



Research Journal of **Microbiology**

ISSN 1816-4935



Academic
Journals Inc.

www.academicjournals.com

Anti-microbial Screening of *Streptosporangium nondiastaticum* TBG-75A20, Isolated from the Forest Soil of South India

Sugathan Shiburaj

Applied Microbiology Laboratory, PS and ES Division, Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram, Kerala, Pin-695 562, India

ABSTRACT

An Actinomycetes strain TBG-75A20 was isolated from a forest soil sample collected from Neyyar wild life sanctuary of Kerala state. Cultural properties of this new isolate were studied extensively using ISP media. This isolate showed separate aerial and substrate mycelium and the aerial mycelia produced spherical sporangia of 11-17 μm size with non-motile spores. The morphological, biochemical, physiological characters and 16s rDNA sequence analysis of the isolate indicated that this strain belongs to the genus *Streptosporangium* and identified as *S. nondiastaticum*. Interestingly, this isolate secreted antifungal compounds against *Aspergillus niger*, *A. fumigatus*, *Fusarium oxysporum*, *F. solani* and *Rhizoctonia solani* and antibacterial substances which are active against *Bacillus subtilis*, *B. cereus*, *Staphylococcus aureus*, *Escherichia coli*. Antimicrobial properties of *S. nondiastaticum* are reported for the first time.

Key words: Actinomycetes, *Streptosporangium*, antimicrobial, 16s rDNA

INTRODUCTION

Actinomycetes are the largest producers of antibiotics and other bioactive compounds and estimates show that 66% of the known metabolites are produced by this group of organisms (Kieser *et al.*, 2000). In recent years the rare Actinomycetes members have helped in the understanding of the basic biology of secondary metabolite biosynthesis also. *Streptosporangium* is one of the rare Actinomycetes genera that have been attracting wide attention as a new and important source of various secondary metabolites with antimicrobial and antineoplastic activities. There are about thirty different bioactive molecules, which were shown to be produced by this group of organisms, including derivatives of chloramphenicol, platomycins, sibiromycin and others (Lazzarini *et al.*, 2000). The genus *Streptosporangium* is the type genus of the family Streptosporangiaceae (Couch, 1955). The family Streptosporangiaceae contains several genera, including *Streptosporangium*, *Herbidospira*, *Microbispora*, *Microtetrastroma*, *Planobispora*, *Planomonospora*, *Planotetrastroma*, *Nonomuraea* and *Acrocarpospora* (Tamura *et al.*, 2000). In the course of research on unknown microbial resources, some strains of actinomycetes were isolated from samples collected in the forest soils of Neyyar wild life sanctuary of Kerala, south India. This area is one of the 12 known mega diversity centers in the world (Nayar, 1996). The present work has described the isolation of a *Streptosporangium* strain, designated TBG-75A20, its identification by a polyphasic taxonomy approach and demonstration of antimicrobial activities elaborated by the strain.

MATERIALS AND METHODS

Isolation and maintenance of microbial strains: Glucose asparagine agar consisting of 1% Glucose, 0.05% L-Asparagine, 0.05% di-Potassium hydrogen Phosphate and 2% Agar (pH 6.8) was

used for the isolation following standard dilution plate method. The strain was purified by streak plate method and stock culture was maintained by periodical sub-culturing on Sabouraud's agar slants and stored at 4°C. The fungal strains used for antifungal screening were maintained on Potato-Dextrose-Agar (PDA) plates and bacterial strains on nutrient agar with periodical sub-culturing.

Taxonomic characterization: The isolate TBG-75A20 and other strains of *Streptosporangium* (*S. nondiastaticum* JCM 3114^T, *S. roseum* JCM 3005^T, *S. longisporum* JCM 3106^T and *S. fragile* ATCC 31519^T) used for the comparative study were cultured on International *Streptomyces* Project (ISP) media (Shirling and Gottlieb, 1966), such as oatmeal agar (ISP3) and HV agar (Hayakawa and Nonomura, 1987). Cultural characteristics were studied using 14-day-old cultures grown at 28°C on various agar media. Colour determinations were made by comparing the culture with standard colour charts. Physiological characteristics of the strains were tested according to the *Streptosporangium* standard (Nonomura, 1989) on various ISP media, HV agar, nutrient agar and Sabouraud's agar. The microscopic slides were prepared following the cover slip culture method (Chakraborti, 1998) and observed by Nikon Optiphot-II phase contrast microscopy. Chemotaxonomic characteristics were determined as described previously. Biomass for chemotaxonomic analysis was obtained from a culture grown in shake ISP2 medium (Shirling and Gottlieb, 1966) and incubated at 28°C for 14 days. Diaminopimelic Acid (DAP) isomers and whole-cell sugar pattern were analyzed according to the methods of Becker *et al.* (1965).

16S rDNA sequence analysis: Total DNA preparation from strain TBG-75A20 was carried out using the procedure described by Marmur (1961). The 16S ribosomal DNA was amplified using the primers 8-27F, 5'-AGAGTTTGATCCTGGCTCAG-3' and 1495R 5'-CTACGGCTACCTTGTACGA-3'. Amplification was performed in automated thermocycler (MJ Research) using 30 ng of genomic DNA as template with 10 pmol of each primers, 1 U of Taq DNA polymerase and the recommended buffer system, in a 25 µL reaction volume. The temperature profile followed was 95°C (3 min) for initial denaturation followed by 35 cycles of denaturation at 95°C (1 min), annealing at 56°C (1 min) and extension at 72°C (1.5 min), followed by a final extension at 72°C (10 min). The PCR product was detected by agarose gel electrophoresis and was visualized by UV fluorescence after ethidium bromide staining. The 1.5 kb amplified 16S rDNA fragment was purified by 0.8% low-melting-point agarose gel electrophoresis and was sequenced in an automated sequencer (ABI Prism) with 8-27 F primer. A BLAST (NCBI) homology search was performed with the resultant sequence. The 16S rDNA sequences of the strains were aligned manually using the Clustal W program (Thompson *et al.*, 1997) against corresponding nucleotide sequences of representatives of the genus *Streptosporangium* retrieved from GenBank. Evolutionary distance matrices were generated as described by Jukes and Cantor (1969) and a phylogenetic tree was inferred by the neighbour-joining method (Saitou and Nei, 1987). Tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings of the neighbour-joining dataset and a phylogenetic tree was constructed using TREECON® software package (Van de Peer and De Wachter, 1993).

Antimicrobial screening: The strain TBG-75A20 was screened for antimicrobial activity by dual culture method and cylinder plate method. TBG-75A20 was grown on Sabouraud's agar plates at 27±1°C for five days and then agar cylinders of 5 mm in diameter with profuse growth were cut

out and placed on fresh Sabaraud's agar plate for antifungal assay. Agar cylinders with test fungal strains prepared on same conditions also placed on the plates at 2.5 cm apart. Control plates with fungal agar discs were also prepared and incubated at 27±1°C for seven days. The antifungal activity were scored in terms of percentage of inhibition using the formula $(A1-A2)/A1 \times 100$, where A1 is the Area in diameter of fungal colony in control plate and A2 is diameter of fungal colony in dual culture plates. For antibacterial screening, 5 mm agar cylinders with profuse growth of TBG-75A20 were placed on Luria Bertani (nutrient agar) plates seeded with test bacterial strains, *Bacillus cereus* MTCC 430, *B. subtilis* MTCC 441, *Escherichia coli* MTCC 443, *Klebsiella pneumoniae* MTCC 109, *Pseudomonas aeruginosa* MTCC 741, *Staphylococcus aureus* MTCC 740. The plates were further incubated at 37°C for 24 h and a zone of inhibition around the isolate was examined.

RESULTS

Colonies of TBG-75A20 strain grown on various media at 28°C for 14 days were observed to be 4-6 mm in diameter, with a wrinkled appearance. The strain showed good growth on most of the media used but failed to grow on nutrient agar. The mycelium is stable and not fragmented. The mass-aerial mycelium is pinkish brown in colour and substrate mycelium is red-orange to brown on most of the media used (Table 1). Substrate mycelia are well developed, branched and mostly un-fragmented. Some spindles shaped crystals were also observed along with substrate mycelia. Sporangia were observed on aerial mycelium on HV agar and oatmeal agar. Sporangia are spherical and of 11-17 µm size (Fig. 1). Sporangia are short and of 9-10 µm long and spores are spherical and non-motile. The colonies did not produce any soluble pigments. Table 1 shows the results of morphological characters and physiological tests of strain TBG-75A20 in comparison with other *Streptosporangium* spp.

The TBG-75A20 strain does not grow in Bennett's agar containing NaCl concentrations of up to 20% (w/v). Optimal growth temperature was 28°C and pH 7-8; growth occurred at 10 and 42°C but no growth occurred at 50°C. The strain shows negative starch hydrolysis reaction but shows positive reaction for reduction of nitrate and gelatin liquefaction. The strain utilized carbohydrates such as L-arabinose, cellobiose, D-fructose D-galactose, D-glucose, D-mannose, D-mannitol, D-raffinose, sucrose, D-xylose but not Meso-inositol and Rhamnose. This organism degraded cellulose effectively. The G-C content of the DNA of TBG-75A20 was 71.5 mol%. The chemotaxonomic study showed the presence of meso-diaminopimelic acid (meso-DAP) isomer in the cell-wall, whole-cell hydrolysates contained madurose, glucose, ribose and rhamnose. This strain had a type III cell wall.

Table 1: Cultural characteristics of TBG-75A20 on different media

Medium	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
ISP-2	Good	Pink	Orange red	Nil
ISP-3	Good	Pink	Orange red	Nil
ISP-4	Moderate	Pinkish brown	Brown	Nil
ISP-5	Good	Pink	Orange red	Nil
HV	Good	Pink	Orange red	Nil
Nutrient agar	No	-	-	-
Sabarauds agar	Good	Pink	Orange red	Nil

Table 2: Comparison of characteristics of TBG-75A20

Characteristics	TBG-75A20	<i>S. nondiastaticum</i>	<i>S. roseum</i>	<i>S. longisporum</i>	<i>S. fragile</i>
Sporangium size 11-15 µm	+	+	Rare	+	+
Short sporangiophores (10 µm)	+	+	+	+	+
Spherical spores	+	+	+	-	-
Spore mass colour					
Pink	+	+	+	+	+
Substrate mycelium					
Red, Orange	+	+	+	+	-
Yellowish brown	+	+	+	-	-
Brown-Black	-	-	-	-	+
Soluble pigments	-	-	+	-	+
Starch hydrolysis	-	-	+	+	+
Nitrite from nitrate	+	+	+	+	+
Gelatin liquefaction	+w	+	+	ND	-
Utilization of rhamnose	-	-	+	ND	+
Inositol	-	-	+	ND	ND
Growth at 42°C	+	+	-	-	+

+: Positive reaction, -: Negative reaction, +w: Weak, ND: Not determined

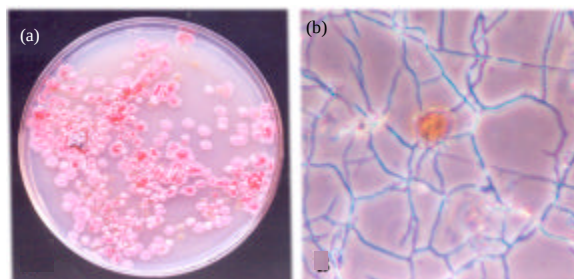


Fig. 1(a-b): Morphological characters of strain TBG-75A20. a: Colony morphology of strain grown on ISP-4 media for 14 days at 28°C and b: Phase contrast microphotograph showing hyphae and sporangia after 14 days growth at 28°C (Scale bar-20 µm)

Through 16S rDNA sequencing, a partial sequence of 580 nucleotides was obtained and the homology search showed more than 95% homology with other *Streptosporangium* 16s rDNA sequences. The sequence was aligned with corresponding sequences of the representative reference strains of Streptosporangiaceae. The results show that strain TBG-75A20, *S. nondiastaticum*, *S. pseudovulgare* and *S. viridalbum* clustered into a group. The similarity level ranged from 97 to 98% with *S. nondiastaticum* having the closest match. The phylogenetic tree obtained by applying the neighbor joining method is illustrated in Fig. 2. Based on this phylogenetic and morpho-physiological data the strain TBG-75A20 is identified as *S. nondiastaticum*. The strain is deposited to Microbial Type Culture Collection and Gene Bank, Chandigarh, India with an accession number MTCC 4149 and the partial 16s rDNA sequence to NCBI GenBank (EF608222).

The antimicrobial activity of the strain TBG-75A20 against various target microorganisms is shown in Table 3. The strain exhibited a good activity against fungal strains such as *A. niger*

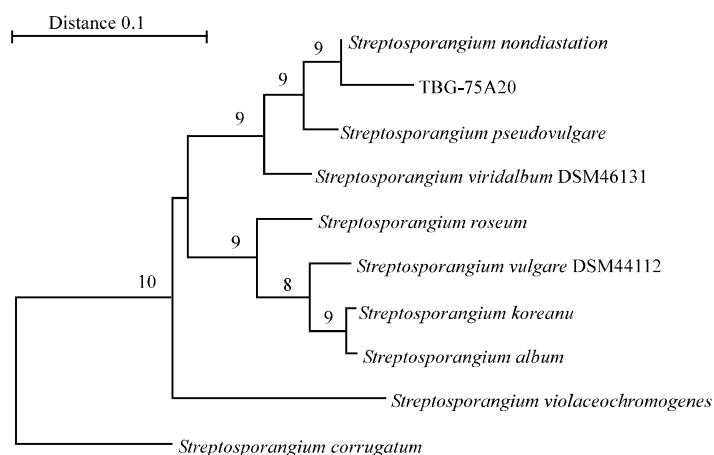


Fig. 2: Neighbour-joining tree, based on 16S rDNA sequences, showing the relationship between strain TBG-75A20 and members of the genus *Streptosporangium*. The numbers at the nodes indicate the levels of bootstrap support based on neighbour-joining analyses of 1000 resampled datasets; only values over 50% are given. Bar, 0.1 nt substitution per nt position

Table 3: Antimicrobial activity elaborated by TBG-75A20

Fungal strains	Percentage inhibition (%)	Bacterial Strains	Zone of Inhibition (mm diameter)
<i>Aspergillus niger</i> MTCC 1344	41.17	<i>Bacillus cereus</i> MTCC 430	10.5
<i>Aspergillus fumigatus</i> MTCC 1811	148.3	<i>Bacillus subtilis</i> MTCC 441	16
<i>Fusarium oxysporum</i> MTCC 1756	48.52	<i>Escherichia coli</i> MTCC 443	7.2
<i>F. solani</i> MTCC 284	41.17	<i>Klebsiella pneumoniae</i> MTCC 109	No activity
<i>Rhizoctonia solani</i> MTCC 2162	28.67	<i>Pseudomonas aeruginosa</i> MTCC 741	No activity
		<i>Staphylococcus aureus</i> MTCC 740	18

Antibacterial assay was done by cylinder plate method. Activity was scored as zone of inhibition around agar cylinder with profuse growth of TBG-75A20. Antifungal assay was done by dual plate culture technique and activity was scored in terms of percentage of inhibition using the formula $(A1-A2)/A1 \times 100$, where A1 is the area in diameter of fungal colony in control plate and A2 is diameter of fungal colony in dual culture plates

MTCC 1344, *A. fumigatus* MTCC 1811, *F. oxysporum* MTCC 1756, *F. solani* MTCC 284 and *R. solani* MTCC 2162. The antibacterial activity was moderate and exhibited a good activity against tested Gram-positive bacteria and no activity against Gram-negative bacteria, except for *E. coli* (weak activity).

DISCUSSION

On the basis of its morphological and chemical properties, the strain TBG-75A20 was classified in the genus *Streptosporangium*. The distinction of *Streptosporangium* species is mainly based on the color of aerial and substrate mycelia and of soluble pigment, the size of sporangia, the shape of spores and some physiological characters (Nonomura, 1989). Comparison of cultural and physiological characteristics of the strain TBG-75A20 with those of *Streptosporangium* known species indicated that *S. nondiastaticum* and *S. roseum* were the nearest species (Table 2). The three strains have the same aerial and substrate mycelia colors, sporangia size, spore shape. However, significant differences in physiological characteristics were recorded between strain TBG-75A20 and *S. roseum*. The differences consisted of utilization of Rhamnose, Inositol and growth at 42°C.

The 16S rDNA sequence of strain TBG-75A20 was compared with those of other *Streptosporangium* species. The similarity level ranged from 97 to 98% with *S. nondiastaticum*, the most closely related species. It is clear from phylogenetic analysis that strain TBG-75A20 cluster with *Streptosporangium nondiastaticum* on a distinct phyletic line, suggests the identity as the same.

On antimicrobial screening, the strain TBG-75A20 showed good activity against filamentous fungi like *Aspergillus niger*, *A. fumigatus*, *Fusarium oxysporum*, *F. solani*, *Rhizoctonia solani*. While it is showing moderate antibacterial activity against gram positive bacteria. The results suggests that the *Streptosporangium nondiastaticum* strain TBG-75A20 is having promising antifungal properties against phytopathogenic fungi. The literature survey showed the only bioactive metabolites reported are the angiotensin converting enzyme inhibitors, SF2513 A, B and C, from *S. nondiastaticum* strain SF2513 (Shokichi *et al.*, 1988). Moreover, the known bioactive compounds produced by *Streptosporangium* are other than antifungal agents only (Boudjella *et al.*, 2006; Inahashi *et al.*, 2011).

CONCLUSION

Data presented in this report clearly indicate that the *Streptosporangium nondiastaticum* strain TBG-75A20 exhibited significant antifungal activity against filamentous fungi. The literature survey shows that this is the first report on a strain of *S. nondiastaticum* elaborating antimicrobial compounds, eventhough some angiotensin converting enzyme inhibitors, SF2513 A, B and C, were reported from *S. nondiastaticum* strain SF2513.

ACKNOWLEDGMENT

S. Shiburaj acknowledge Kerala State Council for Science Technology and Environment (KSCSTE) for financial support and Director, TBGRI for providing facilities.

REFERENCES

- Becker, B., M.P. Lechevalier and H.A. Lechevalier, 1965. Chemical composition of cell-wall preparations from strains of various form-genera of aerobic actinomycetes. *Applied Microbiol.*, 13: 236-243.
- Boudjella, H., K. Bouti, A. Zitouni, F. Mathieu, A. Lebrihi and N. Sabaou, 2006. Taxonomy and chemical characterization of antibiotics of *Streptosporangium* Sg. 10 isolated from a Saharan soil. *Microbiol. Res.*, 161: 288-298.
- Chakraborti, T., 1998. Actinomycetes: Isolation, Screening, Identification and Gene Cloning: Laboratory Manual. IMTECH, Chandigarh, India.
- Couch, J.N., 1955. A new genus and family of the *Actinomycetales*, with a revision of the genus *Actinoplanes*. *J. Elisha Mitchell Sci. Soc.*, 71: 148-155.
- Felsenstein, J., 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, 39: 783-791.
- Hayakawa, M. and H. Nonomura, 1987. Humic acid-vitamin agar, a new medium for selective isolation of soil actinomycetes. *J. Ferment. Technol.*, 65: 501-509.
- Inahashi, Y., A. Matsumoto, S. Omura and Y. Takahashi, 2011. *Streptosporangium oxazolinicum* sp. nov., a novel endophytic actinomycete producing new antitrypanosomal antibiotics, spoxazomicins. *J. Antibiot. (Tokyo)*, 64: 297-302.
- Jukes, T.H. and C.R. Cantor, 1969. Evolution of Protein Molecules. In: *Mammalian Protein Metabolism*, Munro, H.N. (Ed.). Academic Press, New York, pp: 21-132.

- Kieser, T., M.J. Bibb, M.J. Buttner, K.F. Chater and D.A. Hopwood, 2000. Practical *Streptomyces* Genetics. John Innes Foundation, Norwich, England.
- Lazzarini, A., L. Cavaletti, G. Toppo and F. Marinelli, 2000. Rare genera of Actinomycetes as potential producers of new antibiotics. *Antonie van Leeuwenhoek*, 78: 399-405.
- Marmur, L.J., 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. Mol. Biol.*, 3: 208-218.
- Nayar, M.P., 1996. Hot Spots of Endemic Plants of India, Nepal and Bhutan. Tropical Botanic Garden and Research Institute, Kerala, India, ISBN: 9788190039710, Pages: 252.
- Nonomura, H., 1989. Genus *Streptosporangium*. In: Bergey's Manual of Systematic Bacteriology, Williams, S.T., M.E. Sharpe and J.G. Holt (Eds.). Vol. 4, Williams and Wilkins, Baltimore, pp: 2545-2551.
- Saitou, N. and M. Nei, 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, 4: 406-425.
- Shirling, E.B. and D. Gottlieb, 1966. Methods for characterization of *Streptomyces* species. *Int. J. Syst. Evol. Microbiol.*, 16: 313-340.
- Shokichi, O., K. Kuniyuko, S. Akiko, T. Tamako and Y. Junko *et al.*, 1988. New angiotensin converting enzyme inhibitors SF2513 A, B and C, produced by *Streptosporangium nondiastaticum*. *Meiji Seika Kenkyu Nenpo*, 27: 46-54.
- Tamura, T., S. Suzuki and K. Hatano, 2000. *Acrocarpospora* gen. nov., a new genus of the order *Actinomycetales*. *Int. J. Syst. Evol. Microbiol.*, 50: 1163-1171.
- Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougi and D.G. Higgins, 1997. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.*, 25: 4876-4882.
- Van de Peer, Y. and R. De Wachter, 1993. TREECON: A software package for the construction and drawing of evolutionary trees. *Comput. Applied Biosci.*, 9: 177-182.