ANTAGONISTIC ACTIVITY OF *STREPTOMYCES* VITDDK1 spp. (GU223091) ISOLATED FROM THE COASTAL REGION OF TAMIL NADU, INDIA

Lakshmipathy Deepika and Krishnan Kannabiran*

Division of Biomolecules and Genetics, School of Biosciences and Technology, VIT University, Vellore- 632014, Tamil Nadu, India.

Summary

Our continuous searches for antagonistic actinobacteria lead to the isolation of *Streptomyces* VITDDK1 spp.from the soil sample collected at the Ennore saltpan region, Tamil Nadu, India. The isolate exhibited significant antibacterial activity against the gram negative pathogenic bacteria, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and antifungal activity against pathogenic *Aspergillus flavus.* Also the isolate possessed fairly moderate chitinolytic activity. Morphological and chemotaxonomic studies showed that the isolate belonged to the genus *Streptomyces*. Based on polyphasic taxonomy and phylogeny the isolate was designated as *Streptomyces* VITDDK1 spp. Further the isolate showed 99% similarity with the strain *Streptomyces sp. J-1.*

Keywords: *Streptomyces* VITDDK1 spp., antagonistic activity, chitinolytic activity, polyphasic taxonomy

* *Corresponding author*
Dr. K.Kannabiran
Professor& Division Head,
Division of Biomolecules and Genetics,
School of Biosciences and Technology,
VIT University
Vellore-632014, Tamil Nadu, India.
Tel.: +91-0416-2202473; Fax: +91-0416- 2243092 / 2240411.
E -mail: kkb@vit.ac.in

Introduction

Actinobacteria are a distinct group of gram positive, branching filamentous microorganisms with high G+C content of 69-78% in their DNA (1). They are unicellular prokaryotic microorganisms (2) and omnipresent in nature (3). *Streptomyces,* initially referred to as *Actinomyces* was identified by Waksman and Henrici (4). *Streptomyces* are free-living, aerobic, saprophytic in nature, predominantly found in soil and normally playing a crucial role in the degradation of complex biopolymers (1, 5, 6). At maturity they produce long chains of non-motile arthrospores and are highly oxidative (7) and they contain LL-Diaminopimelic acid, glycine and possess no characteristic sugar in their cell wall (8).

Actinobacteria are well acknowledged for their ability to produce various essential metabolites such as antibiotics, enzymes and immunomodulators (9-11). The history of antibiotics from actinobacteria dates back to 1943 when streptomycin was isolated for the first time from *Streptomyces griseus* by Waksman and Henrici in the year 1943 (4). Since then actinobacteria are looked upon as a source of remedy for almost all types of ailments. Of all the genera, the genus *Streptomyces* is the major producer of antibiotics and other vital secondary metabolites (12-14). About 75 to 80% of the antibiotics discovered in the 20th century were derived mainly from the genus *Streptomyces* (2). Hence the genus *Streptomyces* is considered a boon to the scientific and medical world.

The demand for new and safe drugs is increasing at an alarming rate and at present researchers are showing more attention towards the actinobacteria especially *Streptomyces* from marine environment which is a potential producer of secondary metabolites with large chemical diversity (15). Hence the present study was planned to isolate antagonistic *Streptomyces* species from marine soil sample collected at the Ennore saltpan region of India.

Materials and methods

Sample collection and isolation of actinomycetes

Soil sample was collected from the Ennore saltpan, the coastal region of Tamil Nadu (Lat. 13°.14’ N, Long. 80°.22’ E) from its top layers at a depth of 5-15 cm in sterile

polyethylene bags, transported to the laboratory aseptically and stored in the refrigerator at 4°C until further use. The Ennore coast is located about 24 km north of Chennai on a peninsula and is bound by the Korttalaiyar river, Ennore creek and the Bay of Bengal (16). Soil sample was serially diluted and plated for the isolation of actinobacteria colonies. Filtered sea water was used for both media preparation and serial dilution. 1ml from each dilution was plated on Starch Caesin agar (SCA) by pour plate technique (17). The plates were incubated at 30° C for 7-10 days. The colonies were purified by quadrant streaking technique and maintained for further studies.

Antibacterial and antifungal assay

A single colony of the isolate from the pure culture plate was inoculated in ISP1 broth (International Streptomyces Protocol) and incubated at 30° C for 7 days on rotary shaker at 150rpm. After the specified time period the culture was centrifuged at 10,000rpm and the cell free supernatant was collected in a fresh eppendorf tube. Bacterial cultures were raised overnight in nutrient broth. Bacterial cultures used for the study included *Staphylococcus aureus, Staphylococcus epidermidis, Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* A lawn culture of the bacteria was made from the 24hrs old culture on nutrient agar. Wells were cut using a sterile well borer on the agar surface seeded with the test organisms. From the cell free supernatant 200µl was aseptically added to each well and the plates were incubated at 37° C overnight. Penicillin G (antibiotic disc (10µg), Himedia, Mumbai, India) was used as the positive control. The plates were examined for zone of inhibition around the wells (Kirby Bauer method).

Pure culture of *Aspergillus flavus, Aspergillus niger* and *Aspergillus fumigatus* were raised in sabourauds' dextrose broth. Cell free supernatant (200µl) was aseptically added to each well made on the agar surface seeded previously with test fungal cultures. Nystatin (100µg/ disc, Himedia, Mumbai, India) was used as the positive control. The plates were incubated at 30° C for 72hrs and examined for zone of clearance around the well as described in the standard protocol (NCCLS M38-A2).

Chitinolytic assay

Chitinolytic activity of the isolate was tested by using Chitin agar. Chitin agar plates (chitin 5 g, yeast extract 0.5 g, KH_2PO_4 0.7 g, K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.3 g, $FeSO_4.7H_2O$ 0.1 g and NaCl 0.1g in 1000ml) were prepared. Spot inoculation of the isolate was made at the centre of the agar plate and the plate was incubated for 7 days at 30° C. The plate was examined for zone of inhibition around the colony as described earlier (18).

Polyphasic taxonomic identification

The morphological, cultural and chemotaxonomic characterization of the isolate was carried out as described earlier (19). The growth pattern and colony characteristics of the isolate VITDDK1was 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 agar plates were incubated at 30°C and. the cultural characteristics of the isolate were recorded periodically on the 7th, 14th and 21st days. The morphology of the isolate was studied as described in the Bergey's manual (20). The spore surface morphology and its orientation were studied using Scanning electron microscopy technique (SEM, S3400, 20KV, 5.00µm) (21). The cell wall amino acid and whole cell sugar was determined as described by Lechevalier and Lechevalier (22).

From the pure culture plate, DNA was isolated as per the protocol reported by Kieser et al. (23). The isolated genomic DNA was amplified using Actino specific forward and reverse primers as designed by Stach et al, 2003 (24). The PCR conditions were used as described earlier by Farris and Olson (25). An initial denaturation at 95°C for 4 min was followed by 40 cycles of denaturation at 95°C for 45 sec, primer annealing at 72°C for 60 sec and extension at 72°C for 60 sec, with a 5 min final extension at 72°C. The PCR product was then ligated into the cloning vector pTZ57R/T and subsequently transformed into competent *E.coli* DH5α. The transformed *E.coli* DH5α colonies were confirmed by Blue-white colony selection, mobility shift and restriction digestion. Recombinant plasmid was isolated and the

inserted DNA was sent to Macrogen (Seoul, South Korea) for 16 S rRNA partial gene sequencing.

Phylogenetic analysis

The 16S rRNA partial gene sequence data was subjected to BLAST search using NCBI data bases and the homologous sequences was identified. Multiple sequence alignment and subsequent phylogenetic tree construction by neighbour joining method were accomplished by using DDBJ ClustalW and Treeview software (26). A bootstrap value of 100 was used for tree construction. The 16S rRNA sequence was then submitted to the GenBank, National Centre for Biotechnological Information (NCBI), USA.

Results and discussion

The sampling site, Ennore saltern is situated on the north side of Chennai in the east coast region of India. The saltern soil is a mixture of sandy-silt and silty-sand with the percentage of sand ranging from 37% to 63% and silt from 33% to 53% (27). The soil sample collected at the ennore saltpan was processed under laboratory conditions aseptically and serial dilution followed by plating on starch caesin agar yielded 110 strains. All the 110 strains were screened for antibacterial and antifungal activity. It resulted in the identification of a potent antagonistic actinobacteria and the strain was named as VITDDK1. The isolate VITDDK1 produced an inhibition zone of 15-25 mm against Gramm negative bacteria (Table 1), *Escherichia coli* (20 mm), *Klebsiella pneumoniae* (25 mm) and *Pseudomonas aeruginosa* (15 mm) whereas no activity was exhibited against *Staphylococcus aureus, Staphylococcus epidermidis* and *Bacillus subtilis.* The degree of inhibition was in the order of *K. pneumoniae* > *E. coli* > *P. aeruginosa.* The standard drug, Penicillin G (10 µg /disc) also produced an inhibition zone of 20 mm. This shows that the isolate VITDDK1 is effective against tested gram negative bacteria and not antagonistic against tested gram positive bacteria. *Aspergillus flavus* was the only fungal pathogen susceptible to VITDDK1 with an inhibition zone of 15 mm when compared to the standard Nystatin (100 µg /disc)

which produced a zone of clearance of 16-20 mm (Table 1). However no activity was observed against fungal pathogens *Aspergillus niger* and *Aspergillus fumigatus*.

Table 1. Antimicrobial activity of *Streptomyces* VITDDK1 spp. culture supernatant against Gram negative bacteria

Test organism	Zone of inhibition (mm)*	
	Culture supernatant (100 µl/ well)	Penicillin (10 µg /disc)
Gram negative bacteria		
Escherichia coli	20	20
Klebsiella pneumoniae	25	-
Pseudomonas aeruginosa	15	-
		Nystatin (100 µg /disc)
Fungal strains		
Aspergillus flavus	15	19
Aspergillus niger	-	16
Aspergillus fumigatus	-	20

*Average of three independent experiments

Recent reports from our laboratory substantiate the results obtained in the present study (22). Several marine actinomycetes isolated till date have been reported to possess potent antimicrobial activity against human pathogens (28, 29). *Streptomyces* GCA 0001 spp. isolated from warm soil collected at the roadside, riverbank and construction sites have been reported to possess antimicrobial activity (30). *Streptomyces* RSP9 spp. (31) isolated from terrestrial soil was reported to have antimicrobial activity. Antimicrobial activity of methanolic crude extract obtained from *Streptomyces* ERI-26 spp. isolated from the Nilgiri forest soil of Western Ghats have already been reported (32). Isolation of antagonistic secondary metabolites from actinomycetes was reported by several researchers (33, 34). After the discovery of Penicillin (1928) from the fungus *Penicillium notatum,* natural

products from microorganisms have been considered as an alternative source for synthetic drugs. The strain VITDDK1 was also screened for chitinolytic activity. A clear zone was observed around the colony of the isolate inoculated at the centre of the chitin agar plate (Fig. 1). It was reported that *Streptomyces* spp. isolated from the Pindari glacier region of Indian Himalayas have been shown to possess chitinolytic activity (18). Also reports on the chitinolytic activity of Antarctic strain, *Verticillium lecanii* are available (35).

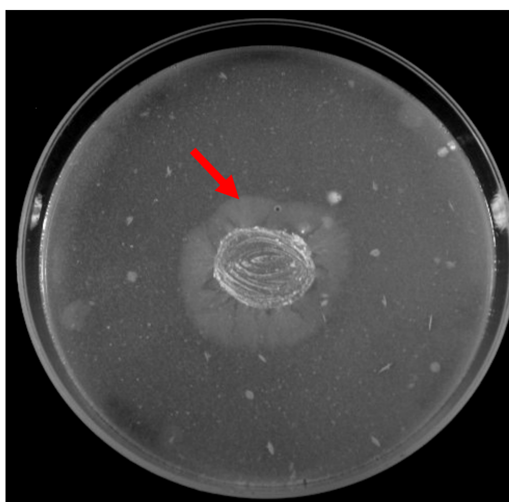

Figure 1. Chitinolytic activity of *Streptomyces* VITDDK1 spp. The ability to break down chitin was determined for the isolate *Streptomyces* VITDDK1 spp. Chitinolytic activity was studied using Chitin agar. A clear zone around the colony indicates the lytic activity exhibited by the isolate.

The first step in the identification of the organism by polyphasic approach includes examining the micro and macro morphology, analyzing the cell wall composition with a combinatorial study of the 16S rRNA gene sequence of the organism. The isolate VITDDK1 is a gram positive, aerobic organism with oxidative type of metabolism. The colony morphology and the growth pattern of the strain VITDDK1 are provided in the Table 2. The isolate VITDDK1 produced a spore chain with 9-17 smooth spores per chain in a rectiflexibles fashion. Chemotaxonomic analysis of the isolate showed the occurrence of LL-2, 6 Diaminopimelic acid (LL-DAP) along with glycine in the cell wall. No characteristic sugar was detected in the whole cell fraction. Hence the strain VITDDK1 has cell wall type I

which is characteristic to *Streptomyces* species. Chemotaxonomic analysis has been adopted by several workers for the successful identification of the organism. Rabah et al (33) have identified the strain SK4-6 as belonging to the genus *Streptomyces* based on the cell wall composition. Similarly the antagonistic actinomycete DPTD-5 isolated from the Vellar estuary was identified as *Streptomyces* spp. by studying the cell wall composition (36). Abbas has followed the cell wall fraction analysis for the identification of 3 actinomycetes strains namely *Actinopolyspora* spp, *Microbispora* spp. and *Amycolatopsis* spp. isolated from saline soil collected from different locations in Kuwait (37).

Table 2. Cultural and morphological characteristics of *Streptomyces* VITDDK1 spp.

Medium	Growth pattern*	Aerial mycelium	Reverse pigmentation
ISP1	Good	White	Yellow
ISP2	Moderate	White	Dark yellow
ISP3	Moderate	White-yellow	Pale yellow
ISP4	Good	White	Yellow
ISP5	Good	White	Pale yellow
ISP6	Good	White	Dark yellow
ISP7	Good	White	Light brown
Starch Caesin agar	Good	White	Yellow
Marine Zobell agar	Abundant	White	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

* Based on the dry weight of the mycelium

The morphological, cultural and chemotaxonomic results indicated that the isolated potent strain VITDDK1 belonged to the genus *Streptomyces.* PCR amplification with actino specific forward and reverse primers resulted in 657 bp amplicon. The 16S rRNA sequence of the strain obtained was subjected to BLAST search using the sequences in the NCBI data base. The BLAST search result showed that VITDDK1 was 99% similar to the isolate *Streptomyces sp. J-1*(FJ797601). A phylogenetic tree constructed based on neighbor-joining method also confirmed the BLAST results (Fig. 2). Based on molecular taxonomy and phylogeny the strain was designated as *Streptomyces* VITDDK1 spp. The 16s rRNA sequence of the strain *Streptomyces* VITDDK1 spp. was submitted to the GenBank under the accession number, **GU223091**. The BLAST search and phylogenetic analysis showed that VITDDK1 has significant genomic relatedness with *Streptomyces sp. J-1* (FJ797601).

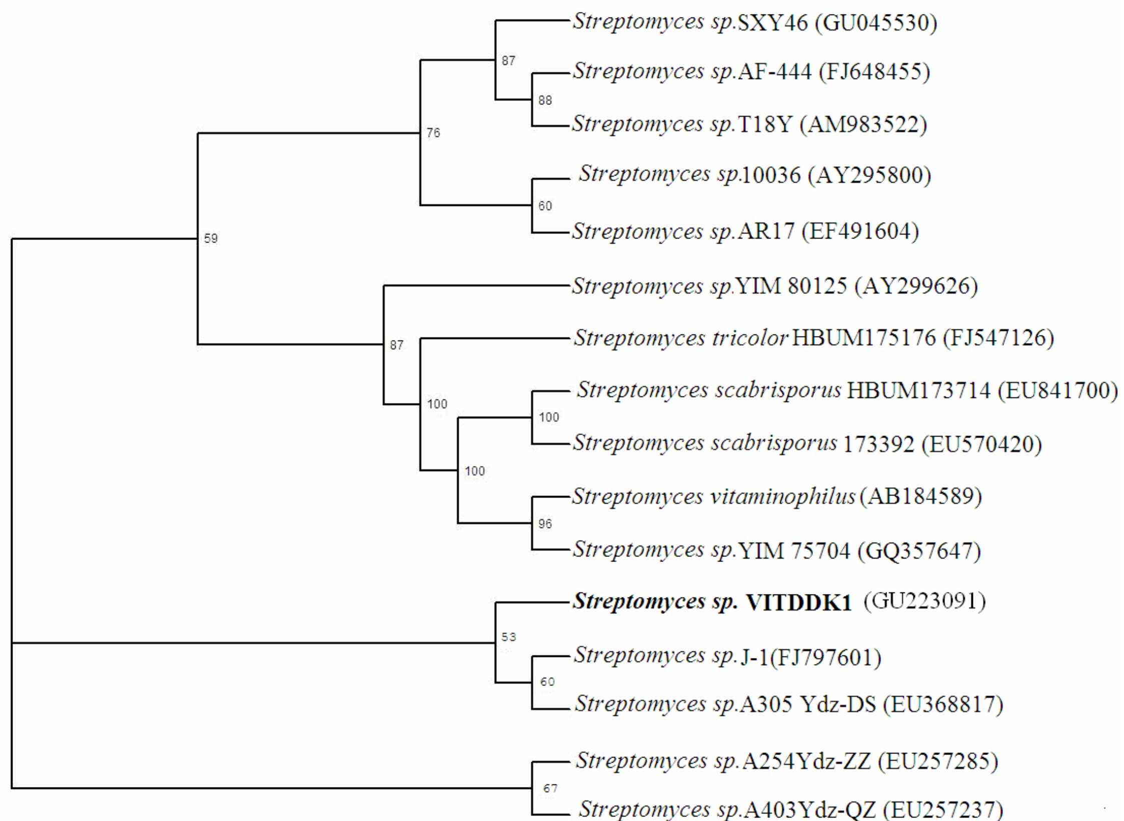

Figure 2. A phylogram indicating the taxonomic position of *Streptomyces* VITDDK1 spp. The taxonomic position of *Streptomyces* VITDDK1 spp. was determined based on 16S

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.

Since the phenotypic data for *Streptomyces sp. J-1* (FJ797601) and few other closest neighbours are not available in the databank, a comparative study with *Streptomyces sp. J-1* (FJ797601) was not carried out. However, comparison of phenotypic characteristics of the isolate was done with one of the closest neighbour *Streptomyces scabrisporus sp. nov* (EU570420) (Table 3).

Table 3. Characteristics that distinguish *Streptomyces* VITDDK1 spp. from its closest phylogenic neighbor- *Streptomyces scabrisporus sp. nov.*

Characteristics	***Streptomyces* sp. VITDDK1**	***Streptomyces scabrisporus sp. nov.****
Gram staining	+	+
Aerial mycelium on ISP3	White-yellow	Grey
Melanin	-	-
Spore surface	Smooth	Rugose
Spore ornamentation	Rectiflexibles	Spiral
ISP2	White	Light ivory
ISP3	White-yellow	Colourless
ISP4	White	Pearl
ISP5	White	Pearl
ISP6	White	Pearl
ISP7	White	Bamboo

* Ping et al, 2004 (38).

Marine ecosystem encompasses a wide range of diverse microbial species than the terrestrial environment. These microbial species are a rich source of chemically diverse

entities with potent applications which can be exploited for human welfare. One of the best approaches in the identification and isolation of novel bioactive compound is the isolation of new species of microorganism from the marine environment. This is possible only with an extensive study of the marine systems. In the present study an attempt was made to identify the *Streptomyces* species with fairly good antibacterial and antifungal activity. This search resulted in the identification of *Streptomyces* VITDDK1 spp. with potent antimicrobial activity. Morphological, cultural and chemotaxonomic studies have shown that VITDDK1 belongs to the genus *Streptomyces.* Phylogenetic study suggests that the strain is identical to *Streptomyces sp. J-1.* At present extraction, identification and characterization of the bioactive secondary metabolite from *Streptomyces* VITDDK1 spp. responsible for antagonistic activity is under progress. Studies involving the exploration of unexplored and rare environments may pave the way for the discovery of several actinomycetes species and diverse secondary metabolites for potential therapeutic applications.

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