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Research Article

BIOACTIVE POTENTIAL OF AN EPIBIOTIC *STREPTOMYCES* STRAIN, CN3 FROM THE SEA FAN *JUNCELLA JUNCEA*

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ABSTRACT

Objective: To screen and isolate peptide antibiotics producing epibiotic bacteria from the surface of the sea fan *Juncella juncea*, and partially purify the active compound.

Methods: In this study, epibiotic bacteria were isolated by swabbing a small area of the coral surface with a sterile cotton swab, which was then directly swabbed on to Zobell marine agar plates. Antibiotic production was screened using agar overlay method and the active peptide was partially purified using anion exchange chromatography and reversed phase high-performance liquid chromatography (RP-HPLC). Phylogenetic identification based on comparative sequence analysis of 16Ss rRNA gene indicated that the stain fell under the genera *Streptomyces*.

Results: In the initial screening using agar overlay method the strain was found to exhibit broad spectral activity exhibiting both antibacterial and antifungal activities. Protease digestion of the crude extract resulted in the loss of activity indicating the proteinaceous nature of the active molecules. Bioassay guided purification using anion exchange chromatography, and RP-HPLC was carried and the active peptide was partially purified. An epibiotic *Streptomyces* strain CN3 producing potent broad spectral peptide was isolated.

Conclusion: This study highlights the importance of epibiotic bacteria associated with corals as a potential source for the discovery of novel antimicrobials and other natural products.

Keywords: Coral, Matrix-assisted laser desorption/ionization time-of-flight, Selected pathogens.

INTRODUCTION

All marine organisms bear epibiotic biofilms, which range from sparse to dense and from monospecific to highly diverse. These epibiotic biofilms have a huge potential to affect the biology, ecology, and fitness of their host. Many direct and indirect effects of epibiotic biofilms have been described; many more can be expected to exist. Density and composition of epibiotic biofilms vary at different scales: Among host species, among nonspecific host individuals, among body regions of a host individual, among habitats, and among seasons [1]. Most bacteria, and particularly those associated with the surface of other organisms, occur in biofilms [2]. Biofilms on the surface of marine organisms are usually dominated by prokaryotes (bacteria) while eukaryotes such as diatoms, fungi, and protozoa can be present at lower abundance [3-8].

Marine epibiotic bacteria live in a highly competitive environment where they encounter a limitation for space. In order to colonize a surface and to ward-off competition, they have been reported to produce bioactive compounds. Antimicrobial compounds have been isolated from many marine macro organisms, and there is increasing evidence that the bacteria associated with such marine biological forms are responsible for the production of antimicrobials isolated from them [9]. Various studies have shown that a higher percentage of marine epibiotic bacteria produce antimicrobial metabolites compared with the number of planktonic isolates [10,11]. This microbial antagonism, widespread as it is in the marine environment, has been suggested to be a potential avenue for natural product discovery [12-14]. In the last three decades, there has been a surge in reports of bioactive secondary metabolites from marine microorganisms. Scientists and health care workers often stress the need for new antibiotics due to the increasing prevalence of multi-drug resistant pathogens; so new avenues for drug discovery must be explored to discover new chemical classes. In the present study, we have focused on epibiotic bacteria from the surface of the sea fan *Juncella juncea* with an objective to discover new antimicrobials.

METHODS

Collection of samples

The gorgonian coral (sea fan) *J. juncea*, (Cnidaria: Anthozoa: Gorgonacea: Ellisellidae) was collected by Scuba diving from a depth of 5-10 m from Tuticorin coastal waters, Gulf of Mannar region, south-east coast of India. A single branch of the coral was gently cut-off, and care was taken not to disturb the whole organism. The collected samples were then placed inside sterile ethylpolythene bags underwater and transferred to the laboratory aseptically in iceboxes.

Isolation of bacteria

The coral sample was initially washed gently with sterile seawater to remove sand particles. Then epibiotic bacteria were isolated by swabbing a small area of the coral surface with a sterile cotton swab, which was then directly swabbed onto Zobell marine agar plates. Plates were incubated at room temperature for 7 days and from the 5th day onwards colonies of different morphotypes were isolated and repeatedly streaked on to Zobell marine agar plates to obtain pure cultures. The pure cultures were then stored at 4°C in marine agar slants until further studies. This isolation procedure was carried out using five samples collected randomly from Tuticorin coastal waters.

Screening for antibiotic production

Antibiotic production by marine bacteria was carried out by following the standard agar-overlay method. Except *Escherichia coli* all other test strains were purchased from microbial type culture collection (MTCC), Institute of Microbial Technology, Chandigarh, India. Initially, the marine strain was spotted on Zobell marine agar plates and allowed to grow for 5 days. Test strains *E. coli* (DH5α), *Bacillus subtilis* (MTCC 1134),

Pseudomonas aeruginosa (MTCC 4727) *Staphylococcus aureus* (MTCC 3381), *Salmonella typhi* (MTCC 3216), *Vibrio parahaemolyticus* (MTCC 451), *Shigella flexneri* (MTCC 1457), *Saccharomyces cervisiae* (MTCC 3418), *Candida albicans* (MTCC 3958) were gently overlaid using soft agar over the marine strain. The soft agar was prepared by inoculating 1 ml of test strain in 100 ml of soft agar (0.75% agar) and mixing thoroughly. For marine strains, 1.5% NaCl was added to the soft agar. The overlaid plates were then incubated at 37°C for 24 hrs and the zones of inhibition (measured from the edge of the colony to the edge of the clear zone) were recorded.

Cold-ethanol precipitation

The cold-ethanol precipitation of the culture broth of the potent *Streptomyces* strain CN3 was carried out following the slightly modified method of Schubert and Finn (1981) [15]. To the supernatant, two volumes of ice-cold ethanol w*ere* added gradually while agitating with a magnetic stirrer. When the solvent addition was complete, the culture was agitated at 4°C for at least 60 *min*utes. 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 7000 *rpm* for 30 *min*utes in 4°C. The precipitate was dried at room temperature to remove the ethanol and then dissolved in 5 *ml* 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 method of Chellaram (2014). Tryptic soy agarose (TSA) was used as the assay medium. TSA was prepared by adding 3 *g* Tryptic soy broth powder (Hi-media, Mumbai, India) and1g of low electroendosmosis agarose in 100 *ml* of double distilled water. 100 µL of the extracts (ethanol precipitate/crude biofilm) were poured into wells (6 mm diameter) of TSA plates previously seeded with the test strains. Plates were placed at 4°C for 4-6 hrs to allow diffusion of the substance into the agar, and their contents were subsequently incubated for 12-18 hrs at 37°C. The presence or absence of inhibition zones around the wells was recorded. All well diffusion assays were carried out in triplicates.

Proteinase K digestion of the crude extract

In 200 µL of the crude extract proteinase K was added at a concentration of 100 µg/ml. The sample was incubated at 37℃ for 1.5 hrs, and the antimicrobial activity was tested by agar - well diffusion method. A control extract without proteinase K was also incubated at 37℃ for 1.5 hrs and assayed for antimicrobial activity.

Ion exchange chromatography

Preliminary tests using different ion exchange matrices indicated that the crude extracts consist of active components binding to cation exchange matrices. The crude extract was purified Q-Sepharose (Amersham Biosciences Ltd), cation exchange chromatography. The crude extract was loaded onto respective columns (~40 ml bed volume) that had been previously equilibrated with 20 mM Tris-HCl buffer (pH-8). The columns were then washed extensively overnight (20 column volumes) with the same buffer used for equilibration. Components bound to the column were eluted by application of linear salt (NaCl 0-1 M) gradient. The fraction of 5 ml volumes were collected and checked for activity using the standard agar well-diffusion assay (200 µl per well). Fractions containing the active component were pooled and lyophilized.

High-performance liquid chromatography (HPLC) purification

The concentrated active fraction was applied onto a Phenomenex C18 semi-prep column (Jupiter 4 µm Proteo 90A, 250 mm × 10 mm, 4 µ) already equilibrated with 95% solvent A (0.1% TFA in water) and eluted with a linear gradient of solvent B (0.1% TFA in acetonitrile). The flow rate was monitored at 1 ml/minute and the absorbance was monitored at 226 nm. Fractions were collected and checked for antimicrobial activity employing disc diffusion assay (200 µl per disc), assays were carried out in triplicates.

Mass determination

Matrix-assisted laser desorption/ionization time-of-flight spectrum of the crude as well as diethylaminoethyl and HPLC purified active fractions were acquired on an Ultraflex Bruker mass spectrometer, equipped with a nitrogen laser of wavelength 337 nm. Samples were prepared by mixing equal amounts of samples 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 the potential strain

Molecular identification and phylogenetic analysis were done for the potential strain [16]. The analysis of the sequence was done at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) whereas the alignment of the sequence was done using CLUSTALW programmed at European Bioinfomatics site (http://www.ebi.eic.uk/clustalw). Trees were constructed using the MEGA Software version 3.1.

RESULTS AND DISCUSSION

The *Streptomyces* strain CN3 was found to exhibit broad-spectral activity (agar-overlay method), inhibiting the growth of 4 out of 9 test strains. The strain exhibited excellent antifungal activity inhibiting the two fungal test strains, 17 mm and 13 mm against *C. albicans* (Fig. 1) and *Saccromyces cerevisiae* respectively. It also exhibited a 9 mm activity against the bacterial pathogen *B. subtilis*. The ethanol precipitate was found to be active against bacteria, *C. albicans* and *S. cerevisiae* but only mild activity was noted against *B. subtilis*. The crude extract was digested with proteinase K to determine whether the activity was due to some proteinaceous substance present in the crude. The proteinase K digested crude lost its activity, indicating the proteinaceous nature of the active compound. The control extract without proteinase K retained its activity. Partial purification of the active compounds using cation exchange chromatography and reversed phase-HPLC yielded compounds with masses of 2165, 2231, 2247, 2249, 2261, 2165 –2325 Da (Fig. 2). The strain initially designated as CN3 when isolated was identified as a *Streptomyces* spp. employing 16Ss rRNA gene sequence method (Fig. 3).

In the present study, we were able to isolate an epibiotic *Streptomyces* strain exhibiting excellent antifungal activity. The *Streptomyces* strain CN3 of the present study was also noted to produce compounds of proteinacious nature and this was found to be peptides by mass spectrometric study (Fig. 4). Peptide compounds have already been reported from marine *Streptomyces* exhibiting novel structures and diverse bioactivity. Renner *et al.*, (1999) [17], reported cyclomarins A-C, novel anti-inflammatory cyclic peptides produced by a marine *Streptomyces*. Nam *et al.*, (2011); Prem Anand *et al.*, (2014) and Chellaram, (2014) [18-20] reported three highly modified peptides,

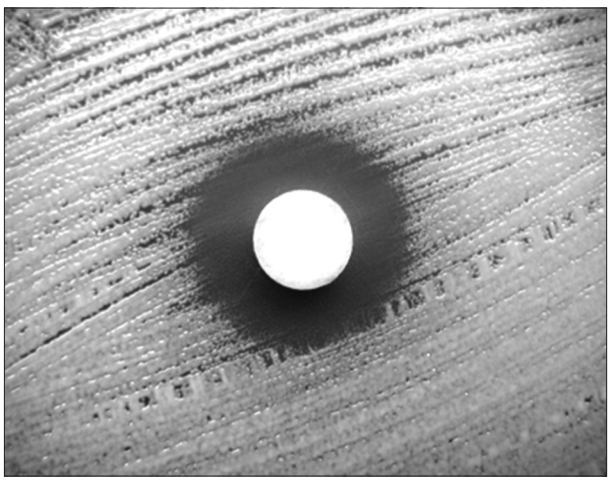

Fig. 1: Activity against *Candida albicans* by active fraction

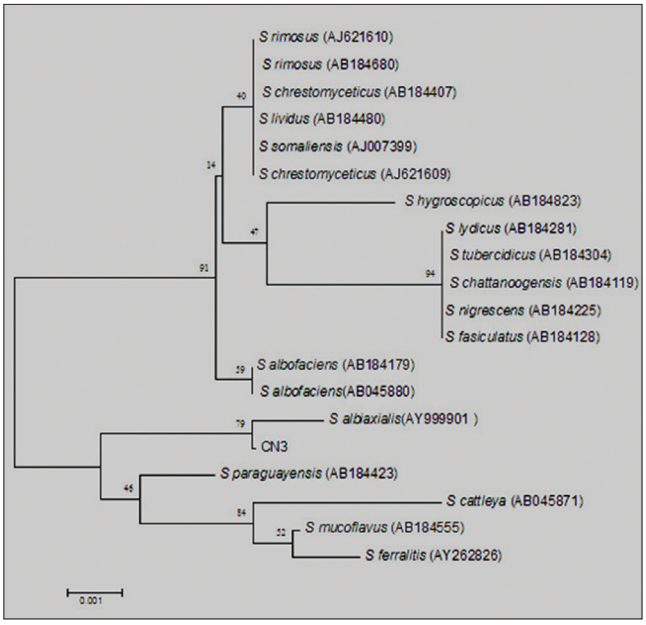

Fig. 2: Phylogenetic tree of the *Streptomyces* strain CN3

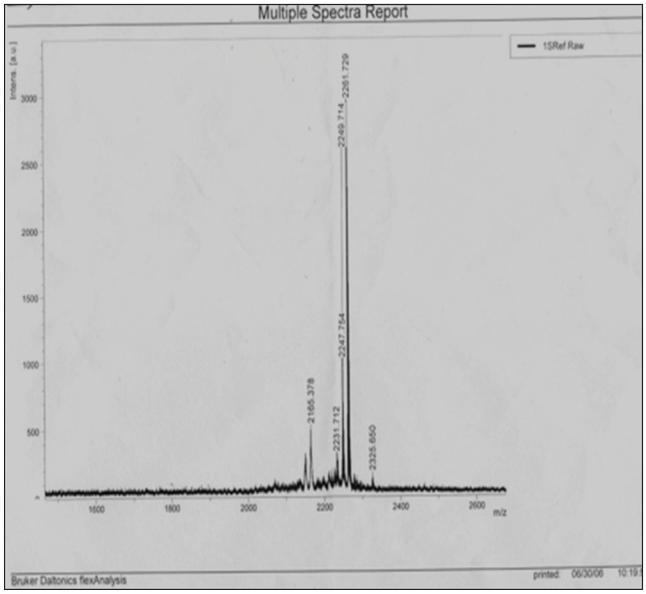

Fig. 4: MALDI-TOF spectra of the high-performance liquid chromatography purified active fraction

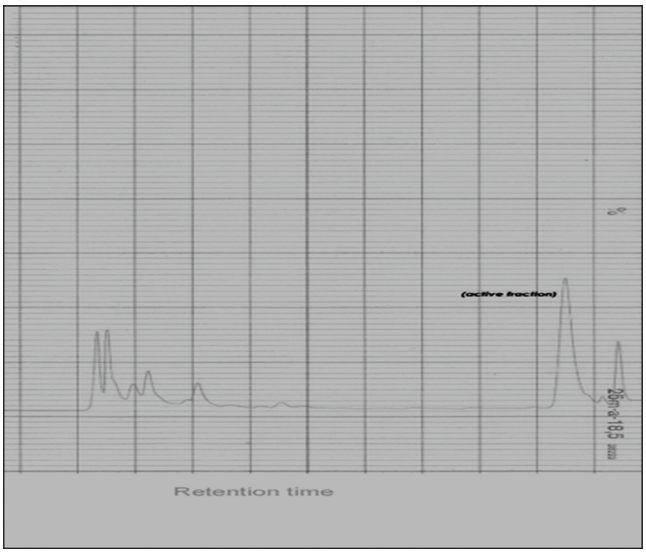

Fig. 3: High-performance liquid chromatography trace of the cation exchange purified active fraction showing the active peak

actinoramides A-C (1-3), which are produced by a marine bacterium closely related to the genus *Streptomyces*.

Valli *et al.*, (2012) [21] isolated 21 actinomyctes strain from the coastal South India and reported two potent strains exhibiting broad spectral antimicrobial activity. Selvakumar *et al.*, (2010) [22] isolated 94 *Streptomyces* spp. from the marine sponges namely *Callyspongia diffusa*, *Mycale mytilorum*, *Tedania anhelans* and *Dysidea fragilis* collected from Vizhinjam port, situated in the South-West coast of India, they identified 7 potent strains. Hu and MacMillian (2012) [23,24] reported a new peptide, L-O-Lac-L-Val-D-O-Hiv-D-Val (1), consisting of D-valine, L-valine, L-lactic acid, and 3-D-hydroxyisovaleric acid from the culture of the marine sediment derived *Streptomyces bacillaris.*

CONCLUSION

In recent years, the importance of antimicrobial peptides (AMPs) has stressed by researchers throughout the world because AMPs do not appear to induce antibiotic resistance. The potent antifungal activity exhibited by the partially purified peptides from this study highlights the importance of marine epibiotic bacteria as a potential source for bioactive compounds and the need to explore this unexplored resource.

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