Antagonistic activity of an epibiotic Bacillus strain SG3 from Gorgonian coral, *Junceella juncea* (Pallas, 1766)

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Abstract: Marine environment continues to be a huge source of pharmacologically active compounds that cure deadly disease. This research investigates the bioactive efficacy of bacteria isolated from surface of the coral, *Junceella juncea* (Pallas, 1766). 128 bacterial strains were isolated from the coral *Junceella juncea* from Tuticorin coast, Gulf of Mannar region, south east coast of India. The strains were tested against selected five human pathogens. Initial screening shows that the strain SG3 was found to exhibit broad spectral activity inhibiting *Staplylococcus aureus*. Also, twenty other strains were found to be active against various pathogens. Based on 16S rRNA sequencing and phylogenetic identification, the stain SG3 was identified to fell under the genera Bacillus. The ethanol precipitated of the culture broth (SG3) was done and its activity was noted. Mass spectrophotometry (MALDI-TOF) analysis has shown that the mass of the molecules ranged from 1225 Da to 1927 Da. Thus the marine bacteria isolated from corals are a potential source of novel bioactive agents and other natural products. Epibiotic bacteria also direct future isolation of peptide anti-MRSA compounds from marine source.

Keywords: Epibiotic, *Junceella juncea, Staplyloocccus aureus*, mass spectrophotometry.

INTRODUCTION

More than 70% of the earth's surface is covered by world's oceans and signify an colossal resource for the discovery of therapeutic agents (Wright, 1998). Microbiologically, the oceans represent indeed the most diverse resource of life with huge dimensions and extreme variations in pressure, salinity and temperature. A unique adaptation strategy has to be acquired to sustain these extreme conditions, leading to new natural products differing from products obtained from terrestrial organisms (Faulkner, 1999). In marine environment, bacteria grow on submerged biotic and abiotic surfaces (Chellaram *et al*, 2012, Prem Anand *et al* 2011 and Dunne, 2002). In particular, a diverse community of surface attached bacteria was found on the surface of marine invertebrates (Rohwer *et al*. 2002 and Chellaram *et al*, 2011). Several factors including surface-specific interactions account for the diversity of these organisms (Rohwer *et al*. 2002; Taylor *et al*, 2005). Majority of chemotherapeutics have been identified from marine invertebrates of which sponges dominate (Lie and Zhou, 2002). Marine sponges remain a rich source of structurally unique compounds, which shows wide variety of biological activities (De Rosa *et al*., 2003). Heterotrophic bacteria that are commonly present in marine environments have not received much attention, although the agar digesters have been investigated extensively. Extensive investigations on marine bacteria in Mandapam were reported (Leifson *et al*., 1964 and Okami *et al*., 1976). Biofilm formation by bacteria that adhere to the implanted medical devices or damaged tissue can become harmful by causing persistent infections (Stewart and Costerton, 2001). Hence, such bacteria have to be removed from tissues and devices. Marine bacteria are of great interest as novel and rich sources of biologically active products. Every year, hundreds of new compounds are being discovered from marine organisms. The number of natural products isolated from marine organisms increases rapidly exceeding 18,000 (Jenson and Fenical, 1994). However, coral reefs provide balance in sea ecosystem thus it is vital to prevent them from extinction.

Here, our aim is to isolate and identify bacteria from sea fan and analyze its antagonistic activity against selected human pathogens. The isolated marine bacteria was identified as *Bacillus vietnamensis* using 16s rRNA sequencing technique. The marine bacterium produced antibodies against *Staphylococcus aureus.*

MATERIALS AND METHODS

Sample collection

The coral (sea fan) *Junceella juncea* was collected by SCUBA diving from 5-10m depth at Tuticorin coastal waters, Gulf of Mannar region, south east coast of India during the month of December, 2013. A single branch of the coral (about 2cm in length) was gently cut off and care was taken not to disturb the whole organism. This species was identified by one of the authors and senior marine biologist, Dr. C. Chellaram. The collected samples were

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then placed inside sterile ethylpolythene bags underwater and transferred to the laboratory aseptically in iceboxes. A voucher specimen (MS-2) was deposited in the School of Biomedical Sciences, Veltech Multi Tech Dr. Rangarajan Dr. Sakunthala Engineering College, Chennai, India.

Isolation of bacteria

The coral sample was first washed gently with sterile seawater to remove sand particles. Isolation of epibiotic bacteria was done by swabbing a small area of the coral surface with a sterile cotton swab. The swab was then directly swabbed on Zobell marine agar (ZMA) plates. ZMA plates were incubated at room temperature for six days and from the fifth day on colonies of different morphotypes were isolated and repeatedly streaked on Zobell marine agar plates to obtain pure cultures. The pure cultures were then stored at 4°C in marine agar slants until further studies.

Screening for antibiotic production by the Bacillus strain SG3

Standard agar-overlay method was followed to carry out antibiotic production test by marine bacteria. Initially, the marine strains were spotted on ZMA plates and allowed to grow for 5 days. Test strains *E. coli*, *K. pneumoniae*, *Pseudomonas aeruginosa, Staphylococcus aureus*, *Candida albicans* were overlaid using soft agar on the spotted marine strain. 1ml of test strain was inoculated in 100 ml of agar solution (0.75% agar) to prepare soft agar. Thorough mixing was done. For marine strains 1.5% NaCl was added to the soft agar. Plates overlaid with soft agar were incubated at 37°C for 24h. After incubation, the zone of inhibition was recorded.

Cold-ethanol precipitation

The cold–ethanol precipitation of the culture broth was carried out following the slightly modified method of Schubert and Finn (1981). The *Bacillus* strain SG3 culture was prepared as mentioned above. To the supernatant two volumes of ice-cold ethanol was added gradually simultaneously agitating with a magnetic stirrer. When the solvent addition was complete, the culture was agitated at 4˚C for at least 60min. The culture was then placed in an ice bucket and left overnight inside a cold room (4˚C). The precipitate was separated from the supernatant by centrifugation at 7000rpm for 30min in 4˚C. The precipitate was dried in room temperature to remove the ethanol and then dissolved in 5ml of MilliQ water. The antimicrobial activity of the ethanol precipitate was carried out using agar-well diffusion method.

Agar well diffusion assay

The agar well diffusion assay was carried out using the modified Stein *et al*., (2002) method. Tryptic Soy Agarose (TSA) was used as the assay medium. TSA was prepared by adding 3g Tryptic Soy broth powder (Hi-media, Mumbai, India) and1g of low electro endosmosis (EEO) Agarose in 100ml of double distilled water. Hundred micro liters of the extracts (ethanol precipitate/ crude biofilm) were poured into the 6-mm wells of TSA plates previously seeded with the test strains. To allow diffusion of the substance into the agar, the plates were placed at 4˚C for 4 to 6h and were subsequently incubated for 12 to 18 h at 37˚C. The presence or absence of inhibition zones around the well was recorded.

Mass determination

Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) spectrum of crude and 7HPLC purified active fractions were acquired on Ultraflex Bruker mass spectrometer, equipped with a nitrogen laser of wavelength 337nm. Equal amounts of samples were mixed with the matrix solution (α-cyano-4-hydroxy cinnamic acid) saturated with 0.1% TFA and acetonitrile (1:1). Measured masses have an error of ~ ±3Da.

Molecular identification and phylogenetic analysis of Bacillus strain SG3

Single colony of the strain SG3 was taken from the agar plate. The strain was suspended in 50µl of lysis solution (10mM Tris-HCl, pH 7.5; 10mM EDTA and 50µl/ml of proteinase K). The mixture was incubated at 50°C for 15 minutes. Proteinase K inactivation was done at 85°C for 10 minutes. The mixture was later centrifuged at 15,000 rpm at 4°C for 15mins. Genomic DNA, present in the supernatant was directly used as template in PCR reaction. PCR amplification of almost full-length 16Ss rRNA gene was carried out with eubacteria specific primer set 16F27N (5'-CCAGAGTTTGATCMTGGCT CAG-3') and 16R1525XP (5'- TTCTGCAGTCTAGAA GGAGGTGWTCCAGGC-3') (Pidiyar *et al*., 2002). 10ng of the genomic DNA, 1X reaction buffer (10mM Tris-HCL, pH 8.8 at 25°C, 1.5mM MgCl2, 50mM KCl and 0.1% Triton X-100), 0.4mM deoxynucleoside triphosphates (Invitrogen), 0.5U DNA Polymerase (New England Labs, UK) was used to perform a 25µl reaction volume PCR. An automated Gene Amp PCR system 9700 thermal cycler was used to perform PCR under the following conditions. The amplification condition was given as follows: 94°C for 1 min (denaturation), 55°C for 1 min (annealing), 72°C for 1.30 min (elongation) at and 72°C for 10 min final elongation. PCR product of around 1.5 Kb was run by electrophoresis with 5µl of the PCR product on 1% agarose gel in 1X TBE buffer and stained with ethidium bromide 0.5 µl/ml. The PCR product was precipitated by PEG-NaCl (20% PEG in 2.5 M NaCl). Precipitation was done at 37°C for 30 min. Centrifugation of reaction mixture was done again at 12,000 rpm for 30 min at room temperature. The resultant pellet was washed twice with 70% ethanol. The pellet was later dried and resuspended in 5µl of sterile nuclease-free water. Later, one microliter (~ 50ng) of purified PCR product was sequenced (Pidiyar *et al*., 2002). The sequence analysis was done at NCBI server (http:// www.ncbi.nlm.nih. gov/BLAST). The alignment of the sequence was done using CLUSTALW programmed at European

Bioinfomatics site (http://www.ebi.eic.uk/clustalw). Phylogenetic tree was constructed using the MEGA Software version 3.1. The sequence of the 16s rRNA gene of the *Bacillus* strain SG3 was deposited in GenBank.

RESULTS

Bioactivity

Testing of *Bacillus* strain SG3 using agar overlay method showed that the strain exhibits broad-spectral activity, inhibiting the growth of 1 out of 5 test strains. The epibiotic strain might have secreted antimicrobial metabolites (fig. 2).

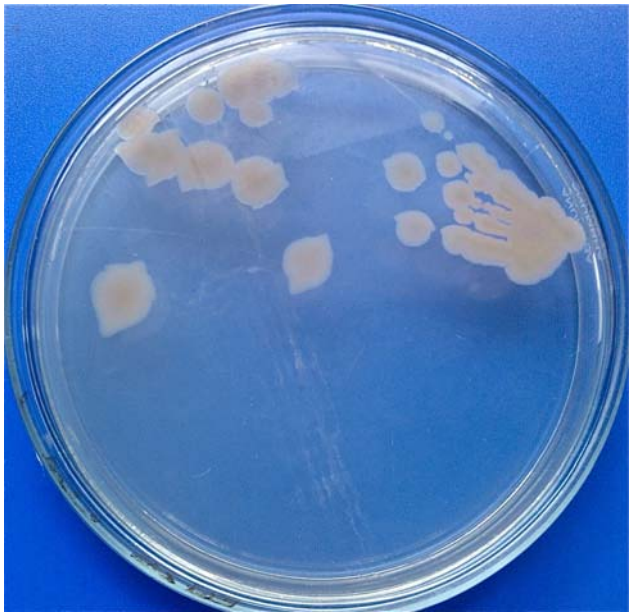

Fig. 1: Strain SG3 (*Bacillus sp*)

The crude extract (ethanol) was found to be active against *S. aureus*. MALDI-TOF spectrums of the crude extract (fig. 3) give the mass of active molecules. Mass of the crude extract molecules ranged from 1225Da to 1927Da.

Molecular identification and phylogeny

The strain SG3, was identified as a *Bacillus sp.* engaging 16Ss rRNA gene sequencing method. Phylogenetic analysis based on comparative analysis of the sequenced 16Ss rRNA indicated that the strain was closely related to *Bacillus vietnamensis* strain (fig. 4).

Sequence was obtained by 16s rRNA sequencing and related sequences were obtained from BLAST. Multiple sequence alignment was done and phylogenetic tree was constructed using European Bioinformatics site (http://www.ebi.eic.uk/ clustalw) and tree view 1.6.6.

Further purification may result in the extraction of active compounds that are novel and efficient. Marine source continue to provide humanity with potential compounds that can be developed into drug against deadly diseases. These compounds can also be tested for its activity against deadly diseases like cancer, malaria, etc.

Fig. 2: Anti – MRSA activity of strain SG3 (*Bacillus sp*.)

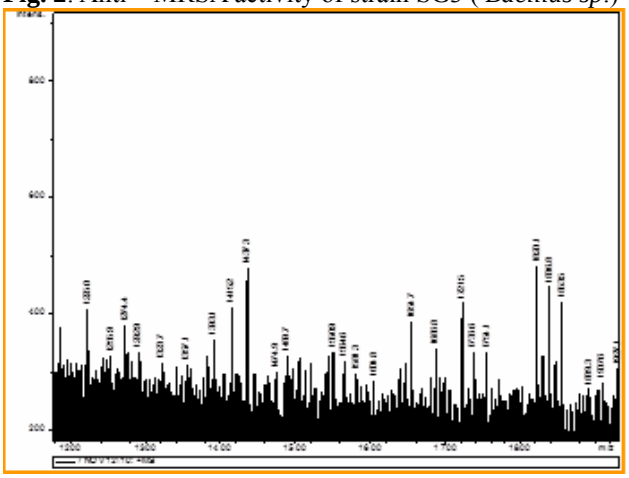

Fig. 3: MALDI- TOF data of crude extract of SG3 (*Bacillus sp)*

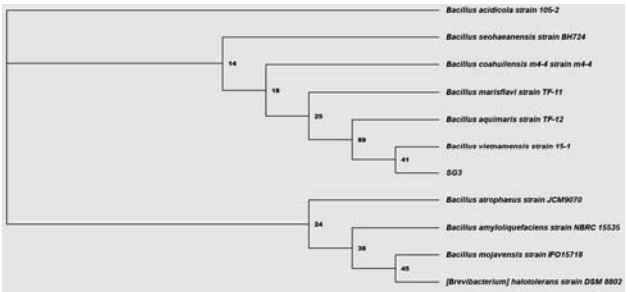

Fig. 4: Phylogenetic tree of the strain SG3 (*Bacillus sp*)

DISCUSSION

Increased incidence of multiple resistances among pathogenic microorganisms to drugs leads to the discovery of new classes of antibiotics (Burgess *et al*., 1999). Marine chemicals often possess quite novel structures, which in turn lead to pronounced biological activity and novel pharmacology. The study of such chemicals therefore is a very promising endeavor (Blunt

2005). Microorganisms will automatically acquire there resistance towards common antibiotics by altering their metabolism and genetic structure. These microbial compounds are most prominent source for discover and production for new drugs (Zhang *et al*, 2009, Yuan *et al*, 2010 and Chellaram *et al*, 2009).

This study showed that the isolated bacterium *Bacillus vietnamensis* has excellent antimicrobial activity against the *Staphyococcus aureus*. It is evident that *Bacillus vietnamensis* constitutively produces a novel compound responsible for antimicrobial activity. Further study is necessary for purification of compound using High Performance Liquid Chromatography (HPLC) and for structure and functional group elucidation of the compound by using Nuclear Magnetic Resonance (NMR) and Infra-red (IR).

CONCLUSION

From the above investigations, the bacterial strain *Bacillus vietnamensis* isolated from the surface of gorgonian coral, *Junceella juncea* was found to produce antibiotics against MRSA. MALDI-TOF spectrum showed the mass of active molecules at 1225Da to 1927Da. The strain isolated showed anti-MRSA activity, thus providing positive results towards the search of novel anti-malarial compounds. Further study is needed to isolate and characterize the active molecule produced by the bacterial strain.

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