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Plasmid DNA of Antibiotic Producing Strains of *Streptomyces sannanensis* Isolated from Different States in Southern India

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Abstract: Soil samples were collected from different states in Southern India to isolate and characterize actinomycetes at molecular levels through plasmid DNA and protein pattern. A total of 12 soil samples were collected from which four strains (AP-1, KN-2, KL-3 and TN-4) were isolated and characterized as *Streptomyces sannanensis*. The antimicrobial activity of these strains was studied against gram-negative and positive-bacteria. It was capable of producing antibiotic against gram-positive, while gram-negative bacteria were not affected. The potential of antibiotic production of these strains is likely to be chromosomally encoded by confirming the detection of plasmid DNA. The strains such as KN-2 and KL-3 showed two plasmids and the other two strains showed only one. This is a preliminary step to correlate the chemotaxonomic relationship among the strains of *Streptomyces* spp. for secondary metabolite production.

Key words: Actinomycetes, *Streptomyces sannanensis*, antimicrobial activity, plasmid DNA

INTRODUCTION

Actinomycetes population has been identified as one of the major groups of the soil population. They have peculiar characteristics that are transitional between bacteria and fungi and are sometimes called as fungi-like bacteria. They are phylogenetically and chemotaxonomically related with gram-positive bacteria with a high G+C content in their DNA (60-70%) reported by Locci (1994). They are capable of producing spores (Berdy, 1995), which facilitate their rapid dispersal in aquatic habitats (Actinoplane zoospores), air/soil (*Streptomycete* arthrospores) and may even ensure viability over many decades (Thermoactinomycetes). It has been reported that they are nutritionally versatile being able to grow both on rich substrates and on those containing a minimum or even an apparent lack of nutrients (Wellington *et al*., 1992).

Actinomycetes have characteristic biological aspects such as mycelial forms of growth that accumulates in sporulation and the ability to produce an array of secondary metabolites many of which have antibacterial or antifungal properties (Vasavada *et al*., 2006). In fact, most antibiotics developed for human pharmaceutical use are actinomycetes secondary metabolites, many of the being derived from *Streptomyces* species (Ponmurugan *et al*., 2007). Complex morphological development in the genera is phenotypically related to secondary metabolism (Ishibashi, 1992). The most promising role for secondary metabolites from actinomycetes relies upon deference mechanisms and inhibiting other competing cells would leave more nutrients for the survival of the secondary metabolites producing strain. Moreover, few marine halophilic and alkaliphilic actinomycetes have also been recently reported for their secondary metabolites production (Kokare *et al*., 2004; Vasavada *et al*., 2006).

Many *Streptomycetes* carry detectable extrachromosomal elements (plasmids) and in most cases, plasmids are present abundance in the form of Covalently Closed Circular (CCC)-DNA, but, occasionally, linear elements are also found (Saadoun and Blevins, 1997). The economic importance has led to tremendous interest in the genetic aspects of antibiotic biosynthesis by these organisms are encoded by either small or giant linear plasmids (Stutzman-Engwall *et al*., 1992). So far, number of different *Streptomycetes* has been investigated for plasmids and genes encode for proteins supposedly involved in the genetic control of the production of antibiotics (Bonjar *et al*., 2005). However, more detailed analyses have shown that antibiotic biosynthetic structural genes reside mostly on the chromosome. The findings in earlier studies indicated that the genetic diversity in terms of plasmid DNA and protein pattern was greater among strains of *Streptomyces* spp. in soil origin (Saadoun *et al*., 1998).

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These recent examples from the literature highlight the fact that despite extensive exploration of the actinomycetes for their antimicrobial products in the past, the search for novel molecules having unique therapeutic properties and phylogenetic relationship among strains continues to be an active area of research (Etebarian, 2006). To keep in mind, the present study was undertaken to isolate and characterize the biologically diverse strains of *Streptomycetes* from soil samples for the production of bioactive secondary metabolites. Studies were also conducted to characterize the strains at molecular level in terms of extracting plasmid DNA.

MATERIALS AND METHODS

Isolation of *Streptomyces* spp.: Soil samples were collected from vegetable fields at different states in Southern India such as Andhra Pradesh (Karim Nagar), Karnataka (Mysore), Kerala (Kottayam) and Tamil Nadu (Coimbatore) from a depth of 6-10 cm using an open-end soil borer. A total of 12 soil samples (three samples per state/area and pooled together) were obtained for isolation of actinomycetes by serial dilution plate technique using casein nitrate agar (g L^{-1}: 10 soluble starch, 0.3 casein, 2 potassium nitrate, 2 sodium chloride, 2 dipotassium hydrogen orthophosphate, 0.05 magnesium sulphate and 0.02 calcium carbonate). These soil samples were also subjected to analyze various parameters like pH, total organic carbon (Walkley and Black, 1934), nitrogen (AOAC, 1990) and available phosphorous (Jackson, 1973) and subsequently correlated with actinomycetes distribution. Single linear regression analysis was adopted and the data were analysed with SPSS statistical software, where actinomycetes population density was kept as dependent variable and the individual soil nutrient parameters were kept as independent variables.

Identification of *Streptomyces* spp.: There were four strains obtained from these soils and designated as AP-1, KN-2, KL-3 and TN-4 based on the name of the state. Identification of these strains was carried out based on morphological, physiological and biochemical tests to the genus level following the direction mentioned in the Manual of International cooperative project for description and deposition of *Streptomyces* cultures and the method of Bergey's manual of systemic Bacteriology (Holt, 1989). Biochemical characterization such as pigment production, starch hydrolysis, casein hydrolysis, catalase test, oxidase test, urease test, nitrate reduction, indole production, gelatin hydrolysis, citrate utilization and hydrogen sulphide production were carried out to identify the name of actinomycetes. Similarly, morphological characterization such as gram staining, motility, nature of colony and mycelium and spore morphology were studied. In addition, the effect of different pH and temperature regimes on the growth of these strains was studied.

Isolation of plasmid DNA from *Streptomyces* spp. (Kieser, 1984): All the four strains were grown on yeast-malt extract broth (g L^{-1}: 3 yeast extract, 5 bacto-peptone, 3 malt extract, 10 glucose, 30 sucrose, 5 glycine and 2 mL of 2.5 M $MgCl_2.6H_2O$ solution, the latter added after autoclaving) at 28°C under shaking at 200 rpm for 5 days. Actinomycetes-hyphae were harvested by filtration and then washed several times in sterile distilled water. It was suspended in 1 mL of TE buffer (40 mM Tris acetate, 2 mM EDTA, pH 7.9) and lysed by the addition of 2 mL of freshly prepared lysis buffer (3 g SDS, 0.6 g Tris, 6.4 mL 2 N NaOH in 100 mL distilled water). It was incubated for 1 h at 55°C and extracted with 6 mL of phenol-chloroform (1:1 V/V). After centrifugation, the supernatant was subjected to agarose gel electrophoresis using 0.7% agarose gels. Gels were viewed under an UV Transilluminator (Bangalore Genei, India) and then photographed using a Gel Documentation system (Alpha Digitoc, USA).

Screening of *Streptomyces* spp. for antimicrobial activity: Each actinomycete strain was lawn cultured on casein nitrate agar and incubated at 28°C for 5 days. From well grown cultures, 5 mm agar disks were prepared as described by Boyd (1995) using a sterile cork borer and transferred to fresh lawn cultures of gram-negative organisms such as *Escherichia coli*, *Shigella dysentery*, *Pseudomonas fluorescence*, *P. aeruginosa* and *Salmonella enteritidis* and positive organisms such as *Staphylococcus aureus*, *Bacillus amyloliquefaciens*, *B. cereus*, *B. megaterium* and *B. subtilis*. After incubation at 37°C for 24 h, the activity was recorded by measuring the diameter of inhibition zones for each test organism. The data obtained were subjected to analysis of variance (ANOVA) and the significant means were segregated by Critical Difference (CD) at 5% level of significance (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

Survey of actinomycetes diversity: In the present study, total number of actinomycetes population present in soil samples collected from different states in southern India indicated that it was found to be 12.5×10^{-3} g^{-1} soil dry wt. in samples collected from Andhra Pradesh followed by Karnataka (10.9) and lesser in Tamil Nadu (6.7) regions.

There was a positive correlation between soil nutrients and population density (Table 1). The relationship was significant at 5% probability, which is coincided with the report of Krishnakumari *et al.* (2006). Regression equation was developed from the study may be useful to find out the population density of actinomycetes of a particular locality. Actinomycetes, particularly *Streptomyces sannanensis*. by virtue of their wide distribution and antibiotic production, may participate activity in establishing the microbiological equilibrium in soil (Moreno *et al.*, 2003). It has been reported that most of the isolates tend to grow in acidic soils which is an important characteristic feature of *Streptomyces* spp. and with adequate source of carbon and nitrogen present in it that enhance the rate of multiplication (Etebarian, 2006). The survey on actinomycetes diversity in rhizosphere soil samples collected from different districts of Tamil Nadu was carried out by Krishnakumari *et al.* (2006) which revealed the population density was found to be more in Coimbatore district than the other districts such as Erode, Salem and Namakkal.

Characterization of actinomycetes: The results on morphological, physiological and biochemical activity revealed all the strains of actinomycetes belonged to *Streptomyces sannanensis*. They showed good sporulation with compact, chalk-like dry colonies of different colour variations from chalky white (AP-1) to chalky orange (KN-2). KL-3 and TN-4 strains showed grey white and pale orange colony, respectively (Table 2). All the strains were found to be gram-positive and showed branched mycelium in their morphology similar to fungal characters (Holt, 1989). Aerial mycelium was observed in KL-3 and TN-4 strains. Pigment production, hydrogen

Table 1: Population density of actinomycetes and soil nutrients

	Name of the states			
Parameters	Andhra Pradesh	Karnataka	Kerala	Tamil Nadu
Designation of strains	AP-1	KN-2	KL-3	TN-4
Population density (cfu $\times 10^{-3}$ g^{-1} soil dry wt.)	12.5	10.9	10.0	6.7
Soil pH	5.7	5.6	5.1	6.6
Total organic carbon (%)	0.0068	0.0057	0.0034	0.0036
Total nitrogen (%)	0.0003	0.0002	0.0002	0.00008
Available phosphorous (ppm)	16.23	14.88	14.07	12.22
Coefficient of variance	4.90	4.10	5.72	5.28
Regression equation	Y = 0.13x - 2.29 (R^2 = 0.886)**	Y = 0.10x - 2.15 (R^2 = 0.868)**	Y = 0.16x - 2.17 (R^2 = 0.747)*	Y = 0.17x - 4.05 (R^2 = 0.738)*

**Significant at 1% level, *Significant at 5% level

Table 2: Characterization for identification of *Streptomyces sannanensis* strains

	Strains of *Streptomyces* spp.			
Characterization	AP-1	KN-2	KL-3	TN-4
Gram's reaction	++	++	++	++
Motility	-	-	-	-
Nature of colony colour	Chalky white	Chalky orange	Gray white	Pale orange
Spore morphology	Spiral	Rods	Spiral	Rods
Aerial mycelium	-	-	+	+
Growth in pH 5.0	++	++	++	++
Growth in pH 6.0	++	++	++	++
Growth in pH 7.0	-	+	-	+
Growth in pH 8.0	-	-	-	-
Growth at temp. 25°C	++	++	++	++
Growth at temp. 30°C	++	++	++	++
Growth at temp. 35°C	-	+	-	+
Growth at temp. 40°C	-	-	-	-
Pigment production	++	++	++	++
Starch hydrolysis	++	++	++	++
Casein hydrolysis	++	++	++	++
Gelatin hydrolysis	++	++	++	++
Catalase	-	-	-	-
Oxidase	-	-	-	-
Urease	++	++	++	++
Nitrate reduction	++	++	++	++
Indole production	-	-	-	-
H_2S production	++	++	++	++
Voges-Proskaur test	-	-	-	-
Citrate utilization	++	++	++	++

++Positive reaction, -Negative reaction, +Weakly positive reaction

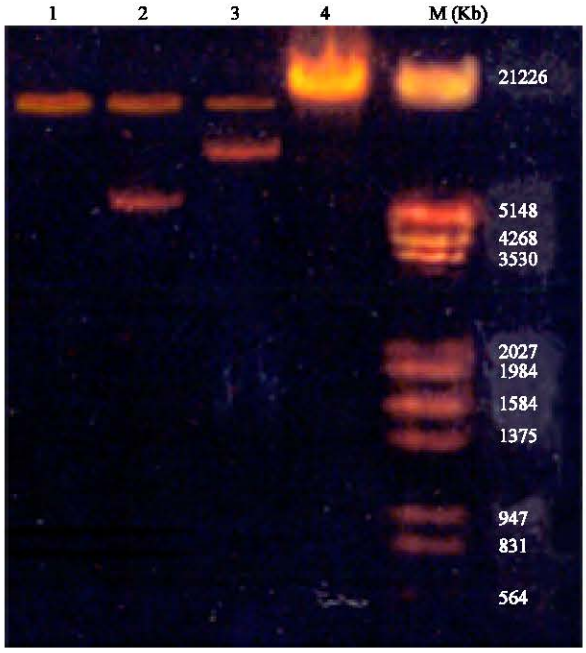

Fig. 1: Plasmid DNA profile of *Streptomyces sannanensis* strains [Lane 1 - 4: Different strains of *Streptomyces sannanensis* (1: AP-1, 2: KN-2, 3: KL-3, 4: TN-4); M- marker DNA]

sulphide, gelatin, casein and starch hydrolysis, urease, nitrate reduction and citrate utilization were given positive result but catalase, oxidase, Voges-Proskaur test and indole production were negative (Table 2). Similar results were reported recently by several investigators (Krishnakumari *et al*., 2006; Vasavada *et al*., 2006). The effect of pH and temperature on the growth of actinomycetes strains were studied that revealed the optimum pH and temperature were found to be 5-6 and 25-30°C, respectively (Table 2). This study may be further useful for the production antibiotics very effectively.

Molecular characterization of *Streptomyces sannanensis*: Lower molecular weight CCC-DNA were detected from all the strains, but there was no similarity between the strains in their profile (Fig. 1). The strains such as KN-2 and KL-3 exhibited two plasmids and the other strains had only one. The molecular weight of plasmids of all the strains was ranged between 21226 and 5148 kb. All the four strains produced one unique band at 21226 kb (Fig. 1). Saadoun *et al.* (1998) observed only CCC-DNA not linear DNA in their samples containing the genus of *Streptomyces*. They were further suggesting that

Table 3: Antimicrobial activity of *Streptomyces sannanensis* strains against various test organisms.

	Zone of inhibition (mm)*			
	Strains of *Streptomyces* spp.			
Culture used	AP-1	KN-2	KL-3	TN-4
Gram-negative bacteria	-	-	-	-
Escherichia coli	-	-	-	-
Shigella dysentery	-	-	-	-
Pseudomonas fluorescence	-	-	-	-
P. aeruginosa	-	-	-	-
Salmonella enteritidis	-	-	-	-
Gram-positive bacteria				
Staphylococcus aureus	30.0	40.7	40.5	31.5
Bacillus amyloliguefaciens	30.3	53.7	52.5	38.0
B. cereus	40.5	52.3	35.5	32.5
B. megaterium	32.0	52.3	53.5	30.7
B. subtilis	40.7	50.3	50.7	40.7
SE±	2.27	2.87	1.94	1.32
CD at p = 0.05	1.87	1.07	2.32	2.47

*Average of three replicates

antibiotic production in these strains is likely to be chromosomally encoded. Four different extraction methods of small plasmid DNA from antibiotic-producing *Streptomyces* isolates and from the positive control *S. lividans*, containing the pIJ702 plasmid, were standardized. Among these, only one procedure allowed the detection of plasmid DNA from the positive control very effectively that was the Kieser (1984) method. This method is widely used now for the extraction of plasmid DNA from actinomycetes (Saadoun *et al*., 1998).

From these results, we could discriminate all the four strains at molecular level. However, we have to generate genetic markers for these strains through amplification of genomic DNA using oligonucleotide primers (RAPD analysis), as this analysis is generally applicable and powerful for screening for bioactive principles.

Antimicrobial activity of *Streptomyces sannanensis*: With the increasing use of antibiotics, the serious problem of antibiotic resistance is gradually increasing. Therefore, intensive search for new antibiotics is going on worldwide. Production of antibiotic as secondary metabolite is controlled by genetic make up that imparts fullest expression and is profoundly influenced by biotic and abiotic factors. This is substantiated by our results presented here. All the strains of *Streptomyces sannanensis* were able to produce antibiotic against gram-positive bacteria but not against gram-negative one. *Staphylococcus aureus*, *Bacillus amyloliguefaciens*, *B. cereus*, *B. megaterium* and *B. subtilis* showed positive response while *Escherichia coli*, *Shigella dysentery*, *Pseudomonas fluorescence*, *P. aeruginosa* and

Salmonella enteritidis showed negative impact (Table 3). Similar results were observed by Vasavada *et al.* (2006) and Krishnakumari *et al.* (2006). The formation of inhibition zone around the pathogenic strains is due to the production of secondary metabolites by *Streptomyces* spp. Recently actinomycetes isolated from the Sundarbans region of the Bay of Bengal, India, which exhibited potent antimicrobial activity against gram-positive and gram-negative bacteria, moulds, yeast and several multiple-drug resistant bacteria (Saha, 2005).

It may be concluded that out of four strains of *Streptomyces sannanensis*. used, two of them such as KN-2 and KL-3 were found to be of potential antagonists against test organisms that has the potential to control variety of pathogenic organisms *in situ*.

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