ANTIMICROBIAL ACTIVITY OF *STREPTOMYCES ALBOFACIENS* VITBRK1 Spp. ISOLATED FROM THE BAY OF BENGAL COAST OF TAMIL NADU, INDIA

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Summary

The aim of the present study was to screen the marine actinomycetes for antimicrobial activity against drug resistant clinical isolate, *Staphylococcus aureus* (methicilin resistant *Staphylococcus aureus,* MRSA) and to characterize the potential isolate. Hundred and twenty strains of actinomycetes were isolated from marine sediments collected at the southeast coast of Bay of Bengal, India. Ten percent of the actinomycetes isolates were found to be antibiotic producers and exhibited antagonistic activity against drug resistant standard ATCC strains by agar plug method. The biochemical, morphological and physiological characterization of the isolate revealed that it was Gram-positive rod, produced grey aerial mycelium, sporulating, Spira-Spirales type of spore chain morphology, smooth surface morphology and non motile in nature. The isolate utilized the following carbon sources arabinose, xylose, inositol, mannitol, fructose, sucrose and raffinose for their growth and L-asparagine, L-phenylalanine, L-histidine and L-hydroxyprolone as nitrogen sources. Based on Nonomura's key for classification of *Streptomyces* and Bergey's Manual of Determinative Bacteriology, the isolate was identified as *Streptomyces albofaciens* and designated as *Streptomyces albofaciens* VITBRK1spp.

Keywords: Actinomycetes, *Streptomyces albofaciens* VITBRK1 spp., antimicrobial activity
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Introduction

Structurally and functionally diverse bioactive compounds isolated from marine actinomycetes have been used as antibiotics with antimicrobial, antiviral, and antitumor activities (1, 2). Recently, the rate of discovery of new compounds from existing genera obtained from terrestrial sources has decreased, while the rate of re-isolation of known compounds has increased. The rise in the number of drug-resistant pathogens and the limited success of combinatorial chemistry in providing new agents is a proof that the discovery of new anti-infective agents is indispensible (3, 4). The exploration of new groups of actinomycetes from unexplored and unique habitats is pursued as sources of novel antibiotics and other small-molecules as therapeutic agents (5). *Streptomyces* species have been reported to be producers of more than half of the 10000 documented bioactive compounds (6). The genus *Streptomyces* was classified under the family *Streptomyceteae,* which includes Gram-positive aerobic members of the order *Actinomycetales* and sub order *Stretomycineae* within the new class *Actinobacteria* (7) and have a DNA G-C content of 69±78 mol% (8). *Streptomyces* are a prolific source of secondary metabolites yielded many antibiotics (9) including, streptomycin, neomycin, tetracycline and chloramphenicol.

The emergence and spread of resistant nosocomial and community-acquired pathogens is becoming a great menace to global public health. Therefore, there is an urgent need to find out new classes of antimicrobials. The search for new pharmacologically active agents obtained by screening of natural sources has led to the discovery of many clinically useful drugs that play a major role in the treatment of human diseases. Nearly 60% of the anti-infective agents that are commercially available or in different phases of clinical trials today are of natural product origin. Natural products are still major sources of innovative therapeutic agents for infectious diseases (both bacterial and fungal) and immunomodulation (10, 11).

Over the last few years, intrinsic or acquired resistance of *Enterococci* and *Staphylococcus aureus* to many antibiotics, in particular, to β-lactams and glycopeptides, has become a major cause of concern. MRSA (methicilin resistant *Staphylococcus aureus*) and VRE (vancomycin resistant *Eenterococci*) cause a health risk, especially in patients with severe underlying disease or immunosuppression (12).This study was planned to screen the antibacterial activity of marine actinomycetes against drug resistant bacterial pathogens.

Materials and methods

Sample collection and isolation of actinomycetes

Marine sediment samples were collected as part of an expedition to the Marakkanam coast, (Latitude [N] 128200; Longitude [E] 798950) at a depth of 400 cm in the southeast costal region of the Bay of Bengal, India. The sediment samples were dried in laminar air flow for 8-12 h and then kept at 42°C for 10-30 days in a sterile Petri dish and these preheated samples were used for the isolation of actinomycetes. The International Streptomyces Project (ISP) No. I media, Starch casein agar and Bennett's agar with 25% sea water, 25% soil extract was used for the isolation of actinomycetes and the growth media was supplemented with antibiotics, cycloheximide (25 mg/ml) and nalidixic acid (25 mg/ ml) (Himedia, Mumbai, India). Plates were incubated at 28 °C for 7-18 days. All the media were prepared with varying salt concentrations (3, 5, 7, 9, 12, 15, 18 and 21% [w/v]) to isolate the halophilic actinomycetes. The isolates were sub

cultured and maintained in slant culture at 4 °C as well as at 20% (v/v) glycerol stock at -80 °C (13).

Bacterial strain

The clinical isolate *Staphylococcus aureus* (MRSA) was obtained from Sri Narayani Hospital, Tamil Nadu, India. The drug resistance profile of the MRSA clinical isolate was tested against a spectrum of standard antibiotics, erythromycin (30 µg/disc), cefoxitin (5 µg/disc), ampicillin (10 µg/disc), gentamycine (10 µg/disc), ceftriaxone (30 µg/ disc), cefazolin (30 µg/disc), cefotaxime (30 µg/disc), Linezolid (30 µg/disc) and vancomycin (30 µg/disc). The zone of inhibition was interpretated in accordance with CLSI guidelines (2000) (14).

Assay of antibacterial activity

Primary screening was done to detect the production of antibiotics by the cross streak method and cylinder plate method (15, 16). Inhibition zones were expressed as diameters and measured after incubation at 37°C for 24h.

Characterization and identification of the potential strain

The classical method described in the identification key by Nonomura (17) 27) and Bergey's Manual of Determinative Bacteriology (18) was used for the identification of the isolate. The morphological, cultural, physiological and biochemical characterization of the isolate was carried out as described in ISP (19). The morphology of the spore bearing hyphae with the entire spore chain with the substrate and aerial mycelium of the strain was examined by light microscope (1000x magnification). Media used were those recommended in the International Streptomyces Project (ISP) (20). Mycelium was observed after incubation at 28 °C for 2 weeks and colours were also determined (21). Carbohydrate utilization was determined by growth on carbon utilization medium (ISP 9) (22) supplemented with 1% carbon sources at 28 °C. Temperature range for growth was determined on inorganic salts starch agar medium (ISP 4) using a temperature gradient incubator. Hydrolysis of starch and milk were evaluated by using the glucose starch agar and skim milk agar respectively. Reduction of nitrate and production of melanin pigment were determined by the method of ISP (23). All cultural characteristics were recorded after 14 days.

Optimization of nutritional and cultural conditions

To determine the optimal nutritional and cultural conditions and to identify the suitable media for growth, the strain was inoculated in different culture media (SCA, ISP 2, ISP 3, ISP 4, ISP 5, ISP 6, ISP 7, modified Bennett's agar, sucrose/nitrate agar, and nutrient agar) and the growth was investigated. The effect of cultural conditions like different incubation temperatures (15, 25, 37 and 50 °C), different pH (5.0, 6.0, 7.4 and 9.0) and NaCl concentrations (2, 5, 7, 9 and 12%) on the growth of the isolate was also studied. The carbon and nitrogen sources required were also studied by inoculating the isolates into mineral salt agar with different sugars substituted to starch (D-glucose, sucrose, starch, D-xylose, D-galactose, maltose, L-arabinose, fructose, lactose, and glycerol), organic nitrogen sources like peptone, yeast extract, casein and inorganic sources like ammonium sulphate, ammonium nitrate and urea. The concentrations of carbon sources and carbon utilization tests were done as described earlier (24, 25) 34, 35). After incubation the dry weight of the mycelium was measured and correlated with

the growth of the isolate. Based on the growth of the isolate in different media the cultural conditions were optimized.

Results

Isolation and screening

A total of hundred and twenty strains were isolated from the sediment samples out of which 12 actinomycetes strains showed antibacterial activity against a wide range of bacteria. The organism which produces white powdery and dried colonies suspected to be actinomycetes were sub cultured on ISP-1 agar with sea water. Microscopic identification was carried out to confirm the isolates as actinomycetes before screening for antibacterial activity. All the three screening methods, cross streak, cylinder plate and agar diffusion method employed were found to be effective in detecting antibacterial activity. The multidrug resistant profile of the clinical isolate was confirmed with testing of the actinomycete isolate with different standard antibiotic discs (Figure 1).

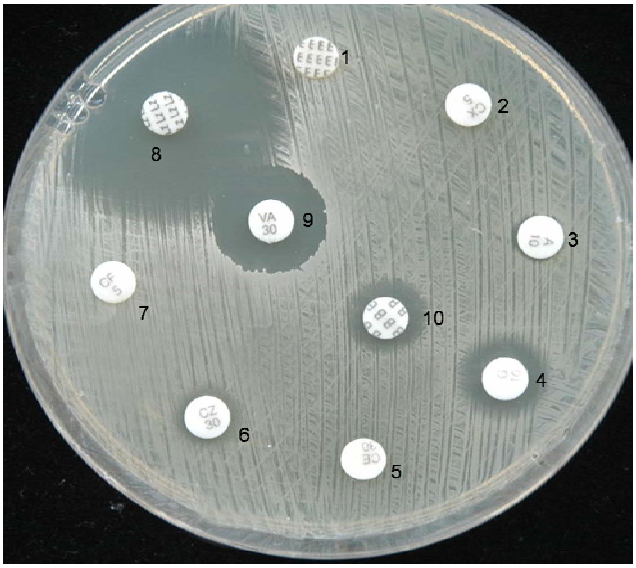

Figure 1. Effect of standard antibiotics on *Staphylococcus aureus* (MRSA) clinical isolate. Antibiotics marked as 1- Erythromycin (30 µg/disc), 2- Cefoxitin (5 µg/disc), 3- Ampicillin (10 g/disc), 4- Gentamycine (10 µg/disc), 5- Ceftriaxone (30 µg/ disc), 6- Cefazolin (30 µg/disc), 7- Cefotaxime (30 µg/disc), 8- Linezolid (30 µg/disc) and 9- Vancomycin (30 µg/disc).

The antibacterial activity of the potential isolate against methicillin resistant *Staphylococcus aureus* (MRAS) was given in Figure 2. The isolate produced the inhibition zone of 19 mm and 21mm against *Staphylococcus aureus* (MRSA).

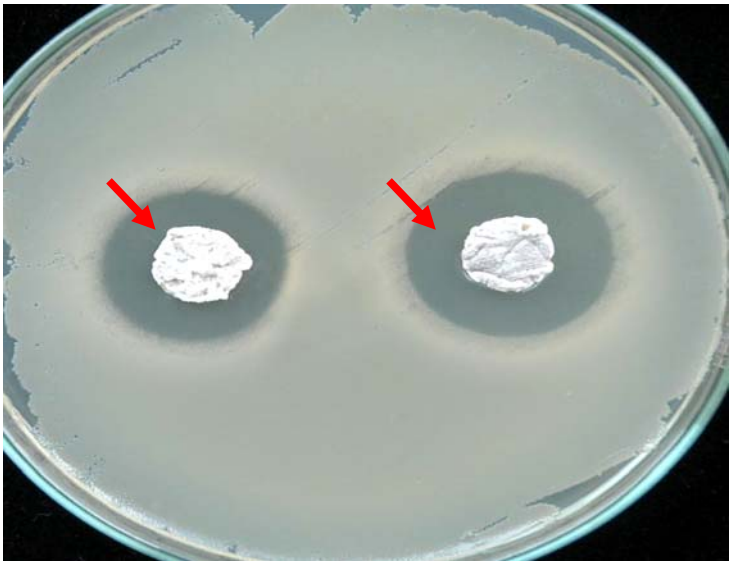

Figure 2: Antibacterial activity of *Streptomyces albofaciens* VITBRK1 spp. against drug resistant *Staphylococcus aureus* ATCC 29213 by cylinder plate method. The zone of inhibition produced by the isolate was indicated by arrow.

Phenotypic characterization of the isolate

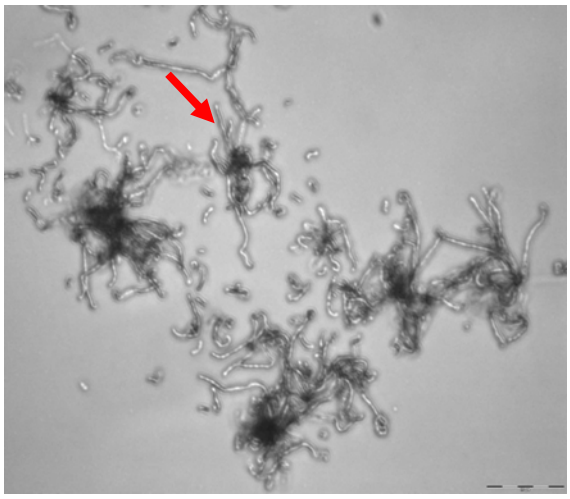

Figure 2: Spiral spore chain and surface morphology of *Streptomyces albofaciens* spp. VITBRK1 (1000X magnification)

The isolate was grown on oat meal agar medium (ISP 3), Yeast extract malt extract agar (ISP 2), and Inorganic salt starch agar (ISP 4) and it was observed that the mature sporulating aerial mycelium was greyish white. Reverse side and melanin pigments were absent. Spira-Spirales spore chain morphology was observed under optical microscope at 1000X magnification (Figure 2) and smooth spore surface morphology was observed.

Cultural, physiological and biochemical characterization

The growth of the strain was maximal in ISP1 medium supplemented with sea water (Table 1) with grey white spore and pale yellow reverse colour. In actinomycetes isolation agar abundant growth with grey spore and yellowish brown reverse colour.

Table 1: Cultural characters of *Streptomyces albofaciens* VITBRK1 spp.

Medium	Growth*	Spore	Reverse colour
Starch Casein agar	Good	White	Pale yellow
ISP medium 1	Good	Grey white	Pale yellow
ISP medium 2	Good	White	Yellowish brown
ISP medium 3	Moderate	Grey white	Pale yellow
ISP medium 4	Moderate	Grey white	Yellowish brown
ISP medium 5	Good	Grey white	Yellowish brown
ISP medium 6	Moderate	White	Pale yellow
ISP medium 7	Good	Grey	Yellowish brown
ISP medium 1 + Sea water	Abundant	Grey white	Yellowish brown
Modified Bennett's agar	Moderate	White	Pale yellow
Nutrient agar	Moderate	White	No colour
Marine agar	Moderate	Grey	Yellowish brown
Actinomycetes isolation agar	Abundant	Grey	Yellowish brown
Muller-Hinton agar	Good	Grey white	No colour
Sabouroud Dextrose agar	Poor	White	No colour
Potato Dextrose agar	Poor	White	No colour

*Growth of the strain was measured as dry weight of the mycelium

The strain showed maximum growth when cultivated at temperature 28°C; pH 7.4, with sea water 25%.The strain assimilated arabinose, xylose, inositol, mannitol, fructose, sucrose and raffinose, however the strain did not utilize rhamnose (Table 2). The strain utilized 0.1% of L-asparagine, L-phenylalanine, L-histidine and L-hydroxyprolone as nitrogen source. The strain was halophylic in nature tolerated Na Cl concentrations between 2% to 12%. The strain showed β-haemolysis on blood agar containing 5% sheep blood.

Based on the results of physiological, biochemical and cultural characterization as well as matching the keys given for classification of 458 species of actinomycetes included in International Streptomycetes Project the isolate was identified as *Streptomyces albofaciens* and designated as *Streptomyces albofaciens* VITBRK1 spp.

Table 2. Biochemical and physiological characteristics of *Streptomyces albofaciens* VITBRK1 spp.

Tests	**VITBRK1**
Gram stain	+
Endospore staining and motility	-
Aerial mycelium	White
Aerial spore mass colour	White grey
Colony colour	White
Production of pigments	-
Spore chain	Spiral
Spore surface	Smooth
Range of temperature for growth	25-28°C
Optimum temperature for growth	28°C
Range of pH for growth	6-8
Optimum pH	7.4
Starch hydrolysis	+
Haemolysis of 5% sheep blood	β-haemolytic
Catalase	+
Amylase	+
Production of amylase	+
Utilization of carbon source (1% w/v)*	
Arabinose	+
Xylose	+
Inositol	+
Mannitol	+
Fructose	+
Rhamnose	-
Sucrose	+
Raffinose	+
Growth in the presence of NaCl *	
2%	+
5%	+
7%	+
9%	+
12%	+

*Growth of the strain was measured as dry weight of the mycelium

Discussion

Our screening resulted in isolation of potential actinomycetes, *Streptomyces albofaciens* VITBRK1 spp. having antagonistic activity against drug resistant *Staphylococcus aureus* (MRSA). Several reports are available on antibacterial activity of marine actinomycetes (26, 27). The secondary metabolites produced by marine actinomycetes capable of inhibiting *Staphylococcus aureus* (MRSA) clinical isolates were reported by several workers. A *Streptomycete* BT-408 producing polyketide antibiotic SBR-22 effective against methicillin resistant *Staphylococcus aureus* was already reported (28). The compound

2-(2',4'-Dibromophenoxy)-4,6-dibromophenol isolated from the marine sponge *Dysidea granulosa* (Bergquist) has been reported to possess inhibitory activity against MRSA and VRE strains (29). Lynamicins A-E (1-5), was discovered from a novel marine actinomycete, NPS12745 and has been reported to possess inhibitory activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* (30). More recently TLN-05220 (1) and TLN-05223 (2) Echinosporamicin-type antibiotics from *Micromonospora echinospora* ssp. challisensis NRRL 12255 possessed strong antibacterial activity against a series of gram-positive pathogens including several strains of methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococci* (VRE) (31).

Since *Streptomyces albofaciens* VITBRK1 spp. exhibited strong activity against *Staphylococcus aureus* (MRSA), it is worth to proceed further to extract and to purify the anti MRSA antibiotics(active secondary metabolite) from the isolate and to establish its chemical identity by spectroscopic techniques. Extraction and purification of the active secondary metabolite is currently under progress in the author's laboratory.

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