

ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF *STREPTOMYCES* SP. VITDDK3 ISOLATED FROM ENNORE COAST, TAMIL NADU, INDIA

LAKSHMIPATHY DEEPIKA AND KRISHNAN KANNABIRAN

ABSTRACT

Actinobacteria isolated from soil samples collected at the Ennore saltern, Tamil Nadu was screened for antibacterial and antifungal activity against common clinical pathogens. The isolate VITDDK3 exhibited more promising antagonistic activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Aspergillus flavus* and *Aspergillus fumigatus*. Presence of LL-Diaminopimelic acid and glycine assigned the isolate VITDDK3 to the genus *Streptomyces*. Further 16S rRNA partial gene sequence was carried out to confirm the chemotaxonomic results. BLAST analysis showed that the strain VITDDK3

possessed 98% similarity with *Streptomyces* sp. A254Ydz-ZZ but phylogenetic analysis revealed that its closest neighbour was *Streptomyces tricolor* HBUM175176. The rRNA secondary structure and the restriction sites of VITDDK3 were predicted using Genebee and NEBCutter online softwares respectively.

Keywords: *Streptomyces* sp. VITDDK3, antibacterial activity, antifungal activity, chemotaxonomy, phylogenetic analysis

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INTRODUCTION

Marine ecosystem covers almost 70% of the earth surface (1). Soil is the most extensively studied ecological niche (2). Organisms present in these environments are extremely rich sources of bioactive compounds (3-5). One of the most important and well acknowledged groups of microorganisms in the soil is the actinobacteria (6, 7). Actinobacteria are gram positive, filamentous organisms inhabiting the soil (8). They are wide spread in distribution (9). They produce a vast array of secondary metabolites such as enzymes (7, 10), immuno-modulators (11), antibiotics (12), antihelminthic (13) and anticancer agents (11). Among them *Streptomyces*, the well-known saprophytes of the soil are the major producer of antibiotics (14). About 75-80% of the drugs available commercially

are being derived from *Streptomyces* (15). Reports reveal that the continuous screening of actinobacteria, especially *Streptomyces* will lead to the discovery of 1, 00,000 new compounds with diverse applications (16).

Drug resistance of pathogenic microorganisms is a global issue. The more the increase in the percentage of drug resistance the more the demand for newer drugs with lesser side effects (17). The best way to confront these microbial villains is by switching over to the drugs from natural sources. In order to overcome the disadvantages with the existing drugs and to fight the drug resistant pathogens, there is an urgent need for newer, safer and less expensive drugs from natural sources. In this view, the present study was designed with the aim of isolating and identifying an actinobacteria with potential antimicrobial activity.

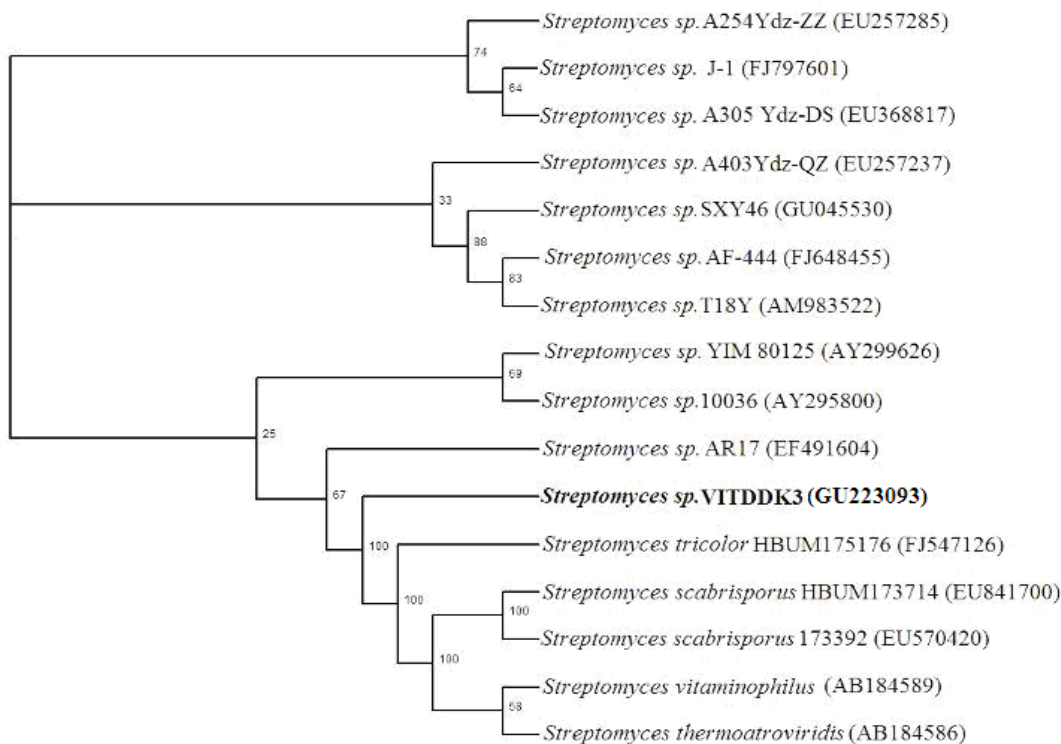


Figure 1. Phylogram indicating the taxonomic position of *Streptomyces* spp. VITDDK

The taxonomic position of *Streptomyces* VITDDK3 spp. was evaluated based on 16S rRNA gene sequencing. Phylogenetic tree was constructed based on neighbour-joining method using the ClustalW and Treeview software. Bootstrap values are represented at the nodes and values of 50 and greater than 50 are considered.

MATERIALS AND METHODS

Sampling site and isolation of actinobacteria

Marine soil sample (500g) was collected from the saltpan at Ennore coast of Tamil Nadu (Lat. 13°.14' N and Long. 80°.22' E). Soil was collected about 5-7 inches from the surface to prevent contamination in sterile bags. The soil sample along with sea water was transported to the laboratory and maintained at 4° C until further use. Ennore is located on the southeast coast of India, bound by the Korttalaiyar river, Ennore creek and the Bay of Bengal (18).

Starch casein agar (SCA) employed for the isolation of actinobacteria was sterilized at 121°C at 15lbs pressure for 15 minutes. Soil samples (1gm) were serially diluted upto 10⁻⁶ dilution. The soil suspensions were plated using starch casein agar by pour plate technique (19). Filtered sea water was used for media preparation as well as serial dilution. The plates were incubated at 30° C for 7-10 days. The colonies obtained were purified by quadrant streaking technique.

Microbial pathogens

Among bacterial pathogens, *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 10273), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923) were used as target organisms. Among fungal pathogens, *Aspergillus fumigatus* (MTCC 343), *Aspergillus flavus* (MTCC 277) and *Aspergillus niger* (MTCC 281) were used as target microorganisms.

Antibacterial and antifungal assay

Spore suspension of VITDDK3 was inoculated in 100ml of ISP1 broth (International Streptomyces Protocol) and incubated at 30° C for 7 days at 150rpm rotation. The culture was centrifuged at 10,000rpm for 10 minutes. The cell free supernatant was separated and transferred to a fresh eppendorf. A lawn culture of the target organisms was

made on the surface of specific agar medium. Nutrient agar was used for bacteria and sabourauds dextrose agar was used for fungi. Using well borer of (7mm width) wells were made and 200 µl of the cell free supernatant was aseptically added to each well. The plates were incubated and examined for zone of inhibition around the wells. The incubation period and temperature for bacteria is 37° C overnight (Kirby Bauer method) whereas for fungi 30° C for 72hrs (CLSI M38-A2). Penicillin G and Nystatin were used as the positive controls for bacteria and fungi respectively.

Cultural characterization

The cultural properties and growth characteristics of the isolate VITDDK3 were studied on various defined 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 recorded on the 7th, 14th and 21st days (20).

Chemotaxonomy

The cell wall amino acid and whole cell sugar of the isolate VITDDK3 was studied as proposed by Lechevalier and Lechevalier (21).

Molecular taxonomy

DNA was isolated using the protocol as reported by Kieser et al. (22). The isolated genomic DNA was amplified using actino specific forward and reverse primers as designed by Stach et al (23). The PCR conditions were adapted from Farris and Olson (24). 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 was subjected to BLAST search in the NCBI data bases. The DNA sequences were

aligned and phylogenetic tree was constructed by neighbor joining method (Treeview software) using ClustalW (25).

The 16S rRNA sequence was then submitted to the GenBank, NCBI, USA.

Free Energy of Structure = -226.3 kkal/mol

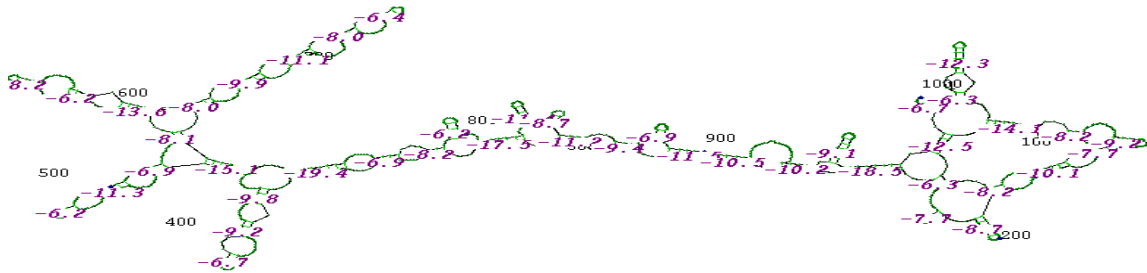


Figure 2. Secondary structure of 16S rDNA sequence of *Streptomyces* spp. VITDDK3

16S rDNA secondary structure and restriction sites analysis

Genebee and NEBCutter (version 2.0) online softwares respectively.

The 16s rRNA secondary structure and the restriction sites on the DNA sequence were predicted using

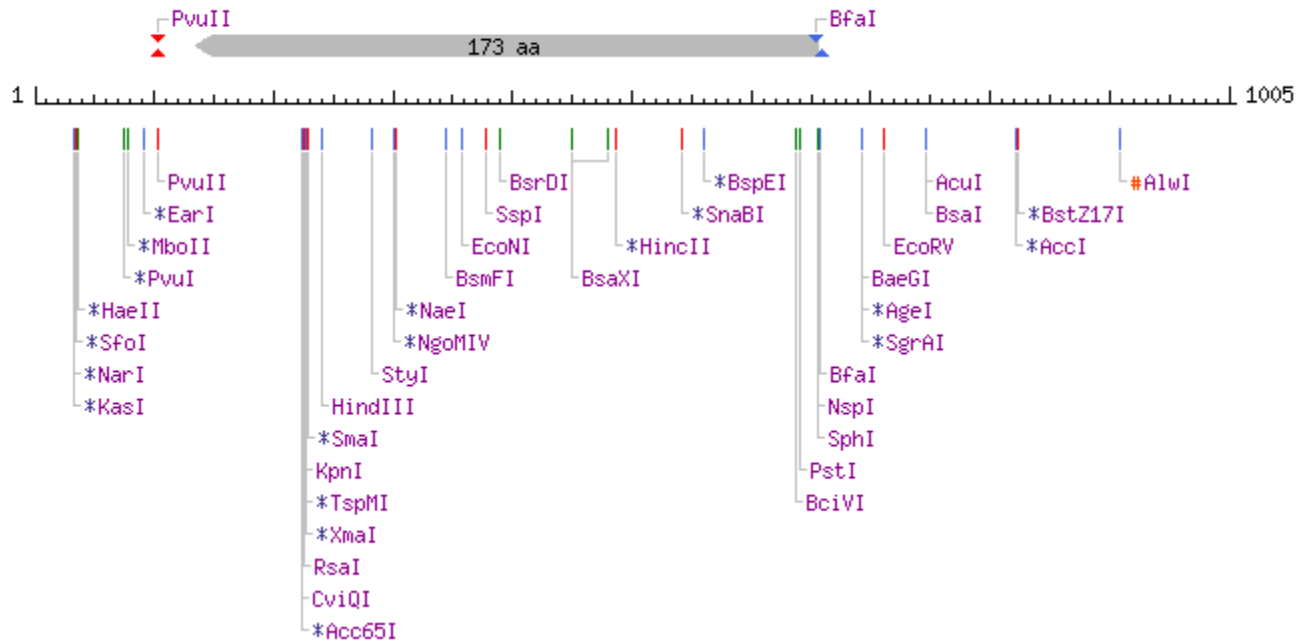


Figure 3. Restriction sites on the 16S rDNA sequence of *Streptomyces* spp. VITDDK3

RESULTS AND DISCUSSION

Actinobacteria with potential activity against bacteria and fungi was isolated from the saltern soil sample collected at Ennore, Tamil Nadu. The Ennore soil is saltern and darkly coloured due to its rich organic content. The soil is a mixture of sandy-silt and silty-sand (26). Literature survey revealed that not many reports are available on actinobacteria isolated from Ennore region and more over not been fully explored for potential marine actinomycetes. Based on aforesaid reasons the Ennore region was chosen for our study. About 75 isolates were obtained from the systematic serial dilution and plating of the collected soil sample.

Among the 75 isolates screened for antimicrobial activity, the strain VITDDK3 showed significant activity against tested bacterial and fungal pathogens. The isolate, VITDDK3 exhibited moderate antagonistic activity (zone of inhibition) against *E. coli* (22 mm) and *P. aeruginosa* (15 mm) and compared with standard drug Penicillin G (10 µg /disc). Similarly VITDDK3 showed mild to moderate antifungal activity against *A. flavus* (10 mm) and *A. fumigatus* (25 mm) and compared to the standard drug

Nystatin (100 µg /disc) which produced an inhibition of 16-20 mm (Table 1). Isolation of a broad spectrum antibacterial secondary metabolite from *Streptomyces* sp. ANU 6277 was reported from the laterite soil collected at the Acharya Nagarjuna University, Guntur (27). Kiran et al. (2009), have demonstrated the antibacterial activity of *Streptomyces* sp. No. 2 isolated from the Lonar lake at Aurangabad, against gram positive and gram negative bacteria (28). Khamna et al. (2009) have isolated and characterized antifungal secondary metabolite from *Streptomyces spectabilis* CMU-PA101. *Streptomyces spectabilis* CMU-PA101 was recovered from rhizosphere soil associated with *Pandanus amaryllifolius*, a medicinal plant collected at Lumphun Province, Thailand (14). Augustine et al. (2005) have isolated a non-polyene antifungal secondary metabolite from *Streptomyces albidoflavus* PU23 recovered from the water sample collected from Pune (29). *Streptomyces* sp. isolated from saline farmlands of Punjab and Pakistan has been reported to possess both antibacterial and antifungal activity (2)

Table 1. Antimicrobial activity of *Streptomyces* spp. VITDDK3 culture supernatant against bacteria and fungi

Test organism	Zone of inhibition (mm)*	
	Culture supernatant (100 µl/ well)	Penicillin (10 µg /disc)
Gram negative bacteria		
<i>Escherichia coli</i>	22	20
<i>Pseudomonas aeruginosa</i>	15	-
Fungi		
<i>Aspergillus niger</i>	-	16
<i>Aspergillus flavus</i>	10	19
<i>Aspergillus fumigatus</i>	25	20

*Average of three independent experiments

In our previous studies we have reported the morphological, physiological and biochemical properties of the isolate VITDDK3 (9, 15). The cultural and growth characteristics of the strain VITDDK3 are elaborated in the Table 2. The cell wall of the isolate VITDDK3 contained LL isomer of 2, 6 Diaminopimelic acid (LL-DAP) along with glycine. No diagnostic sugar was seen in the whole cell fraction. Based on the chemotaxonomy the strain VITDDK3 was identified and belonged to the cell wall type I. Several actinomycetes were identified based on chemotaxonomy, *Nocardioopsis dassonvillei* was identified from the blood of 3-year old child using cell wall chemistry

as one of the identification methods (30). An actinomycete strain SF2809 with anti-chymase (chymase inhibitor) activity was isolated from the soil sample collected at Hachijo Island in Tokyo, Japan. This strain was identified as *Dactylosporangium* sp. by chemotaxonomical study along with morphological and phylogenetic studies (31). Thermo tolerant strains producing N-acylamino acid racemase were isolated from soil samples collected from the coastal ecosystem of Chonburi, Rayong and Trat Provinces. Based on morphology and chemotaxonomy the strains were identified as *Streptomyces* (32).

Table 2 Cultural and morphological characteristics of *Streptomyces* spp. VITDDK3

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

* Based on the dry weight of the mycelium

An amplicon of 643 bp was obtained on PCR amplification of the DNA with specific forward and reverse primers. Using the BLAST search engine the NCBI data bank, sequences homologous to our isolate VITDDK3 were collected and subsequently aligned using ClustalW (DDBJ). A phylogenetic tree was constructed based on

neighbor-joining method using Treeview software (Fig. 1). The BLAST search analysis revealed 98% similarity of the strain VITDDK3 with *Streptomyces* sp. A254Ydz-ZZ I and 93% similarity with the isolate *Streptomyces* sp. 346 (EU257285) although the phylogenetic tree confirmed *Streptomyces tricolor* HBUM175176 (FJ547126) with 97%

similarity was its nearest neighbour. Based on molecular phylogeny the strain VITDDK3 was designated as *Streptomyces* spp. VITDDK3. The 16 S rRNA sequence of the strain *Streptomyces* spp. VITDDK3 was submitted to the GenBank, NCBI under the accession number, GU223093. Due to the non availability of phenotypic data for *Streptomyces* sp. A254Ydz-ZZ and *Streptomyces tricolor* HBUM175176 from the GenBank we are unable to compare and contrast the phenotypic characteristics and genomic relatedness with our isolate.

The 16S rRNA secondary structure of *Streptomyces* spp. VITDDK3 determined showed the free energy of the predicted structure to be -228.3 kkal/mol, threshold energy to be -4.0, Cluster factor to be 2, Conserved factor also to be 2 and compensated factor to be 4 (Fig.2). Similarly the restriction sites of *Streptomyces* spp. VITDDK3 predicted showed the sites for various commercial and NEB restriction enzymes such as BspEI, Hinc II, Hae II, KpnI, NaeI etc. Also the restriction site analysis showed the GC and AT content to be 58% and 42% (Fig.3).

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CONCLUSION

Microorganisms inhabiting the marine ecosystems are more diverse and unique with the ability to produce unique chemical entities. These ecosystems need to be extensively studied to gain a complete knowledge and unravel its unexhausted reserve of secondary metabolites. Our searches for antagonistic actinomycetes lead to the identification of *Streptomyces* sp. VITDDK3 with fairly moderate antibacterial and antifungal activity. However further studies are needed with respect to the structural characterization and the biological activity of the secondary metabolite.

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Authors Address for communication:

Dr. K. Kannabiran, Ph.D.,
Professor and Head,
Division of Biomolecules and Genetics,
School of Biosciences and Technology,
VIT University,
Vellore- 632014, India.
Tel. +91 4162202473, Fax +91 4162243092
E-mail: kkb@vit.ac.in