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Biota Screening of Potent Isolates of Antibiotic Producing *Staphylococcus* Species from Marine Sediment

Tirupathi Rao A, Srinivas N, Radha Krishna E, Veerendra Kumar B

Department of Biotechnology, GITAM Institute of Science, GITAM University, Visakhapatnam, Andhra Pradesh, India

Email: srinivas.maxplanc@gmail.com

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Abstract:

Microorganisms play critical role in human health and its functions colonizes in the human body and maintain consistent health as well. Protein based antibiotics play a pivotal role in curing the human diseases. Current research aimed to discover, screen and isolate the peptide based antibiotics producing Staphylococci from Visakhapatnam coastal area. Isolate(s) screened were identified as *Staphylococcus* species and subjected for further screening to discover a bacterium with an efficient antibiotic producing activity. 129 strain(s) out of 260 were isolated from marine sediment are *Staphylococcus* species. Eighty-five isolates of marine sediment bacteria were isolated using nutrient agar medium. Among 85 isolates, six exhibited antimicrobial activities against human pathogens such as *Escherichia coli* MTCC443, *Pseudomonas aeruginosa* MTCC424, *Proteus vulgaris* MTCC1771, *Bacillus cereus* MTCC430, *Bacillus subtilis* MTCC441 and *Staphylococcus aureus* MTCC3160. Resulting mean diameter of inhibitory zones (19 mm) revealed isolate (*Staphylococcus epidermidis*) was the most potent among all the isolates. The current investigation revealed that the diversity and antimicrobial activity have increased the scope of finding industrially important marine bacteria from the coast of Visakhapatnam and these organisms could be vital sources for the discovery of industrially useful bioactive molecules.

Keywords **—Isolation, Identification, genotypic analysis, Phenotypic Analysis, Marine sediment bacteria, Peptide Bioactive compounds, *Staphylococcus epidermidis*.**

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I. INTRODUCTION

Staphylococci are ubiquitous in the nature and they are being isolated from vide sources includes air, sand, soil, seawater, fresh water and dust [1]. Few *Staphylococcal* species are frequently encountered in humans to cause an infection. Generally, Coagulase producing bacteria is responsible for the infections in humans. Since last two decades' non-coagulase producing staphylococci have evolved as significant pathogens, especially in medical-device-related infections and in immunocompromised humans. Recent research revealed that *Staphylococcus epidermidis, Staphylococcus haemolyticus* and *Staphylococcus saprophyticus* strains found in human infections apart from other non-coagulase producing *Staphylococcal* species [2]. Recently, it was reported that strains belonging to the species *Staphylococcus hominis, Staphylococcus* species are involved in producing several antibiotics.*Staphylococcus epidermidis* is the coagulase negative species most prevalent in producing secondary metabolites with antibiotic activity against several coagulase producing bacteria [3].

Antimicrobial peptides (AMPs) are the key biomolecule(s) involved in enhancing the host's defense against various human infections. Especially, cathelicidins are classified AMPs used as immune boosters to the susceptibility of skin and other infections, proving as an evidence that AMPs

are significant for developing an innate immune defense [4–8]. The peptides deliver immune protection through chemotaxis and immune enhancers in a broad range of infections caused by bacteria. LL-37, an active cathelicidins, is an amphipathic alpha-helix that subsequently forms pores in the cell wall [9].

Staphylococcus epidermidis (S. epidermidis), is one among the most prevalent microflora, is innocuous. Recent research revealed that S. epidermidis colonized as normal flora under the human skin [10]. Normal flora of human body plays a significant role boosting the immune response. Several recent reports recorded that peptides produced by *S. epidermidis* exhibit antimicrobial properties, potentially acting as an additional antimicrobial shield. The physico-chemical properties of the *S. epidermidis* is comparable to Staphylococcus aureus. Peptides produced by the Staphylococcus shows antimicrobial activity seeks the current focus of the researchers [9,11–12].

Traditional screening methods results in an isolation of common microorganisms capable of producing secondary metabolites already established [13]. Recent methodologies help in screening the potent microorganisms producing bioactive metabolites. In addition, it also able to improve the quality of the screened bioactive products [14].

Traditional methods help in the identification of the organism(s) based on their morphological, biochemical, physiological and growth characteristics. Hence, precise identification of bioactive & potent microbial isolates through existing predictive bioinformatics methods and tools are currently available for chemical characterization of the produced bioactive molecules.

India is one of the potent sources of vide varieties of microorganisms because of its variant geographical changes, nature and its nutritional soil. This intricate environment provides suitable accommodation for the microorganisms to reside and produces novel metabolites. Among sediments microbes, the members of *Staphylococcus sp.,* have been widely exploited for the production of commercially important secondary metabolites and enzymes. Current investigation focused on the screening, identification and characterization of microbes isolated from sediment samples impressed to screen out bacteria capability to produce proteins possessing antibacterial and antifungal activities.

II. Materials and Methods

Sample collection and treatment: Marine sediment samples were obtained from different locations at the Visakhapatnam coast (17.6868°N, 83.2185°E), Bay of Bengal, Andhra Pradesh, India. Each location, 15 g of sample has been collected at 50 to 100 cm depth from the surface of shore after the removal of ~5.0 cm of the soil from the surface. The samples collected in polyethylene bags and sealed, labelled properly and stored in a refrigerator at 2–8^{o}C. All the samples collected were pre-treated with $CaCO_3$ (10:1 w/w) and incubated at 37^{o}C for 4 days and subjected to serial dilution (up to 10^{-6} dilution) by adding 1g of soil sample in 10 mL of distilled water. Bacteria, fungi, actinomycetes and yeast were isolated from marine sediment by serial dilution technique. 1.0 mL of sample was serially diluted using sterile distilled water as diluents and 0.1 mL of the sample was spread over media and incubated [15,16].

Isolation of bacteria from marine sediment: Bacterial isolation was done by taking 0.1 mL of each dilution, was placed onto nutrient agar medium by pour plate technique to obtain pure culture of bacteria. Pure cultures isolated were inoculated in to 10 mL of media and incubated for 24 to 48 h prior to screening. Morphological studies were conducted for isolated pure cultures [17].

Primary screening: Antimicrobial actvity for the isolated pure cultures were primarily studied by perpendicular streak method [18] on Nutrient Agar (NA) by in vitro screening of isolates. Nutrient agar plates were prepared, pre-incubated and inoculated with isolates by a single streak of inoculum in the centre of the Petri dish. The plates were seeded with test organisms by a single streak at a 90° angle after the completion of 24 h incubation at 37^{o}C. The microbial interactions were analysed by the determination of the diameter of the zone of

inhibition (Figure 1). Primary screening for evaluating the antimicrobial potential of the axenic cultures was performed by perpendicular streak method against the bacterial strains of Bacillus subtilis MTCC 441, Staphylococcus aureus MTCC 96, Escherichia coli MTCC 64, and Candida albicans MTCC 183. Isolates were screened for antagonism studies by inoculating a single streak of the pure standard organism in the centre of the testing media plates and incubated at 28°C for 5 days and subsequently seeded with "test" organism by a single streak at a 90◦ angle to the streak of the "producer strain" and finally the plates were incubated for 2 days at 28°C. All the microbial interactions were analysed by determining the distance of inhibition measured in mm. Microbial strains showing "moderate" to "good" antibiotic activity were selected for secondary screening, which was performed by agar well method, using 100 µl of their fermented broth against *B. subtilis* MTCC 441, *S. aureus* MTCC 96, *E. coli* MTCC 1304, *Salmonella typhi* MTCC 734, *Pseudomonas aeruginosa* MTCC 741 and *Candida albicans* MTCC 183. All the experiments were performed in triplicate and the average values were considered statistical analysis [19].

Secondary screening:

Selected isolates of primary screening were further screened for antimicrobial property using the agar-well diffusion method [20]. Pure cultures of 14 pathogenic bacterial strains and 2 fungal strains were selected in current investigation (Table 1). Freshly sub-cultured of pure culture of each isolate as screened from the primary screening was inoculated in nutrient broth and incubated at 37°C for 24 h in an incubator. Turbidity observed in the culture tubes indicates the growth of an isolates. Bacterial cells separated from nutrients by centrifuging for 15 min at 7000 rpm. Pellet and supernatants were separated and the supernatant was used in the determination of antimicrobial activity against the standard test microbes. The respective well(s) (5 mm each) made by the cork borer were loaded by 50µL of sample and the plates were incubated subsequently at 37^0C for 24 h, whereas, fungi are incubated at room temperature. Antimicrobial activity was estimated by zone of inhibition and the results exhibiting significant zones of inhibition against test bacteria were chosen and considered for secondary screening of current desired microorganism(s). The above screenings were repeated thrice and analysed the results statistically [21, 22]. The isolated pure culture of desired organism has been submitted to NCL, Pune bearing an accession number - NCIM- 5755.

Characterization of Microbial Strain(s) from the Selected Cultures

Isolated, screened strains were identified and characterized by streaking the culture(s) nutrient agar medium plates and observed after 3–5 days of incubation at 37±2.5°C for the given characteristics. Physiological and biochemical tests were performed [23] and classification by Bergey's manual [24], and results were observed after 3–5 days of incubation at 37±2.5◦C. Subsequently, the potential strain screened has been subjected for the strain phenotypic identification by VITEK-2 and genotypic identification of the strain by 16s rRNA analysis has been completed at Nation Collection of Industrial Microorganisms, Pune. Finally, on the basis of macroscopic, biochemical, physiological, phenotypic and genotypic characterization, hierarchical dendrogram tree has been constructed using NCBI database by BLAST tool [25].

III. RESULTS AND DISCUSSION

A total of 260 single clones were obtained from the marine sediment samples bears a total heterotrophic bacterial load ranged from $1.7x10^6$ to $4.5x10^6$ CFU/g of sediment. Sixty-eight isolates of marine sediment bacteria were isolated and primarily screened based on the significant zone of inhibition. Six bacterial isolates (Table I) selected for secondary screening and further analysis. Out of the screened 6 potential bacterial strains, GB-51 showed the best anti-microbial activity (zone of inhibition 19±0.2mm) against *Bacillus subtilis, Aeromonas veronii* and *Proteus vulgaris*, whereas remaining strains shows antibiotic activity but not

considerable. The inhibitory effect shown by these strains shows clear and distinct zones around the well might be because ofantibacterial compounds production [26].

In due course of study, marine sediment sample(s) from coastal region of Bay of Bengal, bacterial strains isolated present in sediments were screened for potent antimicrobial activity. Many members of the group continue to be dominant bacterial workhorses in microbial fermentation for the production of novel proteins [27]. Hence, the present investigation, we focused and investigated the sequence of study for isolating a bacteria showing the highest zone of inhibition against standard organism(s), were subjected to morphological, biochemical, phenotypic and genotypic conformation.

Fig.1Clear zone of inhibition was observed against the standard organisms which checking with the potent strain (GB-51) identified from marine sediment sample.

Strain Identification

The strain identification has been performed biochemically by VITEK2 and it was identified as Staphylococcus epidermidis (Table II). The strain was confirmed further through genetic analysis by 16s rRNA analysis. The RNA sequences of strain GB51 were amplified and the product were test in 1% agarose gel electrophoresis to confirm the PCR amplification fragment and the 16S rDNA sequence was recovered and analyzed by the ABI3730-XL sequenator, submitted the result to Genbank database at National Center for Biotechnology Information (NCBI). Partial rDNA sequences of strain GB51 showed high homology with (>99%) *Staphylococcus epidermidis*.

Staphylococcus genus consisting of >30 species, including *S. aureus, S. pasteuri* and *S. epidermidis*[28]. *S. epidermidis* are usually persists as normal flora in the human body and it produces wide range of antibiotic secondary metabolitesincluding peptide antibiotics, bacteriocins, bacteriolytic enzymes and polythiazoles [29]. *S. epidermidis* specially secretes cytotoxins and enzymes, such as staphyolysin, enterotoxins, leukocidin, Toxic Shock Syndrome Toxin and coagulase. Furthermore, it also produced the Staphylokinase, Staphylococcal fibrinolysin, Heat-stable nuclease, hyaluronidase and lipase [30].

Since, the significance of *Staphylococcus* species associated with the peptide antibiotics production. We focused further for the genotypic analysis to confirm the strain (GB51). The procedure includes, rRNA was extracted [31]. Bacterial 16S rRNA gene (1500 bp) [32]was amplified using polymerase chain reaction in a thermal cycler and were purified using Exonuclease I -Shrimp Alkaline Phosphatase (Exo-SAP) [33]. Purified amplicons were sequenced by Sanger methodin ABI 3500xL genetic analyzer (Life Technologies, USA). Sequencing files (.ab1) edited using CHROMASLITE(version 1.5) and further analyzed by Basic Local Alignment Search Tool(BLAST) with closest culture sequence retrieved from the National Centre for Biotechnology Information (NCBI) database that finds regions of local similarity between sequences [34]. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches [35]. TheBLAST algorithms used to infer functional and evolutionary

TABLE I
RESULTS OF ANTIMICROBIAL ACTIVITY OF SIX BACTERIAL ISOLATES FROM MARINE SEDIMENT AGAINST STANDARD BACTERIA AND FUNGI

Microorganisms	Zones of inhibition (Mean ± SD) (mm)[a]					
	Crude extracts from bacterial isolates isolated from marine sediments					
	GB-26	**GB-51**	**GB-92**	**GB-96**	**GB-102**	**GB-123**
Gram -ve Bacteria						
Pseudomonas aeruginosa MTCC424	6±0.4	8±0.3	8±0.4	6±0.2	-	-
Proteus vulgaris MTCC1771	18±0.2	19±0.3	14±0.5	7±0.3	10±0.2	-
E. coli MTCC443	15±0.3	18±0.2	9±0.4	18±0.2	11±0.4	5±0.5
Aeromonas hydrophila	8±0.2	10±0.3	8±0.4	8±0.5	-	-
Aeromonas veronii	7±0.4	19±0.2	16±0.3	7±0.6	13±0.6	15±0.5
Pseudomonas putida	8±0.4	17±0.1	13±0.5	8±0.5	16±0.5	12±0.2
Pseudomonas luteola	5±0.7	13±0.2	11±0.3	5±0.6	12±0.2	11±0.3
Vibrio fischeri	6±0.5	13±0.4	-	6±0.5	12±0.5	9±0.6
E. coli	3±0.7	8±0.3	8±0.6	3±0.8	-	-
Gram +ve Bacteria						
Staphylococcus aureus	-	18±0.2	15±0.4	-	15±0.3	12±0.4
Micrococcus luteus	4±0.6	15±0.3	11±0.5	4±0.7	14±0.4	13±0.3
Bacillus cereus MTCC430	3±0.5	-	-	3±0.5	12±0.3	9±0.4
Bacillus subtilis MTCC441	15±0.3	19±0.2	13±0.2	10±0.4	5±0.6	8±0.4
Staphylococcus aureus MTCC3160	-	14±0.4	16±0.2	-	13±0.3	15±0.2
Fungal strains						
Candida albicans	9±0.2	5±0.6	7±0.3	9±0.4	-	-
Candida tropicans	10±0.3	-	8±0.2	6±0.6	-	3±0.5

[a]Values are mean of three replicates ± SD
[b]GB- Marine Sediment Bacteria

relationships between sequences as well as help identify members of gene families. (i) Initial search to find potentially closely related type strain sequences using the BLASTN program [34] (ii) Pairwise alignment to calculate the sequence similarity values between the query sequence and the sequences identified in step (i) [36]. Therefore, each isolate is reported with the first five-ten hits observed in the said database. Further multiple sequence alignment and phylogenetic analysis is therefore recommended for accurate species prediction and evolutionary relationship [37–38]. The phylogenetic tree was constructed, shown in Fig. 3.

From all these observations & results depicted that the bacterial isolates from the marine sediments are found to be most prolific producers of protein antibiotics. In current investigation, we have deduced that the isolated strain (GB51) was identified and confirmed with genotypic analysis as Staphylococcus epidermidis consisting a potent antimicrobial activity. We subjected this strain into further investigation for their ability of producing medically and industrially useful compounds and lead to subsequent purification and characterization are underway.

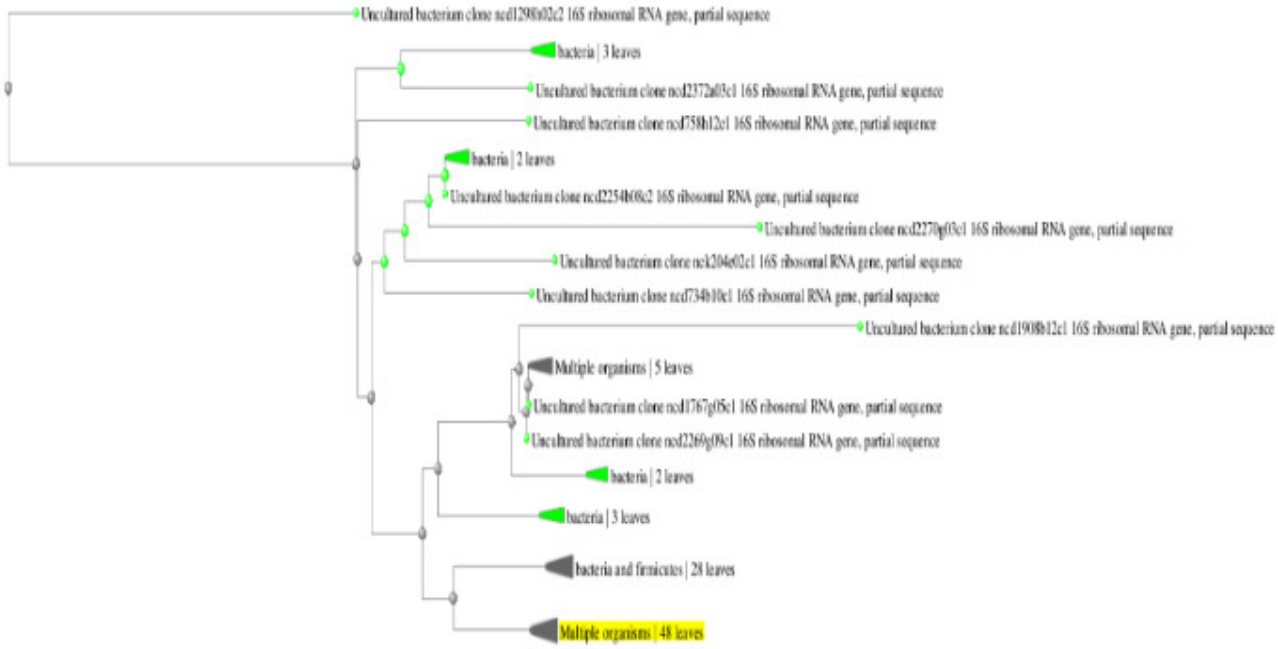

Fig. 2 Dendrogram tree representation of the desired strain (GB-51) through phylogenetic analysis using NCBI–BLAST tool resulted in 100 % similarity search with *Staphylococcus epidermidis.*

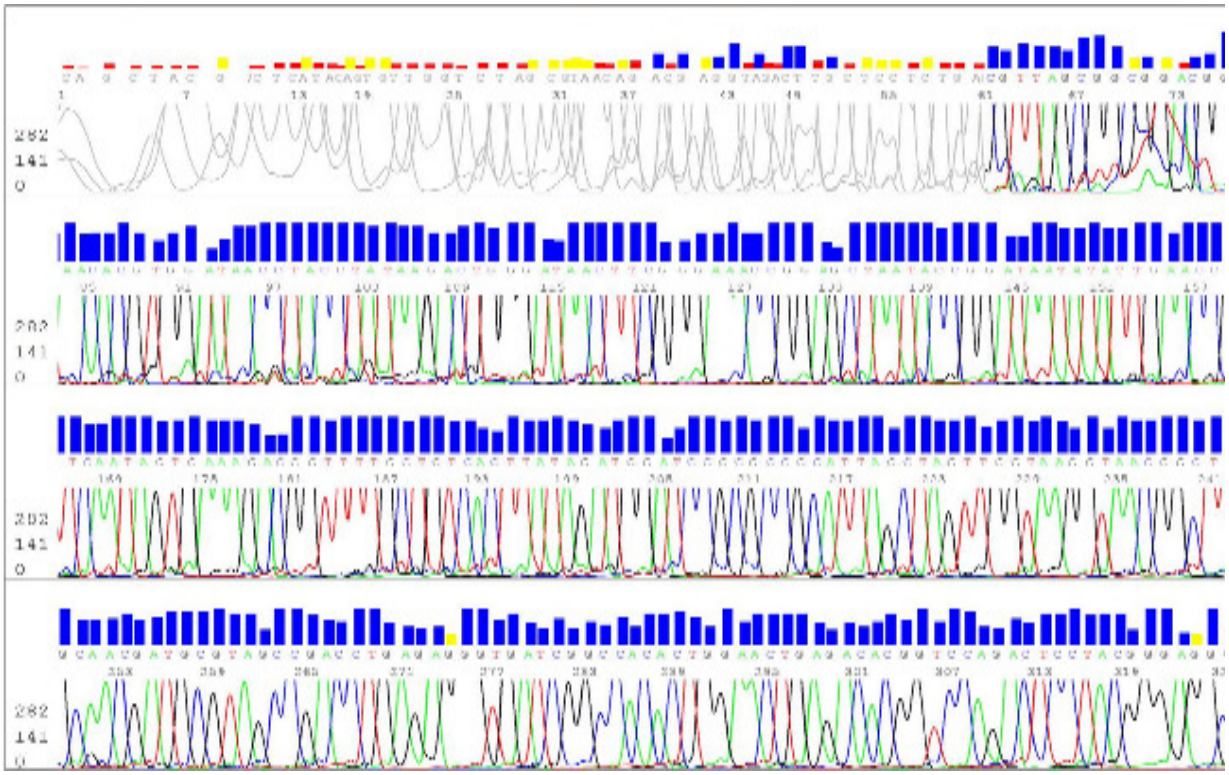

Fig.3Annotation for the genotypic analysis for the potent strain (GB-51) through phylogenetic analysis using NCBI database tool.

TABLE III

Results of biochemical analysis and strain identification of the selected strain GB-51 by VITEK–2 analysis.

Biochemical Details																	
2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	+	11	AGLU	-
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	+
20	LeuA	-	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	-	27	BGUR	-
28	AlaA	-	29	TyrA	-	30	dSOR	-	31	URE	+	32	POLYB	+	37	dGAL	+
38	dRIB	-	39	ILATk	+	42	LAC	+	44	NAG	-	45	dMAL	+	46	BACI	+
47	NOVO	-	50	NC6.5	+	52	dMAN	-	53	dMNE	+	54	MBdG	-	56	PUL	-
57	dRAF	-	58	O129R	+	59	SAL	-	60	SAC	+	62	dTRE	-	63	ADH2s	+
64	OPTO	+															

IV. CONCLUSIONS

In this study, a strain GB51 with antibacterial metabolites screening ability was isolated from the marine sediment source and the fermentation cell-free supernatants of strain GB51 resulted with a visible antimicrobial activity against to standard test organisms. The strain GB51 was identified by 16S rDNA to be Staphylococcus epidermidis and this strain produced broad spectrum antibiotic active substance. This finding maybe the first report about the S. epidermidis isolate from the marine sedimentand have the potential to produce useful antibacterial metabolites. Certainly, the properties of the antibacterial metabolites in details, purification and antibacterial mechanism should be researched in the next studies.

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