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Antimicrobial Screening of Marine Bacterium GS4 (*Bacillus* sp.) Associated with the Sponge, *Mycale mannarensis*

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Eleven marine bacterial strains were isolated from the sponge *Mycale mannarensis* from SCUBA diving at Hare Island, Tuticorin, South East coast of India. Out of the eleven strains 5 strains were found to exhibit antimicrobial activity. Out of these five strains, two strains (GS_2 & GS_6) exhibited antifungal activity against *Candida albicans*. The strain GS_4 exhibited highly potent activity (30mm) against *B.subtilis*. This strain was chosen for further studies. The strain initially designated as GS_4 with wide antimicrobial spectrum was identified as *Bacillus* Sps based on its 16S rRNA sequence analysis.

Key words: Marine bacteria; *Mycale mannarensis*, Antimicrobial Screening.

The world's oceans, which cover more than 70% of earth's surface, represent an enormous resource for discovery of potential chemotherapeutic agents. The Indian coastline measures about 8129 km (Nair, 2003), which is distributed among nine coastal states and four union territories. In South East coast of India alone has 295 species of sponges, 180 species of marine algae and seaweeds, 301 species of gastropods,etc. (Ramadhas *et al.,*1999).

Over the past 30-40years marine organisms has been the focus of worldwide effort for the discovery of novel natural products. A small number of marine plants, animals and microbes have been already yielded more than 12,000 novel chemicals with hundred of new compounds still being discovered every year (Donia and Hamann, 2003). .Several reports have appeared on the characterization of the antimicrobial activity of marine organisms collected off the Indian coastline (Anand *et al.,* 1997;Rajaganapathi*et al.,* 2000; Anand and Edward, 2002; Ely *et al.,* 2004). Marine invertebrates have developed highly specific relationships with numerous associated microorganisms and these associations are of recognized ecological and biological importance (Fenical, W., 1993). It has been reported that the ratio of microorganisms with antimicrobial activity from invertebrates was higher than from other sources (Ivanova et al. 1998; Burgess et al. 1999), which suggests that invertebrate-associated microorganisms might play a chemical defense role for their hosts. This kind of microorganism as a sustainable resource has a high potential to biosynthesize novel biologically active secondary metabolites. Proksch, P (2002) Sponges are primitive marine invertebrates and contain more natural products than any other marine phylum. Many of their products have strong bioactivities including anticancer, antimicrobial and anti-inflammatory activities, and are often applicable for medical use (Finical, W 1992; Freidrich, A.B 1992). The present study is aimed to find the bacterial strains with antimicrobial activity associated with the sponge, *Mycale mannarensis* and further to elucidate the new bioactive compounds from marine microorganisms and to investigate the real origin of natural products.

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MATERIALS AND METHODS

Collection of samples

The sponge species, *Mycale mannarensis* was collected from a depth of 5-10 m by SCUBA diving at Hare Island, Tuticorin, South East coast of India. The sponge samples were placed inside sterile ethyl polythene bags underwater and transferred to the lab aseptically in iceboxes.

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 the tissue was immediately transferred to 99 mL sponge dissociation medium (2.7% NaCl, 0.008% KCl, 0.01% Na_2SO_4, pH 8). 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 °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 °C for further studies.

Screening for antibiotic production by marine bacteria

Antibiotic production by marine bacteria was carried out using a minor modification to the standard agar-overlay method. The marine strains were spotted on Zobell marine agar plates and allowed to grow for 3-5 days depending upon the growth rate of the various strains. Test strains (*B. subtilis, E. coli, C. albicans, S. aureus, P. aeruginosa* and *Shigella flexnari*) were gently overlaid using 3.5 mL soft agar over the marine strains. The soft agar was prepared by inoculating 1 mL fresh cultures of test strain (0.45 in OD at 600nm) in 100 mL of soft agar (0.75% agar) and mixing thoroughly. The overlaid plates were then incubated at 37 °C for 24 h and the zones of inhibition (measured from the edge of the colony to the edge of the clear zone) were recorded.

Mass culture and fractionation

In the initial screening the strain GS4 was highly active against *Bacillus subtilis* (30mm). A seed culture of the strain GS4 was prepared by inoculating in 50 mL Zobell marine broth medium in a 100 mL Erlenmeyer flask in a shaker (30°C/250 rpm) for 48 h. This seed culture (10 mL each) was then transferred to five 2 L Erlenmeyer flask containing 1 L Zobell marine broth and cultured with shaking (250 rpm) for 3 days at 30 °C. Cells were separated by centrifugation (4 °C/7000 rpm/ 20 min) (Wright, 1998).

Ammonium sulphate precipitation

Cells from mass culture were removed by centrifugation (4°C/7500 rpm/30min). The supernatant was separated and was saturated with Ammonium sulphate (90% saturation). This was done by adding Ammonium sulphate very slowly to the supernatant with constant stirring. Then the supernatant was kept in ice and left overnight at 4°C (cold room). The precipitate formed was collected by centrifugation (4°C/7500 rpm/30min). The collected precipitate was re-dissolved in 10ml of MilliQ water. This is considered to be the crude extract.

Anion exchange chromatography

Preliminary tests using different ion-exchange matrices indicated that the active component bound well in the anion exchange matrix. The crude extract was then loaded onto a DEAE-Sephadex (Amersham Biosciences Ltd) anion exchange column (~40ml bed volume) that had been previously equilibrated with 20mM Tris-HCl buffer (pH- 8). The column was then washed extensively over night (20 column volumes) with the same buffer used for equilibration. The active component eluted from the column by application of linear salt (NaCl 0-1M) gradient. Fraction of 5ml volumes were collected and checked for activity using the standard agar well-diffusion assay. Fractions containing the active component was pooled and lyophilized.

RESULTS

Eleven bacterial strains were isolated from the sponge *Mycale mannarensis*. Out of the eleven

strains 5 strains were found to exhibit antimicrobial activity (Table 1). Out of these five strains, two strains (GS2 & GS6) exhibited antifungal activity against *C.albicans*. The strain GS4 (Fig.1.) exhibited highly potent activity (30mm) against *B.subtilis* (Fig.2). This strain was chosen for further studies.

Antimicrobial activity of ammonium sulphate precipitation of GS4

The culture supernatant of GS4 was precipitated using ammonium sulphate (90% cut-off). This precipitate was considered as the crude extract, and it exhibited activity against B. subtilis (Fig.3).

Table 1. Antimicrobial activity of different strains isolated from the sponge sp. *mycale mannarensis* (agar overlay method)

Strain	Pathogens (Zones in mm)					
	Bacillus subtilis	*Staphylococcus aureus*	*Escherichia coli*	*Candida albicans*	*Pseudomonas aeruginosa*	*Shigella flexnari*
GS1	-		-	-	-	-
GS2	-	Trace	-	15 mm	-	-
GS3	-	-		-	-	-
GS4	30 mm	-	-	-	-	Trace
GS5	-	-	-	-	-	-
GS6	Trace	2-3 mm	-	15 mm	-	-
GS7	-	-	Trace	-	-	-
GS8	-	-	20 mm	-	-	-
GS9	-	-	-	-	-	-
GS10	Trace	Trace	-	-	-	-
GS11	8 mm	-	-	-	-	-

Fig. 1. The strain GS4 (*Bacillus* sp.)

Fig. 3. Activity of the crude ammonium sulphate ppt. of GS4 culture against *B.subtilis*

Fig. 2: Activity of GS4 against *B.subtilis* (agar-overlay method)

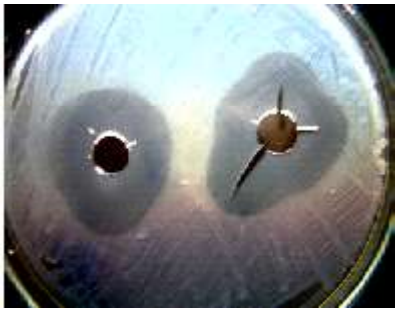

Fig. 4. Activity of crude (left well) and active fraction from anion exchange chromatography (right well)

Antimicrobial activity of active compound purified from Anion exchange chromatography

Preliminary studies using different ion-exchange chromatography matrices indicated that the active component was bound to a DEAE-Sephadex anion exchange matrix (Amersham) (Fig.4). The active fractions purified from the anion exchange chromatography also exhibited activity against *B.subtilis* (Fig.5).

Morphological and physiological characterization of the strain GS4

Morphological and physiological characterization of the strain GS4 showed it to be non-pigmented, Gram positive, non-motile, Catalase and Oxidase positive rod (Tab.2).The strain initially designated as GS4 when isolated

Fig. 5. Activity of the active fraction from anion exchange chromatography against *B.subtilis* (left well is the control well with 1M NaCl)

Table 2. Morphological and physiological characterization of the strain GS4

Test	Result
Gram reaction	+ve
Citrate utilization	-ve
Proteolytic activity	+ve
Motility	-ve
Amino acid decarboxilation:	
Arginine	+ve
Lysine	-ve
H_2S production	+ve
Indol production	-ve
Catalase	+ve
Oxidase	+ve
b-?Galactosidase	+ve
Amylase	+ve
Galactose fermentation	+ve
Sucrose	+ve
Dextrose	V*
Mannose	+ve
Maltose	+ve
Lactose	+ve
Trehalose	-ve
Raffinose	-ve
Xylose	-ve
Fructose	+ve
Salicin (Sa)	+ve
Malibiose (Mb)	+ve
Rhamnose (Rh)	-ve
Arabinose (Ar)	-ve
Sorbital (Sb)	-ve
Adonilol	-ve
Dulatol	-ve
Mannitol (Mn)	+ve
Inulin (In)	-ve
Inositol (Is)	+ve
Cellobiose (Ce)	+ve
Lipolytic activity	-ve

DISCUSSION

Natural products remain either the source of or the inspiration for a significant proportion of the new small molecule chemical entities introduced as drugs (Newman et al. 2003). However, drug discovery strategies changed in the 1990s as techniques in combinatorial chemistry, high throughput screening, and computer assisted design of small molecule ligands created alternatives to traditional drug discovery paradigms (Koehn & Carter, 2005). Although the biology of marine bacteria is beginning to be understood, the chemical activities of these organisms remain largely unexplored (Fenical and Jensen, 2006).Numerous novel chemical structures have been discovered in marine bacteria and are considered as a new resource for the development of medically useful compounds (Long *et al.,* 2003 and Isnansetyo & Kamei, 2003).The discovery of new classes of antibiotics is necessary due to the increased incidence of multiple resistance among pathogenic microorganisms to drugs that are currently in clinical use (Burgess *et al.,* 1999).

It is 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). The terrestrial *Bacillus* sp. is widely recognized as a rich source of antimicrobial agents (Gebhardt *et al.,* 2002).

While we have discovered Gram-positive Bacilli from sponge isolates in the present study, Thiel and Imhoff (2003) reported absence of Gram-positive strains in their study on the phylogenetic identification antibiotic-producing bacteria from Mediterranean sponges. Burja et al. (1999) have reported that Gram-positive bacteria make approximately 10% of the total isolates from sponges. 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). Many antibiotics including cyclic peptides, cyclic lipopepides and novel thiopeptides have been reported from marine *Bacillus* sp. (Nagai *et al.,* 2003). Until 2002, 12 bioactive compounds were reported from marine *Bacillus* sp. (Dobler *et al.,* 2002). GS4, the strain with the highest activity in this study was also found to be a *Bacillus sp*. Marine sponges produce many unique bioactive metabolites and many of them were suspected to be produced by the microbial symbionts associated with them. The present study was undertaken to assess this potential in symbiotic bacteria associated with the sponge species *Mycale mannarensis,* which inhabit the Gulf of Mannar. The results obtained in this study are encouraging, and the isolation and characterization of the active compound from GS4 is in progress.

CONCLUSION

Marine sponges are potential sources of unique bioactive metabolites and many of these compounds are suspected to be of microbial origin. The present study was undertaken to assess this potential in symbiotic bacteria associated with the sponge species *Mycale mannarensis* that inhabit the Gulf of Mannar region. In this present study 11 strains were isolated from the sponge *Mycale mannarensis,* out of which 5 strains were found active. One strain designated as GS4 was found to be potent against *Bacillus subtilis.* This strain was physiologically and biochemically characterized so that this information will help in future for the optimization of media and physical factors to achieve maximum antibiotic production. Partial purification of the active compound was also carried out from the ammonium sulphate precipitate of the culture supernatant, using ion-exchange chromatography. Reports regarding antibiotic production and phylogenetic identification of sponge associated bacteria found off the coastline of India are very few and this study highlights the importance of this resource for natural product discovery.

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