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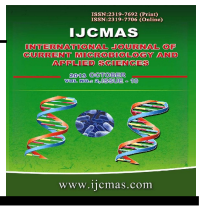


Original Research Article

DNA Based Identification and Characterization of Thermophilic *Streptomyces* sp. From Desert Soil of Rajasthan

Dimple Songara* and Swarnjeet Kaur

Karanja House 2, Dumayne road, Colaba, Mumbai, India
******Corresponding author*

A B S T R A C T

The present study was aimed at to identify the thermal resistant actinomycetes from the desert soil of Rajasthan, India. The morphology, cultural, physiological, biochemical and phylogenetic analysis of isolate stb-1 were characterized. According to the morphology, isolate stb-1 was identified as a representative of the genus *Streptomyces*. The 16S rRNA region of this strain was amplified and sequenced. A Neighbour-Joining algorithm tree of 16S rRNA was constructed. The strain showed growth at high temperatures and were screened for antagonistic potentiality against gram positive and gram negative human pathogenic bacteria. The strain stb-1 showed broad spectrum antibiotic activity against the tested pathogens. According to the observation and phylogenetic analysis, the strain stb-1 was proved to belong to the genus *Streptomyces* with thermal resistance and antibiotic potentiality.

Keywords

Actinomycetes; morphology phylogeny; antibacterial; Streptomyces; thermal resistance; 16s r RNA.

Introduction

Actinomycetes are widely distributed in terrestrial environments and have long been a source of commercially useful enzymes and therapeutically useful bioactive molecules, identifying biological diversity increases the chances of identifying novel molecules. In the case of bacteria, identifying new species and genera increases the chances that any bioactive molecules produced by such organisms are unknown to science (Lazzarini *et al*., 2000). The traditional methods used for the identification of the aerobic filamentous actinomycetes are laborious, time consuming and often require a series of specialized tests (Steingrube *et al*., 1995b, Harvey *et al*., 2001). Chemical criteria, such as the isomer of diaminopimelic acid (DAP) present in the cell wall and the diagnostic sugar(s) present in the whole-cell hydrolysate, have been used to separate the actinomycete genera into broad chemotaxonomic groups. However, determination of these characteristics is time-consuming and, in most cases, cannot identify an isolate to a single genus (Lechevalier, 1989).

Sequence-based identification is an alternative method of identifying clinical isolates that are either slowly growing or

difficult to identify by biochemical profiling.In particular, amplified rDNA restriction analysis (ARDRA) has proved to be very useful (Harvey *et al*., 2001; Alves *et al*., 2002). By conventional isolation methods, members of the genus Streptomyces comprise more than 95 % of the filamentous actinomycete population in soil (Lacey, 1973; Elander, 1987). The streptomycetes produce more antibiotics than any other genus of bacteria and, therefore, have been heavily exploited as a source of novel antimicrobial agents (Watve *et al*., 2001). We investigated the utility of 16S rRNA gene sequence analysis for the identification of actinomycete isolates. Since the accuracy of sequence identification is directly dependent upon the sequence database that is queried, we evaluated the GenBank database. The GenBank database is a public database that contains a large number of sequences, including 16S rRNA sequences.

Materials and Methods

All the chemicals used in the analytical methods and media preparation were of analytical grade with maximum available purity supplied by Hi-media (Mumbai), SISCO (Chennai) and Sigma (USA). The 16S rRNA primers were from Genei (Bangalore) and PCR reagents and Taq Polymerase from Biogene, USA. The Actinomycetes presence soil samples were collected from Jodhpur and Jaislmer region of Rajasthan, India.

In this study, was conducted during the period of September 2009, for that, the soil samples were collected aseptically from the Thar Desert, India at a depth of 6-12 inches. The soil samples were air dried in a hot air oven at 45°C for 1 h to reduce the proportion of bacteria other than actinomycetes (Williams *et al*., 1972). Standard dilution plate technique was followed for the isolation of actinomycetes (Kuster and Williams, 1964). Ten gram each of the soil samples were added to 90 ml distilled water in a 250 ml Erlenmeyer flask under sterile condition and kept in a rotary shaker (120 rpm) at room temperature for 30 min. Vacuum filtration was used for collecting supernatant and then the supernatant was serially diluted to obtain 10^{-4}, 10^{-5} and 10^{-6} dilutions. Each dilution was plated on different media like actinomycetes isolation agar, starch casein agar and ISP 1- ISP 7 agar, to know the better media for the growth of desert soil actinomycetes. After inoculation of 7-9 days at 27±1°C the actinomycetes colonies were selected, counted and made into pure culture following single spore culture technique. The culture was maintained on sabouraud's agar by periodical sub-culturing.

Thermal assay

In screening, different colonies were used to test the growth of isolates on high temperatures. The actinomycetes isolation agar was prepared; 20 ml of the medium was poured in to the petriplates. The isolated actinomycetes were inoculated into different petriplates and incubated at different temperatures i.e., 27^0C, 37^0C, 47^0C. The actinomycete colonies shows growth at higher temperatures (47^0C) was taken for further studies.

Identification and characterisation of isolates

Isolates were identified and characterised on morphological, cultural, physiological, biochemical criteria and antibiotic potential was tested against six different gram positive and gram negative bacteria.

The Morphology of actinomycete isolates were examined by using cover slip culture technique. After the growth of isolate cover slip was taken, left in air to dry, stained with Gram's stain and examined under microscope and structure was compared with Bergey's manual.

Cultural Characters:

Cultural characteristics of actinomycetes isolates were examined by using different media like Actinomycetes Isolation Agar, Starch Casein Agar, Nutrient Agar, Blood agar and ISP media

(1-7). Colour of aerial mycelium on ISP media was observed by using colour scale.

Physiological tests:

The physiological properties were depending on melanoid pigmentation, utilization of different carbon sources; a variety of carbohydrates were be used like mannitol, fructose, inositol and sucrose. Decomposition of casein, xanthin, hypoxanthin, tyrosine and hydrolysis of starch were determined by clear zone around the colonies.

Biochemical tests:

The biochemical tests like Gram's staining, indole formation, MR-VP, Catalase enzyme, H_2S test and Oxidase test etc. were performed. These tests were recorded as negative or positive.

Screening for antibacterial activity

The isolated actinomycetes were screened for antibacterial activity, against six human pathogenic bacteria, including two Gram-positive test organisms *Bacillus subtilis*, *Staphylococcus aureus* and four Gram-negatives bacteria *Escherichia coli*, *Klebsiella* species, *Pseudomonas* species and *Salmonella typhi*. The bacterial cultures were procured from IMTECH Chandigarh and were identified microscopically and biochemically. Bacterial strains were inoculated into 10 ml of sterile nutrient broth, and incubated at 37 °C for 24 h.

Molecular characterization of thermophillic isolates

Isolated strains that show thermophillic and antagonistic activity against human pathogenic bacteria were subjected to molecular analysis, includes genomic DNA isolation, purification by gel analysis, PCR amplification and 16s r RNA sequencing.

DNA extraction, purification and gel analysis

Actinomycetes isolates were propagated in Broth medium for ten days at 30ºC, for DNA extraction. Extraction were done either by method of EL-Fiky (2003) and purified as described by Saito and Miura (1963) and Tamaoka and Komagata (1984). DNA of the selected isolates subjected to randomly amplified polymorphic DNA analysis (RAPD) using short oligonucleotide primers by which multiple amplification products from loci distribution throughout the genome were resulted. RAPD reactions will be assembled and optimized as described by Williams *et al*. (1990).

PCR Amplification of 16S rRNA Sequence

The 16S rRNA gene was amplified from genomic DNA obtained from cultures by PCR with selected primers pA (5'AGA

GTT TGA TCC TGG CTC AG 3') and pH (3'AAG GAG GTG ATC CAG CCG CA 5'). The reaction mixture contained 25 to 50 ng of DNA, Taq PCR buffer, 1.5 mM $MgCl_2$, 10 mM deoxynucleoside triphosphate mixture, 50 pmol of each primer, and 0.5 U of *Taq* polymerase. PCR conditions consisted of an initial denaturation at 80°C for 5 min; 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s; and a final 7-min extension at 72°C. The amplification products were examined by agarose gel electrophoresis and purified from gel.

Phylogenetic Analysis

BLASTN (optimized for megablast) searches were manipulated with the sequences of isolated Streptomyces strain. The corresponding sequences of representative species were used for phylogenetic analyses. Neighbour-Joining (NJ) algorithm was used to construct a graphical phylogenetic tree using software MEGA 4.1 (Tamura *et al.*, 2007). The program was starts with a set of aligned sequences using Clustal W and searches for phylogenetic trees that are optimal according to NJ algorithm.

Results and Discussion

Morphology

In this study, different strains were obtained and only one type of strain was found to shows growth at high temperature i.e. 47^0C. These colonies were blue in colour and show reverse pigmentation i.e. yellow in colour. It was named as Stb- 1.

The isolate Stb-1 gives morphology similar to that of a *Streptomyces* isolate with a distinct substrate and aerial mycelium with conidial chains observed on microscopic examination (see figure 1). The conidial chains with more than 50 spores were observed. The microscopic examinations of the present isolate Stb-1 shows that the conidial chains are Rectus-flexibilis. The cell wall analysis shows the presence of LL-Diaminopimilic acid (LL-DAP) as major cell wall peptidoglycan content. There were no diagnostic sugars detected in cell wall. So the isolate Stb-1 was identified as a representative of genus *Streptomyces* (Waksman and Henrici, 1943).

Table 1 shows the cultural characteristic of the isolate after an incubation period of one week at 28±1°C. The spore mass colour of the *Streptomyces* members may vary from white, gray, red, yellow, green and blue to violet. Colour of the aerial mycelium is one of the prominent identification characters of *Streptomyces* isolates at species level (Pridham and Tresner, 1974). On further examination of the morphological characters the isolate is having a spore mass colour of predominantly blue and occasionally grey and white. The International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966) has recommended recording of aerial mycelial colour in different media for use as a taxonomic character, which was also followed in the present study. ISP-3, ISP-3, ISP-5, 6 and ISP-7 were showed the reverse colony colour i.e. yellow and cream and is not producing any diffusible pigments. The isolate was showing good growth on ISP media and other suggested media (Table 1). The above-mentioned characters suggest that the isolate Stb-1 can be identified as *Streptomyces* groups of blue series (Nonomura, 1974).

The biochemical tests like Gram's staining, indole formation, MR-VP, Catalase enzyme and Oxidase test etc. were performed. These tests were recorded

as negative or positive. The Physiological tests used to characterize the isolates were melenoid pigmentation, degradation of tyrosine, xanthine, urea, and citrate. The hydrolysis of soluble starch and casein was detected by the presences of clear zone around the colonies. The biochemical and physiological characteristics are presented in table 2.

Table 3 shows the antagonistic activity of isolate Stb1 against six selected gram positive and gram negative bacteria, the isolate strain showed significant broad spectrum antibacterial activity against all the test pathogens. Maximum inhibition was reported against *Bacillus subtilis* with 26.0±0.52 and minimum activity was against *Pseudomonas* species *with* 15.6±0.57. Standard antibiotic, Chloramphenicol was taken as a positive control.

Phylogenetic Analysis

Genomic DNA was isolated, purified and amplified by primers and 16s rRNA was sequenced with help of Macrogen Genomics, Korea. Nucleotide sequences were compared to those in the Gene Bank database with the Basic local alignment search tool (BLAST) algorithm to identify known closely related sequences. Sequences were analyzed with MEGA 5.2 software, and thereby trees were generated by the neighbour-joining algorithm implemented in phydit (Saitou and Nei, 1987).The 16s rRNA sequence was submitted to gene bank under accession number JX312806. The 16S rRNA gene showed high similarity with 16S rRNA genes deposited in the GenBank (Table 4). The 16S rRNA gene sequence showed high similarity with 16S rRNA genes deposited in the GenBank. Stb-1 strain 16S rRNA had 99% identity (E value 0.0) with 16S rRNA gene of Streptomyces sp. GB24 (JX965401) followed by 99% identity (E value 0.0) with 16S rRNA gene of S. thermolilacinus (AB184585), 99% identity (E value 0.0) with 16S rRNA gene of Streptomyces sp. YIM 75712 (JQ808019), 99% identity (E value 0.0) with 16S rRNA gene of Actinobacterium ZXY017 (JN049466) and Streptomyces fradiae (FJ486352) and also stb-1had 99% identity (E value 0.0) with Streptomyces sp. N01-1767 (DQ778660), Streptomyces sp. ZZY 2013 (KC336149), Streptomyces sp. MBRC-75 (KC179810), Streptomyces sp. MBRC-55 (KC179796), Streptomyces sp. MBRC-37 (KC179788). In this study, 16S rRNA gene of different *Streptomyces* species (different strains of a species) was obtained by BLASTN search, however 10 strains of *Streptomyces* species were selected on the basis of high identity (%) with good E value for phylogenetic analysis. As shown in Figure 2 all strains belonging to Streptomycetaceae were relatively closely related to *Streptomyces*; strain stb1 had a clade supported with *Streptomyces thermolilacinus.*

The present study is mainly involved in the isolation and identification of actinomycetes based on the cultural, morphological physiological, biochemical and molecular characteristics. The streptomyces stb1 strain has shown broad spectrum antibacterial activity due to production of antibiotics. The 16s rRNA sequence revealed that Streptomyces sp. Stb1 (accession no. JX312806) have close family relationship with 16s rRNA gene sequences of different streptomyces species. Therefore based on experimental studies and genetic DNA sequence analysis, we concluded that present strain is a new species of *streptomyces* having antibacterial and thermophillic activity.

Table.1 Cultural characteristics of isolates stb1

Medium	Growth	Aerial mycelium colour	Reverse colony colour
ISP1	Good	white	None
ISP2	Good	Blue	Yellow
ISP3	Good	Blue	crème
ISP4	Poor	Absent	None
ISP5	Good	Green blue	Bright yellow
ISP6	Good	grey	White
ISP7	Good	Blue	Yellow
AIA	Good	Deep blue	Bright yellow
SCA	Poor	white	None

AIA = Actinomycetes Isolation Agar, SCA=Starch Casein agar

Table.2 shows the biochemical and physiological characteristics (utilisation of carbon source) of isolate stb1

Test	Results
Gram's staining	+
Hydrolysis (%w/v)of:	
Starch	**+**
Casein	**-**
Urea	+
Enzymatic activity:	
Catalase	**+**
Oxidase	**+**
H2S production	**+**
Indole formation	**-**
MR test	**-**
VP test	**-**
Conc. of NaCl(%w/v)	
1%	**+**
2%	**+**
3%	**+**
5%	**-**
Optimum temperature	45-47^{o}C
Optimum pH for growth	**7**
Nitrogen Source (1% w/v)	
Peptone	**+**
Yeast extract	**+**
Casein	**-**
Urea	**+**
Chemical characteristics	
G+C content (mol %)	**59.71%**

Degradation (%w/v)of:	
Xanthine	+
Hypoxanthine	+
Tyrosine	+
Melanin production	+
Soluble pigment	-

Test	**Results**
Carbon source utilization and sugar fermentation (1% w/v)	
D-glucose	+
Sucrose	+
D-xylose	-
D-galactose	+
Maltose	+
L-arabinose	+
Lactose	+
Inositol	-
Inuluin	-
Raffinose	+
Rhamnose	-
Fructose	+
Melibiose	-
Sorbitol	-
Mannitol	+
Mannose	-

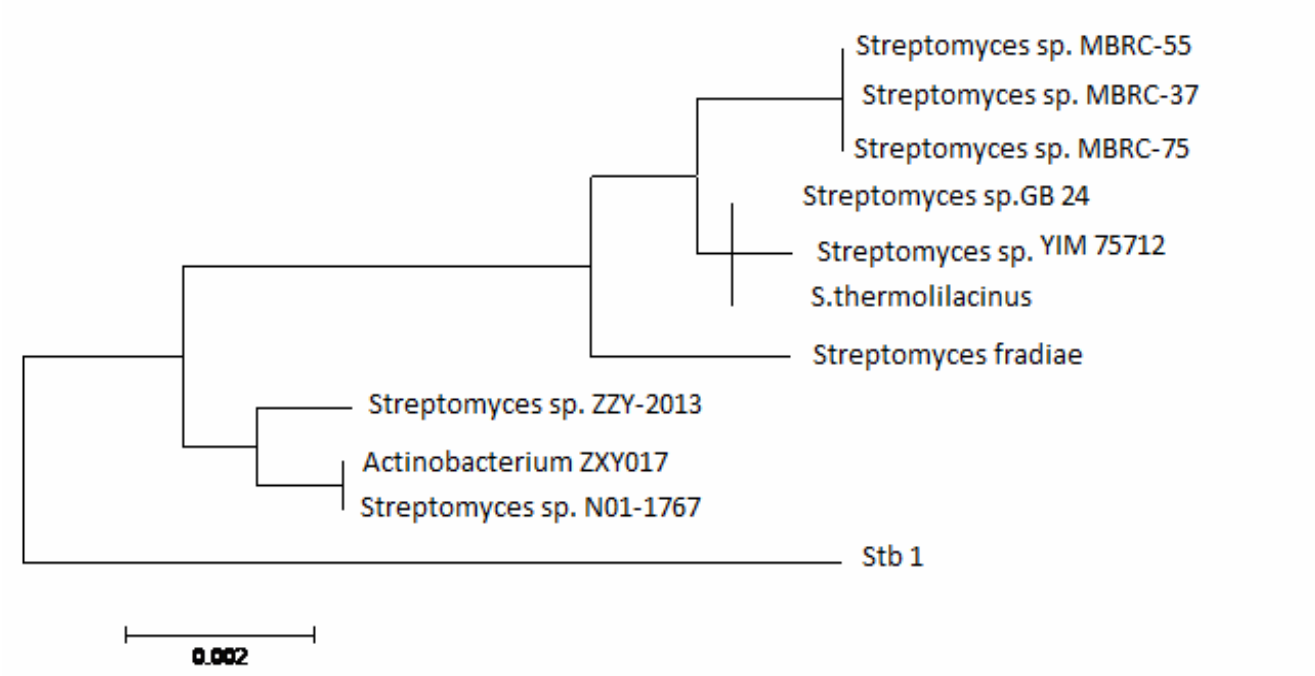
Table.3 Antagonistic activities of *Streptomyces species* against the test pathogen

Test Pathogens	**Zone of Inhibition (mm)**	**Positive control**
Bacillus subtilis	26.0±0.52	18.0±0.57
Staphylococcus aureus	20.0±0.52	16.0±0.57
Escherichia coli	18.9±0.58	12.8±1.0
Klebsiella species	19.0±0.58	12.3±0.57
Pseudomonas species	15.6±0.57	12.8±1.0
Salmonella typhi	16.0±0.57	15.0±1.0

Table.4 Results of similarity searches between 16S rRNA genes isolated in the present investigation and GenBank accessions using BLASTN Algorithm (optimized for megablast)

Highest identical species	Accession number	Sequence identity (%)	E-value
Streptomyces sp. GB24	JX965401	99%	0.0
Streptomyces thermolilacinus	AB184585	99%	0.0
Streptomyces sp. YIM 75712	JQ808019	99%	0.0
Actinobacterium ZXY017	JN049466	99%	0.0
Streptomyces fradiae	FJ486352	99%	0.0
Streptomyces sp. N01-1767	DQ778660	99%	0.0
Streptomyces sp. ZZY 2013	KC336149	99%	0.0
Streptomyces sp. MBRC-75	KC179810	99%	0.0
Streptomyces sp. MBRC-55	KC179796	99%	0.0
Streptomyces sp. MBRC-37	KC179788	99%	0.0

Fig.2 Phylogenetic tree is based on the nucleotide sequence of 16S rRNA genes. The Neighbour-Joining algorithm tree was constructed by MEGA 5.2.



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