *Alteromonas* genera with 90 – 95% sequence similarities. To our knowledge this is the first report on Phylogenetic identification of antibiotic production bacteria associated with fourteen species of sponges from Indian waters.

Keywords: Sponge associated bacteria, antibacterial activity, fish pathogens, Phylogenetic identification.

Introduction

Surface-attached bacteria grow on submerged biotic and abiotic surfaces in the marine environment (Dunne, 2002). Sponges are also known to maintain numerous bacteria which can amount to up to the biomass of a sponge (Vacelet & Donadey, 1977). It has been reported that the ratio of bacteria with antimicrobial activity isolated from marine invertebrates was higher than that from other sources (Burgess *et al*., 1999). Marine invertebrates in particular have diverse communities of attached acteria on their surfaces (Rohwer *et al*., 2002). This diversity may be due to several factors including surface specific interactions (Rohwer *et al*., 2002; Tayalor *et al*., 2004; Webster and Bourne 2007). Numerous natural products from marine invertebrates show striking structural similarities to metabolites of microbial origin, suggesting that microorganisms are the true source of these metabolites or are intricately involved in their biosynthesis (Proksch *et al*., 2002). Bacteria associated with marine invertebrates might be a chemical defense for their cost. Considering the cooperative relationships between bacterium and sponge in chemical defense, there is further potential to seek for natural products in bacteria associated with marine invertebrates.

The marine sponges found in shallow water in the Gulf of Mannar, India, are almost free of fouling organisms and at the same time have proven to be a rich source of interesting metabolites. Many sponge or sponge sumbiont-derived metabolites are potent antibacterial, antifungal, antifeeding and antifouling compounds (Becerro *et al*., 1997). Some marine products originally isolated from macroorganisms, such as sponges, have been subsequently discovered to be present in microbial associates (Bewley *et al*., 1996). The role of sponge associated microorganisms in the synthesis of compounds of biological interest is a subject of scientific debate, as in some case these bacteria are reported to produce bioactive metabolites (Imhoff and Stohr, 2003).

Many previous studies on antimicrobial activity have tested sponge metabolites against terrestrial or medically important strains in order to search for novel antimicrobial agents. But metabolites may affect ecologically relevant bacteria in a different manner. From an

ecological point of vies, it is also important to test sponge surface associated bacteria against bacteria that sponges would naturally encounter in the environment. The aim of this study was to isolate bacteria with antimicrobial actives from marine sponges and to identify potent bacterial strains using a 16S rRNA phylogenetic analysis.

Materials and methods

Collection of samples

Fourteen species of sponges (*Acanthella ramosa, Sigmadocia fibulatus, Mycale mannarensis, Axinella carteri, Callyspongia fibrosa, Epipolasis topsenti, Petrosia testudinaria, Psammaplysilla purpurea, Plakortis simplex, Fasciospongia cavemosa, Stelletta tenui, Dysidea fragilis, Carniella australiensis* and *Dysidea avara*) from a depth of 5 – 10m were collected by SCUBA diving at Hare Island and Rameswaram region, South East coast of India. The samples were placed inside sterile ethyl polythene bags underwater and transferred to the lab aseptically in ice boxes. The sponges were identified as species level in Fisheries College and Research Institute, Thoothukudi, India

Isolation of marine bacteria

Sponge associated bacteria were isolated by following the method outlined by Santavy *et al*. (1990). Initially, the sponge samples were washed with jets of filtered and autoclaved seawater until they were visibly free of debris. Then the sponge surface was sterilized by a rapid wash of 70% ethanol and immediately immersed in autoclaved and filtered seawater and then aspirated. One gram of sponge tissue was removed with a sterile scalpel and then aspirated. One gram of sponge tissue was immediately transferred to 99ml sponge dissociation medium (2.7% NaCl, 0.008% KCl, 0.01% Na_2SO_4, pH 8.0). The samples were soaked for 20 min and then the tissue and diluents were macerated and the homogenate was plated on Zobell marine agar 2216 (Himedia, Mumbai), using a dilution series of 10^{-5}. The plates were incubated at room temperature (approx.27 – 30^o C) for 7 days and isolation of bacteria with different colony characteristics was carried out from the third day onwards up to the seventh day. Day 7 counts were used for the calculation of colony forming units (CFU). The isolated colonies were repeatedly streaked to obtain pure cultures and stored in Zobell agar slants at 4^o C for further studies.

Biochemical identification of sponge associated bacteria

All the isolated bacteria were identified by performing various biochemical tests according to Bergey's manual and Lampert *et al*. (2006). The sponge bacterial isolates were subjected to various morphological and biochemical tests. Carbohydrate tests were performed using the Hicarbohydrate kit (Himedia Laboratories; Cat. No. KB009).

Antibacterial activity of sponge associated bacteria

The bactericidal effects of sponge associated bacterial strain were tested against fish pathogens like (*Aeromonas sobria* (MTCC 1608), *Aeromonas hydrophila* (MTCC 1608), *Vibrio fischer* (MTCC 1738) and *Vibrio vulnificus* (ATCC 29307) by double layer method described by (Riquelme *et al*., 1997). Briefly, MZA plates were spot inoculated with 5 µl of overnight cultures of each bacterial strain to be tested. Following incubation for 24 h at room temperature, the developed colonies were killed with chloroform vapour over a period of 45 min. These plates were overlaid using 6 ml of TSB supplemented with 1% NaCl (salt used depending on growing strain) and 0.9% agar, containing 100 µl of a 1/10 dilution of 12 h culture of fish pathogens. After 24 h of incubation, antagonistic effects of bacterial strains tested were observed by measuring the zone of inhibition, which appeared as clearance zone around these bacterial colonies. A minimum six plates was used for each assay. The results of minimum detectable concentration obtained were based on the means of triplicate.

Genomic DNA extraction from marine organisms associated bacteria

The bacterial cultures grown on Zobell Marine broth overnight at 27 ºC was centrifuged at 4600 g for 3 minutes. Bacterial genomic DNA was isolated according to Babu *et al*. (2009). The pellet was resuspended in 400 µl of Sucrose TE. Lysozyme was added to a final concentration of 8mg/mL^{-1} and incubated for 1 h at 37 ºC. To the tube, 100 µl of 0.5M EDTA (pH 8.0), 60 µl of 10% SDS and 3µl of proteinase K from 20mgmL^{-1} were added and incubated at 55 ºC overnight. The supernatant was extracted twice with phenol:chloroform (1:1) and once with chloroform : isoamylalcohol (24:1) and ethanol precipitated. The DNA pellet was resuspended in sterile distilled water.

Amplification of 16S rRNA gene from bacteria and actinomycetes

Bacterial 16S rRNA gene was amplified from the extracted genomic DNA using the following universal eubacterial 16S rRNA gene primers: forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' (*E. coli* positions 8–27) and reverse primer 5'-ACGGCTACCTTGTTACGACTT-3' (*E. coli* positions 1492–1513). PCR was performed in a 50 µl reaction mixture containing 2 µl (10ng) of DNA as the template, each primer at a concentration of 0.5 µM, 1.5mM MgCl2 and each deoxynucleoside triphosphate at a concentration of 50 µM, as well as 1U of Taq polymerase and buffer as recommended by the manufacturer (MBI Fermentas). After the initial denaturation for 3 min at 95 ºC, 40 cycles consisting of denaturation at 95 ºC for 1min, annealing at 55 ºC for 1min and extension at 72 ºC for 2min, and a final extension step of 5min at 72 ºC were carried out (Mastercycler Personal, Eppendorf, Germany). The amplification of 16S rRNA gene was confirmed by running the amplification product in 1% agarose gel in 1 x Tris-acetate-EDTA.

Amplified ribosomal DNA restriction analysis (ARDRA)

With the objective of determining bacterial diversity, all the 16S rRNA gene amplicons representing various isolates were subjected to ARDRA. To examine the ARDRA profile, 10µl of the PCR product was digested with *Hinf1* at 37^{o} C for 3 h. Digested DNA samples were analysed in 2% agarose gel.

Cloning and sequencing of 16S rRNA gene

The amplified products were purified using GFXTM PCR DNA and Gel Band Purification Kit (Geni, Bangalore) according to the manufacturer's instructions. The 16S rRNA gene amplicon was cloned in pTZ57R/T vector according to the manufacturer's instructions (InsT/A clone^{TM} PCR Product Cloning Kit #K1214, MBI Fermentas). Full-length sequencing of the rRNA gene for all the marine samples associated antagonistic bacterial isolates were carried out in Geni (Bangalore, India).

Nucleotide sequence analysis

The full-length sequences obtained were matched with sequences available in NCBI using BLAST (Altschul *et al*., 1997). Multiple sequence analysis was carried out using CLUSTALW (Thompson *et al*., 1997) and a further neighbor-joining plot (Perriere & Gouy, 1996) and PHYLODRAW (Choi *et al*., 2000) were used to construct the phylogenetic tree. To validate the reproducibility of the branching pattern, a bootstrap analysis was performed.

Results

In the present study, a total of 109 bacterial strains were isolated from fourteen species of sponges. Total count was high in the *Acanthella ramosa* at 3.82 x 10^5 CFU/g and low in *Epipolasis topsenti* at 1.50 x 10^3. The total bacterial counts of 14 species of marine sponges and their representative bacteria (n=109) were given in Table -1.

Table – 1: Total bacterial count in different marine sponges in Gulf of Mannar Coast

Sl. No.	Name of the Sponges	Total count CFU/g	No. of representative bacteria
1.	*Acanthella ramosa* (SAR)	3.82 x 10^5	8
2.	*Sigmadocia fibulatus* (SSF)	2.41 x 10^5	5
3.	*Mycale mannarensis* (SMM)	1.39 x 10^5	9
4.	*Axinella carteri* (SAC)	3.46 x 10^5	7
5.	*Callyspongia fibrosa* (SCF)	2.12 x 10^4	12
6.	*Epipolasis topsenti* (SET)	1.50 x 10^3	10
7.	*Petrosia testudinaria* (SPT)	1.85 x 10^4	6
8.	*Psammaplysilla purpurea* (SPP)	3.45 x 10^5	9
9.	*Plakortis simplex* (SPS)	3.75 x 10^5	6
10.	*Fasciospongia cavernosa* (SFC)	2.75 x 10^5	8
11.	*Stelletta tenui* (SST)	1.73 x 10^5	4
12.	*Dysidea fragilis* (SDF)	2.49 x 10^5	9
13.	*Dysidea avara* (SDA)	1.60 x 10^5	9
14.	*Craniella australiensis* (SCA)	2.79 x 10^5	7

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Taxonomic classification of sponge associated bacteria

Fourteen different marine sponges associated bacteria were identified to generic level along with their percentage incidence are given in Fig. 1. Of the 109 isolates, *Bacillus* sp. (45.87%) was dominant followed by *Alteromonas* sp. (17.43%), *Flavobacterium* sp. (7.33%), *Micrococcus* sp. (6.42), *Vibrio* sp. (5.5%), *Pseudomonas* sp. (5.5%), *Plesiomonas* sp. (4.87), *Corynebacterium* (3.66%), *Photobacterium* sp. (2.75%) and *Staphylococcus* sp. (1%). The high representative members were isolated from *Acanthella ramosa* and *Callyspongia fibrosa*. All the collected marine sponges had *Bacillus* sp. as member. The presence of *Vibrio* sp. was low compared to *Flavobacterium* sp. and *Micrococcus* sp.

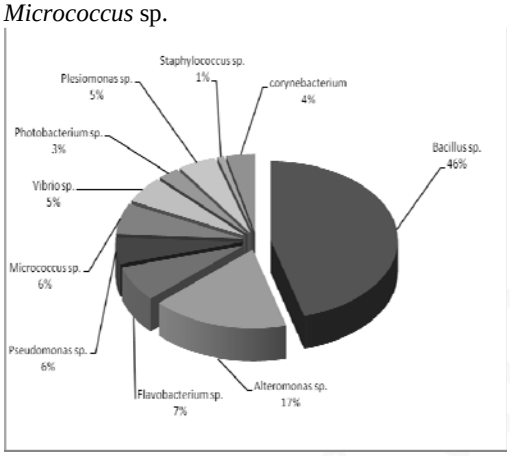

Fig. 1: Pie chart illustrating the diversity of bacterial groups associated with marine sponges

ARDRA analysis

ARDRA showed the presence of different polymorphic groups of bacteria in the sponges. ARDRA analysis revealed 17 polymorphic groups. Among the 17 ARDRA groups of the sponges found, 17 polymorphic patterns for *Hinf1* were observed (Fig 3.).

Antimicrobial activity of sponge isolates

Of the 109 isolates, 17 (15.6%) were found to be antibiotic producers. The antibiotic activity differed from strain to strain and both broad specific and species specific activities were noted (Fig. 2). Of the 17, eight antimicrobial isolates (SAR8, SAR11, SAR17, SAR25, SCF29, SCF37, SET30 and SP42) displayed inhibitory activity against *V. fischeri*, *V. vulnificus*, *V. harveyi*, *A. hydrophila* and *A. sorbia*. The higher activity noted SAR11 against *A. hydrophila* follwed by *V. harveyi*, *V. fischeri*, *V. vulnificus* and *A. sobria*. One of the isolates, SAR36 had activity against *V. harveyi* only. Some of the culture, SAR16, SAR42 and SCF14 were inhibited the growth of *V. fischeri*, *V. vulnificus*, *A. sobria* and *A. hydrophila*. Another antimicrobial isolates, SPT26 and SCF9 has inhibited four test organisms except *V. vulnificus*. The SPS29 and SPS33 had antibacterial activity against the *A. hydrophia* and *A. sobria*. In SPS29 has able to inhibited against *V. fischeri* but not in *V. vulnificus*. The SPS33 had inhibited against *V. vulnificus* not in *V. fischeri*.

Phylogenetic analysis

Full-length 16S rRNA sequence from 17 of the 109 characterized marine sponge isolates. The isolates were identified by 16S rRNA gene sequence. Initial identification was based on around 500 bases sequence at the 5' end gene. The isolates that showed more than 99% similarity with the available data base sequence were not sequenced further, only those with less than 99% similarity were taken for the complete sequencing of the gene. The 17 bacterial isolates from fourteen marine sponges could be assigned to 15 phylogenetically different clusters (Fig. 4). Among the tested isolates majority of them are grouped with genus *Bacillus*, *Alteromonas*, *Pseudomonas* and *Flavobacterium* with 94 - 98% similarity. In the *Bacillus* clusters seven of the isolates SP42, SAR11, SAR16, SAR25, SAR36, SCF9 and SPS29 show close relationship with *Bacillus* sp. However, the strains SPT26 and SAR8 showed a very high homology with *Pseudomonas* sp. The other major cluster, comprising SAR23, SCF29, SCF37 and SET30 was grouped with *Alteromonas* sp. with an almost 85% sequence similarity. The cluster of the strains SCF14 and SAR17 was closely related to *Flavobacterium* sp.

Phylogenetic analysis of the sponge associated strains showed that 7 strains are clustered within the Firmicutes group belonging to several *Bacillus* sp. with 85 - 90% similarity between them. BLAST analysis of the 17 strains showed SP42 and SAR11 are close relatives of *Bacillus* sp. (AJ971873) had 92% similarity. The detailed alignments of DNA sequences between isolate SAR11 and *Bacillus* sp. (accession number AJ971873) are shown in the Fig. 5. (http:// www.ebi.ac.uk/ Tools/ clustalw2/ index.html). The strain SAR16 had 85% similarity with *Bacillus* sp. (EU031762), isolated from municipal waste water sludge,

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India. SAR25, SAR36, SCF9 and SPS29 had close relatives of *Bacillus* sp. with 88% similarity. Other members of the sponge isolates showed that 6 strains are clustered within the Proteobacteria group with 84 - 98% similarity between them.

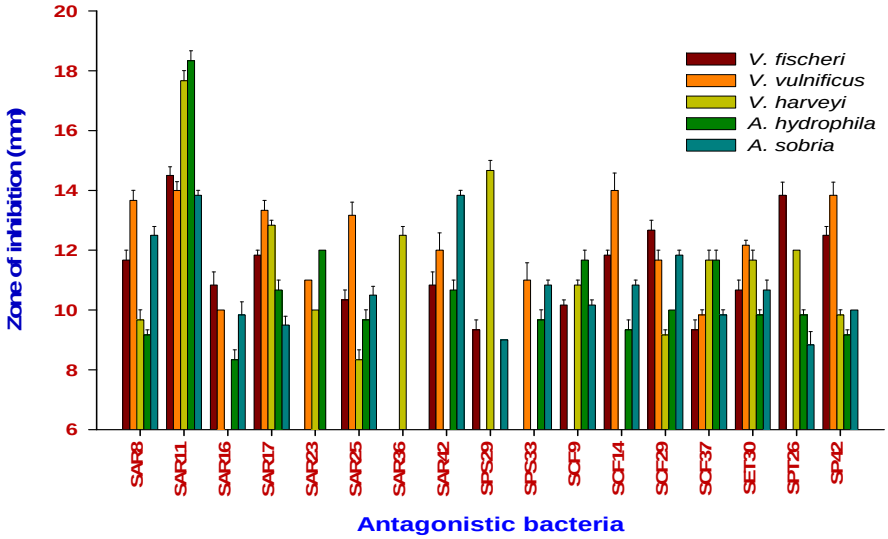

Fig. 2: Antagonistic activity of sponge associated bacteria against different fish pathogens

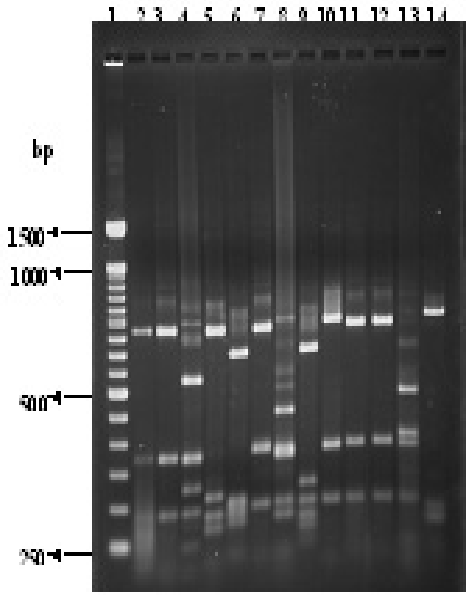

Fig.- 3. ARDRA profile of marine sponge associated bacteria with *Hinf*1 (Lanes 3, 7, 11 & 12: share a similar ARDRA profile, Lanes 2, 4, 5, 6, 8, 9, 10, 13 & 14: each show a distinct ARDRA profile).

Three strains (SAR23, SCF29, SCF37 and SET30) from the Proteobacteria group fall under the family *Alteromonas* with 84 - 96% similarity to *Alteromonas* sp. (FJ461448) and *Alteromonas* sp. (DQ834979). Another member of the proteobacteria group, SPT26, SPS33 and SAR8 had close relatives of *Pseudomonas* sp. with 92% similarity. The SAR8 was closely similarity (92%) to the *Pseudomonas* sp. (GU184339) isolated from ocean water of China. Only three strains, SCF14, SAR42 and SAR17 among the sponge isolates clustered with

Bacteroidetes. It is noteworthy that the strain SAR17 had 100% similarity with *Flavobacterium* sp. (FJ889628), a bacterium isolated from soil of Norway. All the sequences were submitted in GenBank and the accession numbers for all the sequenced producer strains are given in Table -2 (www.ncbi.nlm.nih.gov/ Genbank/).

Table – 2: Identification by 16S ribosomal RNA gene sequencing analysis of antagonistic active sponge associated bacteria from marine sponges based on BLAST analysis

Accession No	Isolates No	Genus	Family	Sequence length	Sponge
GU732413	SP42	*Bacillus sp.*	Firmicutes	800bp	*Dysidea avara*
GU951575	SAR11	*Bacillus sp.*	Firmicutes	345bp	*Acanthella ramosa*
GU951576	SAR16	*Bacillus sp.*	Firmicutes	427bp	*Acanthella ramosa*
GU951577	SAR25	*Bacillus sp.*	Firmicutes	526bp	*Acanthella ramosa*
GU951578	SAR36	*Bacillus sp.*	Firmicutes	193bp	*Acanthella ramose*
GU951579	SCF9	*Bacillus sp.*	Firmicutes	668bp	*Callyspongia fibrosa*
GU951580	SPS29	*Bacillus sp.*	Firmicutes	568bp	*Plakortis simplex*
GU991988	SPT26	*Pseudomonas sp.*	Proteobacteria	670bp	*Petrosia testudineria*
GU991989	SPS33	*Pseudomonas sp.*	Proteobacteria	548bp	*Plakortis simplex*
GU991990	SAR42	*Flavobacterium sp.*	Bacteriodetes	444bp	*Acanthella ramose*
GU991991	SCF14	*Flavobacterium sp.*	Bacteriodetes	551bp	*Callyspongia fibrosa*
GU991992	SAR23	*Alteromonas sp.*	Proteobacteria	554bp	*Acanthella ramose*
GU991993	SCF29	*Alteromonas sp.*	Proteobacteria	429bp	*Callyspongia fibrosa*
GU991994	SCF37	*Alteromonas sp.*	Proteobacteria	614bp	*Callyspongia fibrosa*
GU991995	SET30	*Alteromonas sp.*	Proteobacteria	625bp	*Epipolasis topsenti*
HM014445	SAR8	*Pseudomonas sp.*	Proteobacteria	813bp	*Acanthella ramosa*
HM 014446	SAR17	*Flavobacterium*	Bacteriodetes	704bp	*Acanthella ramosa*

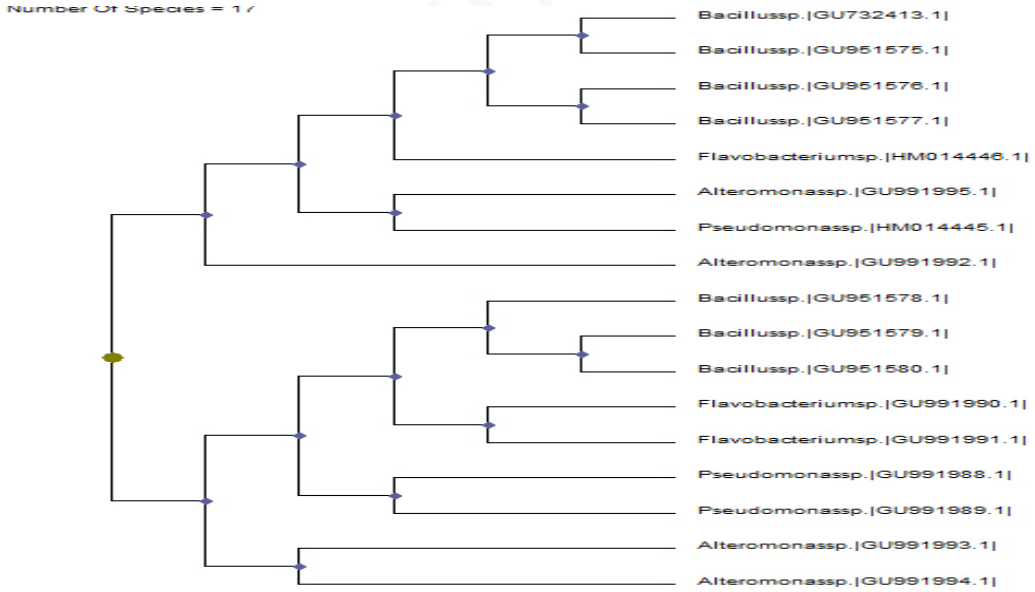

Fig. 4: Neighbour-joining phylogenetic tree from analysis of 16S rRNA gene sequence of marine sponges. The numbers are the percentages indicating the levels of boot strap support, based on a neighbour joining analysis of 1000 resampled data sets.

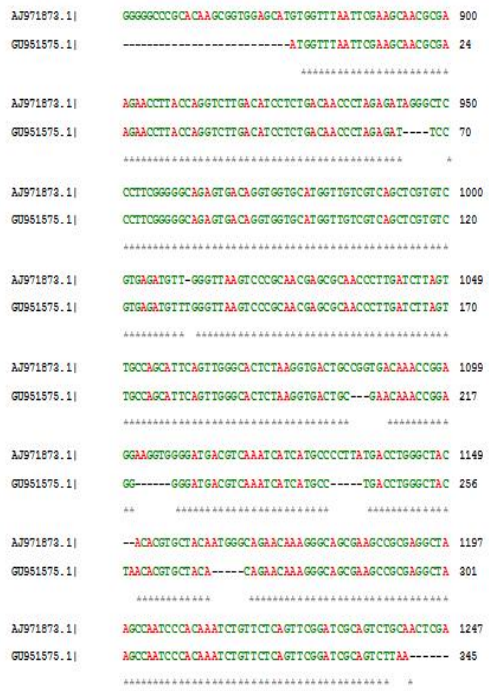

Fig.5: The DNA alignment showing the homology of isolate SAR11 and *Bacillus* sp. (accession number AJ971873) based on ClustalW Multiple sequence alignment

Discussion

The discovery of new classes of antibiotics was necessary due to the increased incidence of multiple resistances among pathogenic microorganisms to drugs that were currently in clinical use (Burgess *et al*., 1999). Peninsular India enjoys a large coastline with diverse marine ecosystems. Present research program to screen the bacterial symbionts associated with marine invertebrates as a source of drugs. As on date, the total 109 bacterial strains from fourteen species of marine sponges were isolated. Preliminary results were encouraging, in that 15.6% of the isolated strains exhibit good antibacterial activity, with several of the strains showed species specificity.

Wilson *et al*. (2009) reported that the ten of the 104 marine isolates possess antimicrobial activity. In the present study found better result, 17 isolates had antimicrobial activity of the 109 sponge associated bacteria. Various studies have been confirmed the predominance of Gram-negative producers in the marine environment (Fenical, 1993). In a study on antibiotic production in marine bacteria, Bernen *et al*. (1997) reported that 36% of the strains were Gram-negative rods. Gram-positive as well as Gram-negative bacteria were more or less equally presented in the producers encountered. . In the present study the most active Gram-positive *Bacillus* sp (SAR11) was found against all the target strains followed Gram-negative bacterium SAR17. The producer strains were molecular and biochemically characterized so that this information will help in future for the optimization of media and physical factors to achieve maximum antibiotic production. These 17 antimicrobial isolates displayed highly potent activity, with abrogation of growth against one or more target strains.

The interactions between sponges and bacteria in the marine environment are poorly understood. It was generally believed that symbiotic interactions exist between sponges and microorganisms. Symbiotic functions that have been attributed to microbial flora include nutrient acquisition, stabilization of sponge skeleton, processing of metabolic waste and secondary metabolite production (Hentschel *et al*., 2002). It has also been suggested that some of these bacteria chemically defend the host against microbial infection (Engel *et al*., 2002). In this study had ascertained that the genera *Alteromonas*, *Pseudomonas*, *Flavobacterium*, *Micrococcus*, *Vibrio*, *Plesiomonas*, *Corynebacterium*, *Photbobacterium* and *Bacillus* were dominantly represented.

In the marine environment the genus *Vibrio* has been reported from biofilms attached to surfaces, as pelagic bacteria and as invertebrate associated bacteria (West and Colwell, 1984). Thiel and Imhoff (2003) reported *Vibrio* in their study on phylogenetic identification of antibiotic producing bacteria from Mediterranean sponges. In the present study there was no active *Vibrio* against the target strains. Santavy *et al*. (1990) found that approximately 78% of the culturable bacteria associated with the sclerosponge *Ceratoporella nicholsoni* were species in the families of Vibrionaceae and Aeromonadaceae. However, in the present study we have not encountered any producer strains from the Aeromonadaceae.

The next cluster consisted of *Pseudomonas* sp. and *Flavobacterium* sp. Bacteria belonging to the *Pseudomonas* species are very common in seawater and have been also reported to be associated with sponges (Thakur *et*

al., 2003; Romanenko *et al*., 2005). The terrestrial *Bacillus* sp. were widely recognized as a rich source of antimicrobial agents (Gebhardt *et al*., 2002). The occurrence of *Bacillus* sp. in the marine environment has been well documented (Ivanova *et al*., 1999). While we have discovered antibacterial active Gram-positive Bacilli from sponge isolates in the present study. Many Gram-positive bacteria are known to generate spores under adverse conditions, such as those encountered in marine ecosystems, and this is thought to ensure their survival within the sponge tissue (Hentschel *et al*., 2001). Interestingly, spore formation is co-regulated with antibiotic production (Marahiel *et al*., 1993). Thiel and Imhoff (2003) reported absence of Gram-positive strains in their study on the phylogenetic identification antibiotic producing bacteria from Mediterranean sponges. Interestingly, in our study, eleven species of Gram-positive bacteria had produced antibiotic substance against the target strains.

Dobler *et al.,* (2002) reported that 12 bioactive compounds were reported from marine *Bacillus* sp. The same result was observed in this study *Bacillus* sp. (SAR11) had higher activity against all the target strains. The diversity of antibiotic producing marine bacteria isolated in the present study suggests that sponges are rich sources of novel bacteria. The phylogenetic diversity may be also due to bacterial - bacterial antagonism inside the sponge tissue (Hentschel *et al*., 2001). The bacterial diversity observed in the present study may be only a fraction of the total diversity of associated bacteria, given that only a small percentage of the bacteria can be cultured using the currently available medium and fermentation techniques (Proksch *et al*., 2002). Lastly, other sponge associated antagonistic active bacterial species found in our study are phylogenetically related to *Pseudomonas, Flavobacterium* and *Alteromonas* species. One possible explanation would be that while these species may not be predominant in sponges, they may be present in significant numbers.

The present study is the first attempt in Gulf of Mannar Coast, India to found to highly active bacteria. In the phylogenetic identification based on comparative sequence analysis of 16S rRNA, majority of the strains fell under the genera *Bacillus* and *Alteromonas* with 90 - 95% sequence similarity. Reports regarding antibiotic production and phylogenetic identification of sponge associated bacteria found off the coastline of India are very few and to the best of our knowledge this is the first study from this region.

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