

16s rRNA Gene Sequencing and Analysis of Marine Bacterium for Biomedical Applications

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Abstract. A marine epibiotic bacterial strain A4 was isolated from the coral *Subergorgia suberosa* from Tuticorin coast, Gulf of Mannar region, south east coast of India. Phylogenetic identification based on comparative sequence analysis of 16S rRNA gene indicated that the stain A4 fell under the genera *Marinobacterium*. The initial screening using agar overlay method the *Marinobacterium* strain A4 was found to exhibit broad spectral activity against *Escherichia coli* and *Candida albicans*. The highest zones of 8mm and 9mm were noted against the strain *Escherichia coli* and *Candida albicans* respectively. The culture broth was ethanol precipitated, and the activity was noted in the precipitate (crude extract). This present study highlights the importance of epibiotic bacteria associated with corals as a potential source for the discovery of novel antimicrobial and other natural products.

Keywords: bacillus, Epibiotic, *Subergorgia suberosa*, *Candida albicans*, Antimicrobial activity

1. Introduction

Surface-attached bacteria grow on submerged biotic and abiotic surfaces in the marine environment [1], [2]. Marine invertebrates in particular have diverse communities of attached bacteria on their surfaces [3], [4]. The microbial compounds are most prominent source for discover and production for new drugs [5], [6]. Antibiotics from marine microorganisms have been reported, including loatins from *Bacillus* produce both antibiotics and several bioactive substances [7]. The ocean remains as an unexploited source for many drugs and for the pharmacologically active substances [8]. The Marine organisms that are present in the environments are extremely rich in source of bioactive compounds [9]-[11]. Marine bacteria are important for maintenance of carbon dynamics in marine ecosystem. The presence of variety of heterotrophic bacteria and their importance is very well recognized for sustained ecological and biogeochemical cycle in marine environment [12]-[14]. In this study, we have isolated and identified a marine bacterium from sea coral *Subergorgia suberosa*, in Gulf of mannar, Southeast coastal region waters of India. The strain was found to possess antimicrobial compounds against *E.coli* and *Candida albicans*. Initial screening was done against five human pathogens by Agar overlay method. Ehanol extract of the strain was obtained and tested against the test organisms by well diffusion method.

2. Materials and Methods

2.1. Collection of coral samples

The bacterial strains were collected from surface of gorgonian coral *Subergorgia suberosa* by SCUBA.

2.2. Isolation of bacteria

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The cotton swab was then directly swabbed on to Zobell marine agar plates. Plates were incubated at room temperature for 7 days and from the 5th day on 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.

2.3. Screening for antibiotic production

Antibiotic production by marine bacteria was carried out by following the standard agar-overlay method. Initially the marine strain were spotted on Zobell marine agar plates and allowed to grow for 5 days. Test strains *E. coli*, *K.pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans* were gently overlaid using soft agar over the marine strain. The soft agar was prepared by inoculating 1ml 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 24h and the zones of inhibition (measured from the edge of the colony to the edge of the clear zone) were recorded.

2.4. 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.

2.5. Agar well diffusion assay

The agar well diffusion assay was carried out using the modified Stein et al., (2002) method. Tryptic Soy Agar (TSA) was used as the assay medium. TSA was prepared by adding 3g Tryptic Soy broth powder (Hi-media, Mumbai, India) and 1g of low electro end osmosis (EEO) Agarose in 100ml of double distilled water. Hundred micro liters 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 to 6h to allow diffusion of the substance into the agar, and their contents were subsequently incubated for 12 to 18 h at 37 °C. The presence or absence of inhibition zones around the wells was recorded.

2.6. Molecular identification and phylogenetic analysis

Single isolated colony of the strain was taken from the agar plate and suspended in 50µl of lysis buffer (10mM Tris-HCl, pH 7.5; 10mM EDTA and 50µl/ml of proteinaseK). The reaction mixture was then incubated at 55 °C for 15min followed by proteinaseK inactivation at 80 °C for 10 min. The reaction mixture was then centrifuged at 15,000 rpm at 4 °C for 15 min. The supernatant that contains genomic DNA 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'-CCAGAGTTTGATCMTGGCTCAG-3') and 16R1525XP (5'-TTCTGCAGTCTAGAAGGAGGTGWTCCAGGC-3') (Pidiyar *et al.*, 2002). A 25µl reaction volume PCR was performed using about 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). The PCR was performed in an automated Gene Amp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, USA) under the following conditions. The amplification conditions were 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. Expected PCR product of around 1.5 Kb was checked 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 at 37 °C for 30 min. The reaction mixture was centrifuged at 12,000 rpm for 30 min at room temperature. The supernatant was discarded and the pellet was washed twice with 70% ethanol. After drying the pellet was resuspended in 5µl of sterile nuclease-free water. One microliter (~ 50ng) of purified PCR product was sequenced as described earlier (Pidiyar *et al.*, 2002). The analysis of the sequence was done at NCBI server ([http:// www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)) whereas the

alignment of the sequence was done using CLUSTALW programmed at European Bioinformatics site (<http://www.ebi.ac.uk/clustalw>). Trees were constructed using the MEGA Software version 3.1.

3. Results

3.1. Bioactivity

The *Bacillus* strain A4 was found to exhibit broad-spectral activity (agar-overlay method), inhibiting the growth of *Escherichia coli* and *Candida albicans* strain. The bacteria secreted metabolites that were both antibacterial (Figure.1) as well as antifungal in activity. Maximum antibacterial activity was found against *Candida albicans* (Figure 2)



Fig. 1: Strain *Serinicoccus marinus*



Fig. 2: Activity of A4 strain against *E. coli*

3.2. Ethanol extract

The crude extract (ethanol) was found to be active against *E. coli* and *Candida albicans*.

3.3. Molecular identification and phylogeny

The strain initially designated as A4 when isolated, was identified in the genus *Marinobacterium*, employing 16Ss rRNA gene sequence method. Phylogenetic analysis based on comparative analysis of the sequenced 16Ss rRNA indicated that the strain was closely related to *Serinicoccus marinus* strain (Figure 3).

4. Discussion

The discovery of new antibiotics is so important due to the increasing incidence of multiple resistant pathogenic microorganisms to drugs that are currently using in clinical treatment. In the marine environment, 90% of bacteria are Gram-negative with different characteristics and the Gram-negative cell wall is better adapted for survival in the marine environment [15-16]. We have successfully culture a strain designated A4, isolated from the surface of a coral. Using molecular phylogeny tools such as 16s rRNA sequencing, which confirmed that the strain A4 was closely related to *Serinicoccus marinus* strain. We have shown that the recently discovered marine natural product A4 possesses potent in vitro antimicrobial activity against strain of *E. coli* and *Candida albicans*. Future studies will further re-find the activity of A4 analogs from marine-derived actinomycetes for characterization and optimization of activity toward further preclinical development. 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) spectroscopy, in order to practice it for pharmacological advantage.

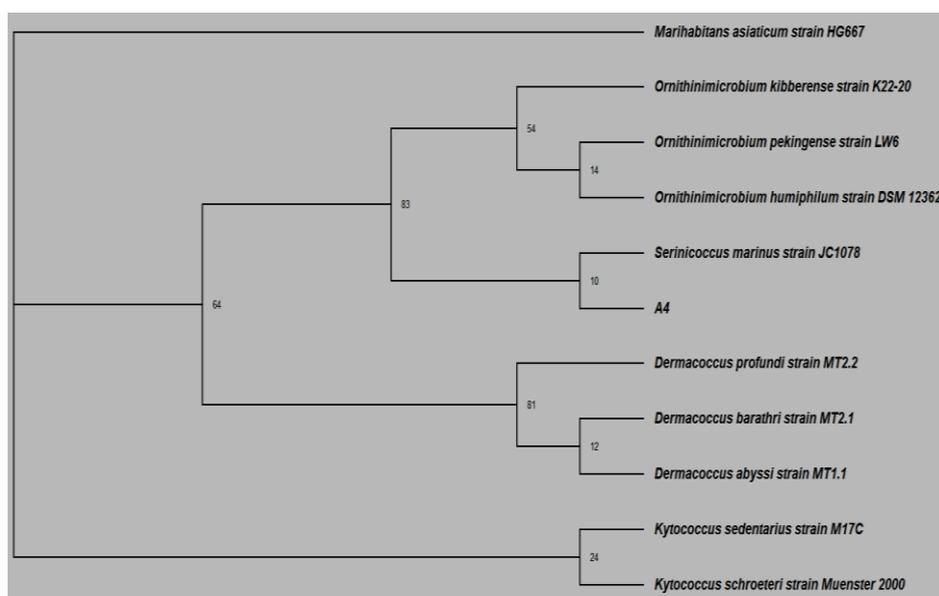


Fig. 3: Phylogenetic tree of the strain A4

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6. References

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