

Isolation and Characterization of Antagonistic Actinomycetes from Marine Soil

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Abstract

Soil sample was collected from the coastal region of Tamil Nadu with the aim of isolating actinobacteria and screen them for antagonistic activity against common bacterial and fungal pathogens. Serial dilution of the soil sample and subsequent screening of the isolates obtained, resulted in the identification of a potential strain VITDDK2 with significant activity against *Klebsiella pneumoniae*, *Aspergillus flavus* and *Aspergillus niger*. In addition, the strain VITDDK2 also possessed chitinolytic activity. Chemotaxonomic analysis showed that the isolate VITDDK2 belongs to cell wall Type I. 16 S rRNA partial gene sequence and phylogenetic analysis showed that the strain VITDDK2 shared 93% similarity with *Streptomyces* sp. strain 346. Also the secondary structure of the rRNA of VITDDK2 and the restriction sites were predicted using Genebee and NEBCutter softwares respectively.

Keywords: *Streptomyces* sp. VITDDK2; Antagonistic activity; Chemotaxonomy; Chitinolytic activity

Introduction

Actinobacteria originally considered as an intermediate group between bacteria and fungi, but latter it has attained a distinct position (Pandey et al., 2004). Actinobacteria are a well defined group of Gram positive, free-living, saprophytic bacteria with high G+C content in their DNA (Pandey et al., 2004). The genus *Streptomyces* was described for the first time by Waksman and Henrici in the year 1943 (Kim et al., 2006). *Streptomyces* are filamentous, aerobic spore formers and omnipresent (Kim et al., 2006; Watve et al., 2001). Presence of LL isomer of 2, 6-Diaminopimelic acid (LL-DAP) and absence of any diagnostic sugar in the cell wall is a salient feature of the genus *Streptomyces* (Debananda et al., 2009).

Marine environment covering almost 70% of the earth surface is an infinite source of novel microorganisms and chemically unique structures which have a wide range of biological applications (Deepika and Kannabiran, 2009a; Deepika and Kannabiran, 2009b). Actinobacteria are a well known source of various secondary metabolites such as antibiotics, enzymes, pesticides, herbicides, immunomodulators, anti-infective and anticancer agents (Newman and Craig, 2007; Takahashi and Omura, 2003). Majority of the antibiotics available till date are derived from the genus *Streptomyces* (Watve et al., 2001). Over 6,000 compounds have been reported to be produced by *Streptomyces* (Takahashi and Omura, 2003). With the drug resistance of pathogenic microorganisms increasing at an alarming rate, there is an increase in the demand for newer and safer antibiotics with lesser side effects (Gupte et al., 2002). Hence the best alternate to meet the increasing demand of safe and cost effective drugs is natural

products from marine actinobacteria (Behal, 2003).

In this long run to combat drug resistant microorganisms, the unexplored and less explored marine environments needs to be studied frequently. In the present study we have reported the isolation and identification of a marine actinobacteria from Ennore saltpan with moderate antibacterial, antifungal and chitinolytic activity.

Materials and Methods

Location and sample collection

Soil samples were aseptically collected in sterile polyethylene bags from the Ennore saltpan (Lat. 13° 14' N, Long. 80° 22' E) at a depth of 5-15 and stored in the refrigerator at 4°C for further study. Ennore coast is situated about 24 km north of Chennai and is bound on either side by the Korttalaiyar river, Ennore creek and the Bay of Bengal (Pulikesi et al., 2007).

Isolation of actinobacteria

Soil samples (1gm) were serially diluted and plated on Starch Caesin agar (SCA) by pour plate technique (Deepika and Kannabiran, 2009b). Filtered sea water was used for media preparation. The plates were incubated at 30°C for 7-10 days and the colonies obtained were purified by quadrant streaking technique.

Screening for antibacterial and antifungal activity

Spores of VITDDK2 from pure culture plate was inoculated in ISP1 broth (International Streptomyces Protocol) and incubated at 30°C for 7 days on rotary shaker at 150 rpm. After adequate growth, the culture was centrifuged at 10,000 rpm and the cell free supernatant was transferred to a new eppendorf tube. Bacterial and fungal cultures were grown in nutrient broth and sabourauds dextrose broth respectively. Bacterial cultures used for the study included the gram negative organisms namely *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 10273) and *Pseudomonas aeruginosa* (ATCC 27853). The fungal cultures used as target organisms were *Aspergillus flavus* (MTCC 277), *Aspergillus niger* (MTCC 281) and *Aspergillus fumigatus* (MTCC 343).

A lawn culture of the target organisms was made on nutrient

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Received January 27, 2010; **Accepted** February 24, 2010; **Published** February 24, 2010

Citation: Deepika L, Kannabiran K (2010) Isolation and Characterization of Antagonistic Actinomycetes from Marine Soil. J Microbial Biochem Technol 2: 001-006. doi:10.4172/1948-5948.1000015

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agar and sabourauds dextrose agar respectively. Wells were cut using sterile well borer on the agar surface seeded with the test organisms and 200 µl of cell free supernatant from the fermentation broth of the isolate was added to each well aseptically. The plates were incubated at 37°C overnight for bacteria (Kirby Bauer method) and at 30°C for 72hrs for fungi (NCCLS M38-A2). Penicillin G and Nystatin were used as the positive controls for bacteria and fungi respectively. The plates were examined for zone of inhibition around the wells.

Chitinolytic activity

Chitinolytic activity of the isolate VITDDK2 was tested using Chitin agar. Chitin agar plates were prepared and the isolate was spot inoculated at the centre of the agar plate. The plate was incubated for 7 days at 30°C and examined for zone of inhibition around the colony (Mukesh et al., 2009).

Polyphasic taxonomy

The cultural and growth characteristics of the isolate VITDDK2 was studied on various culture media such as Tryptone yeast extract broth (ISP1), Yeast extract malt extract agar (ISP2), Oatmeal agar (ISP3), Inorganic salts starch agar (ISP4), Glycerol asparagine agar (ISP5), Peptone yeast extract iron agar (ISP6), Tyrosine agar (ISP7), Starch Caesin agar, Marine Zobell agar, Actinomycetes isolation agar, Nutrient agar, Bennets' agar, Sea water agar and Kusters' agar. The plates were incubated at 30°C and observations were made on the 7th, 14th and 21st days (Shirling and Gottlieb, 1966). The cell wall amino acid and whole cell sugar of the isolate VITDDK2 was determined as proposed by Lechevalier and Lechevalier (Lechevalier and Lechevalier, 1970).

From the strain VITDDK2, DNA was isolated following the protocol reported by Kieser et al. (2000). The isolated genomic DNA was amplified using Actino specific forward and reverse primers as designed by Stach et al. (2003). The PCR conditions were adapted from Farris and Olson (Farris and Olson, 2007). The PCR product was then ligated into the cloning vector pTZ57R/T and sent for sequencing to Macrogen (Seoul, South Korea).

The 16S rRNA partial gene sequence obtained was subjected to BLAST search using NCBI data bases. The DNA sequences were aligned and phylogenetic tree was constructed by neighbor joining method (Treeview software) using ClustalW (Saitou and Nei, 1987). A bootstrap value of 100 was used for tree construction. The 16S rRNA sequence was then submitted to the GenBank, NCBI, USA.

16S rDNA secondary structure and restriction sites analysis

The secondary structure and the restriction sites in the 16s rDNA sequence of the isolate VITDDK2 were predicted using online softwares Genebee and NEBCutter (version 2.0) respectively.

Results and Discussion

An actinomycete having antagonistic activity was isolated from the soil sample and characterized phylogenetically. The east coast of India though well explored compared to the west coast not many reports are available pertaining to diversity of actinobacteria in the Ennore saltpan. The Ennore saltern is situ-

ated to the north of Chennai on the east coast of India (Kasinatha et al., 2004; Remya and Vijayakumar, 2008). The soil composition of this saltern is a mixture of sandy-silt (37% to 63%) and silty-sand (33% to 53%) (Thangadurai et al., 2005). A total of 60 colonies were obtained from soil samples on serial dilution and plating on Starch Caesin agar plates.

Of all the 60 isolates screened for antibacterial and antifungal activity, the strain designated as VITDDK2 yielded promising result. VITDDK2 was antagonistic only towards *K. pneumoniae* producing an inhibition zone of 15 mm and compared with standard drug penicillin G (10 µg/disc). No activity was seen against *E. coli* and *P. aeruginosa*. Antifungal activity was observed against *A. flavus* (10 mm) and *A. niger* (25 mm) when compared to standard drug nystatin (100 µg/disc) which produced a zone of clearance of 16-20 mm (Table 1). Pandey et al. (2004) have reported that a broad spectrum of antibacterial *Streptomyces* species effective against both gram positive and gram negative bacteria were isolated from Lobuche area and Lukla area in Khumbu region of Nepal. Antibacterial activity of culture filtrate obtained from *Streptomyces* sp. No. 87 against gram positive and gram negative bacteria was reported (Charoensopharat et al., 2008). Praveen and Jain, (2007) have isolated *Streptomyces sampsonii* GS 1322 from local garden soil, Sagar, India and reported their antifungal secondary metabolite production. *Streptomyces cheonanensis* sp. nov., a novel *Streptomyces* with antifungal activity was isolated from the soil sample of Cheonan,

Test organism	Zone of inhibition (mm)*	
	Culture supernatant (100 µl/ well)	Penicillin (10 µg /disc)
Gram negative bacteria		
<i>Escherichia coli</i>	-	20
<i>Klebsiella pneumoniae</i>	15 ±0.26	-
		Nystatin (100 µg /disc)
Fungi		
<i>Aspergillus niger</i>	25 ±0.36	16
<i>Aspergillus flavus</i>	10 ±0.79	19
<i>Aspergillus fumigatus</i>	-	20

*Average of three independent experiments

Table 1: Antimicrobial activity of *Streptomyces* spp. VITDDK2 culture supernatant against bacteria and fungi.

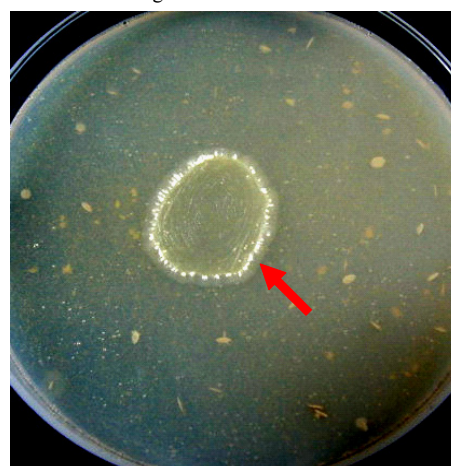


Figure 1: Chitinolytic activity was studied using Chitin agar and a clear zone around the colony (arrow) indicates the lytic activity exhibited by the isolate.

Korea (Kim et al., 2006). Debananda et al. (2009) have isolated a broad spectrum actinobacteria *Streptomyces sindenensis* strain LS1-128 having both antibacterial and antifungal activity from Loktak Lake in Eastern Indian.

The strain VITDDK2 was also studied for its ability to degrade chitin. A clear zone was observed around the colony inoculated at the centre of the agar plate proving its ability to degrade chitin (Figure 1). There are few reports on chitinolytic activity of *Streptomyces* isolated from several locations. Julie et al. (2003) have reported the chitinolytic activity of *Streptomyces melanosporofaciens* strain EF-76 and its ability to reduce the severity of potato scabies symptoms caused by phytopathogen in combination with chitosan. The chitinolytic activity exhibited by our isolate is comparatively lesser to that of *Streptomyces melanosporofaciens* strain EF-76. However in the present study we have tested chitinolytic activity against human pathogens whereas the chitinolytic activity of *Streptomyces melanosporofaciens* strain EF-76 was tested against the phytopathogen *Streptomyces scabies*. Kim et al. (2003) have studied the chitinolytic activity and the effect of various parameters such as pH, temperature, substrates, metals and inhibitors on the activity chitinase enzyme produced by *Streptomyces sp.* M-20 isolated in Mongolian soil. Production of chitinolytic enzyme by *Streptomyces albidoflavus* and its subsequent partial characterization was reported by Broadway et al. (1995). *In-vitro* chitinolytic activity of Iranian strains of *Streptomyces plicatus* and *Frankia sp.* on Olive isolate of *Verticillium dahliae* was already reported (Shahidi and Sonia, 2005). Actinobacteria with chitinolytic activity was isolated from Jordan soil by Tahtamouni et al. (2006).

In our earlier investigation, the morphological, physiological and biochemical properties of the isolate VITDDK2 was reported. These studies proved that the isolate VITDDK2 belonged to the genus *Streptomyces* (Deepika and Kannabiran, 2009a; Deepika and Kannabiran, 2009b). The cultural characteristics and the growth pattern of the strain VITDDK2 are provided in the Table

2. Chemotaxonomic analysis of the isolate showed the presence of LL- 2, 6 Diaminopimelic acid (LL-DAP) along with glycine in the cell wall. No diagnostic sugar was detected in the whole cell fraction. Hence the strain VITDDK2 belongs to cell wall type I. Similar studies related to cell wall chemistry have been utilized by several workers to identify the *Streptomyces* strains. *Actinopolyspora sp.*, *Microbispora sp.* and *Amycolatopsis sp.* isolated from saline soil collected from different locations in Kuwait were reported to be identified based on chemotaxonomy (Ibrhaim, 2006). Suthindhiran and Kannabiran (2009) have isolated VITSDK1 with hemolytic activity from the Marakkanam marine sediments and identified the strain belonging to the genus *Streptomyces* based on the chemotaxonomic results.

PCR amplification of the genomic DNA with actino specific forward and reverse primers resulted in 643 bp amplicon. The 16S rRNA sequence thus obtained was subjected to BLAST search using in the NCBI data base. The BLAST search result showed that VITDDK2 showed 93% similarity to the isolate *Streptomyces sp.* strain 346 (AJ278761). A phylogenetic tree was constructed based on neighbor-joining method (Figure 2). Based on molecular phylogeny the strain VITDDK2 was designated as *Streptomyces spp.* VITDDK2. The 16 S rRNA sequence of the strain *Streptomyces spp.* VITDDK2 was submitted to the GenBank, NCBI under the accession number, GU223092. However due to the unavailability of phenotypic data for the related strain *Streptomyces sp.* strain 346 from GenBank we are not able to do a comparative study of the phenotypic characteristics and genomic relatedness studies with our isolate.

The RNA secondary structure predicted for 16 S rRNA gene of *Streptomyces spp.* VITDDK2 showed the free energy of -150.1 kkal/mol, threshold energy of -4.0 with Cluster factor 2, Conserved factor 2 and Compensated factor 4 (Figure 3). Similarly the restriction sites prediction of the 16S rRNA gene of *Streptomyces spp.* VITDDK2 showed the restriction sites for various commercial and NEB restriction enzymes such as AclI, EcoRI, MboII, EcoRV, BaeI etc. Also the restriction site analysis showed

Medium	Growth pattern	Aerial mycelium	Reverse pigmentation
ISP1	Good	White	Pale -yellow
ISP2	Moderate	White	Dark yellow
ISP3	Moderate	White	Dark yellow
ISP4	Good	White	Pale -yellow
ISP5	Good	White	Pale yellow
ISP6	Good	White	Dark brown
ISP7	Good	White	Pale yellow
Starch Caesin agar	Good	White	Yellow
Marine Zobell agar	Abundant	White	Dark yellow
Actinomycetes isolation agar	Good	White	Pale yellow
Bennets' agar	Poor	White	Cream
Sea water agar	Moderate	White	White
Kusters' agar	Abundant	White	Pale yellow
Nutrient agar	Good	White	Yellow

Table 2: Cultural and morphological characteristics of *Streptomyces spp.* VITDDK2.

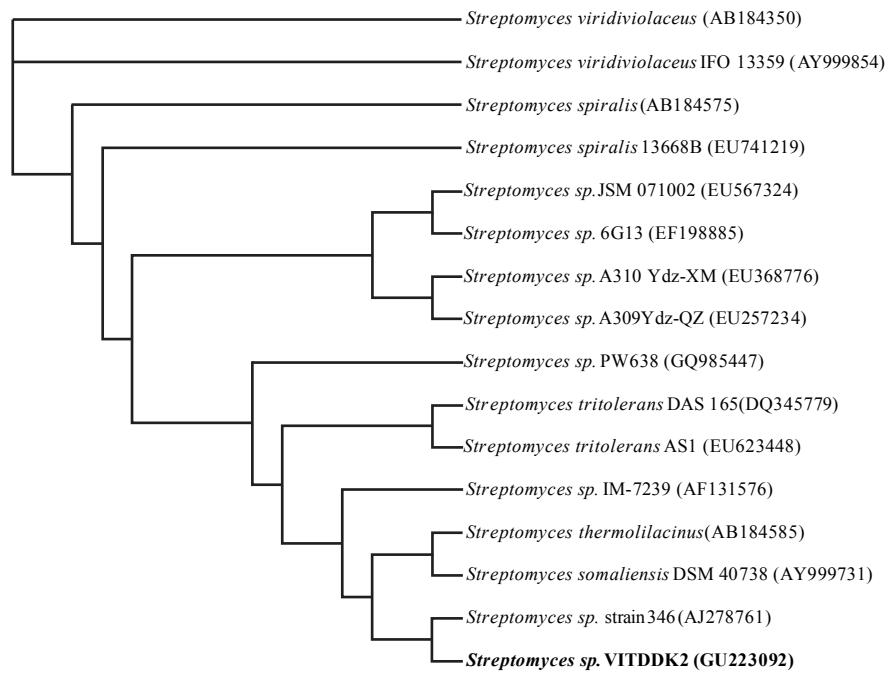


Figure 2: The taxonomic position of *Streptomyces* spp. VITDDK2 was determined based on 16S rRNA gene sequencing. A phylogenetic tree was constructed based on neighbour-joining method using the Treeview software. Bootstrap values are represented at the nodes. Bootstrap values of 50 and above are considered.

Free Energy of Structure = -150.1 kkal/mol

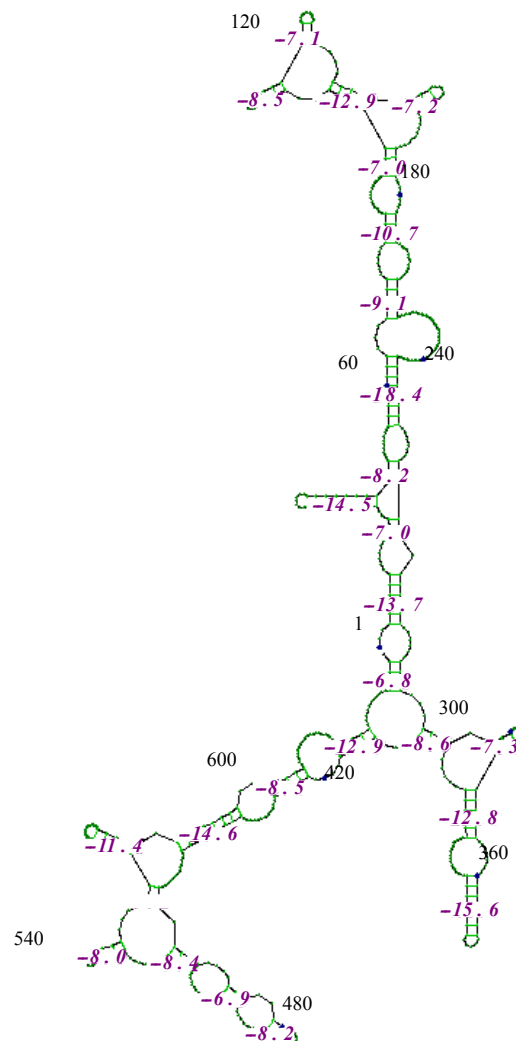
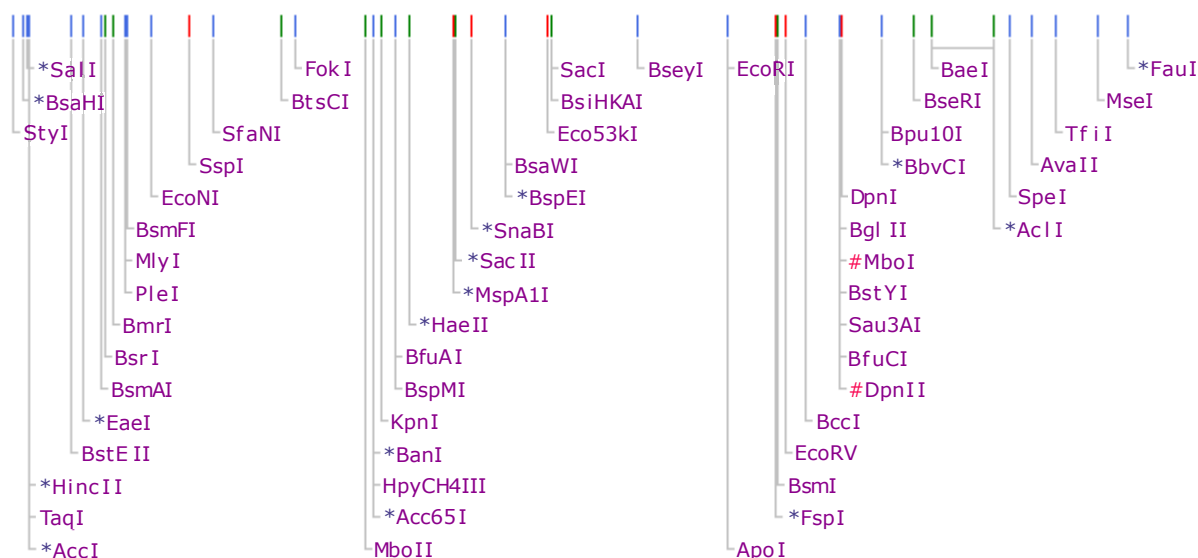


Figure 3: Secondary structure of 16S rDNA of the isolate *Streptomyces* spp. VITDDK2 was predicted by using Genbee software.



GC=58%, AT=42%

Figure 4: Restriction site analysis of 16 S rDNA of the isolate *Streptomyces* spp. VITDDK2 was carried out by using NEB cutter program.

the GC and AT content to be 58% and 42% (Figure 4).

Diverse microorganisms inhabit the marine ecosystem rather than the terrestrial environment. The microorganisms dwelling in this system are put under extremes of physiological conditions. Hence the micro fauna occupying this ecosystem are a reserve source of unique chemical structures. These chemical entities are being exploited for the welfare of mankind. Investigating such stressed environments may lead to the discovery of novel organism as well novel metabolites rather than re-isolating the same organism. In this paper we have reported one such microorganism *Streptomyces* spp. VITDDK2 with antibacterial, antifungal and chitinolytic activity. Further studies on extraction, purification and chemical characterization of the bioactive compound is under progress.

Acknowledgment

The authors wish to thank the management of VIT University for providing the facilities and the International Foundation for Science (IFS, F 4185-1), Sweden and the Organization for the Prohibition of Chemical Weapons (OPCW), The Hague, for financial support to carry out this study. Also thankful to Dr. S. Karutha Pandian, Head, Department of Biotechnology, Alagappa University, Tamil Nadu for helping in molecular work.

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