Antibiotic Production, Activity Spectra and Plasmid Analysis of *Streptomyces* species Isolated from Soil.

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ABSTRACT

Antibiotic producing *Streptomyces* strains were isolated by spread-plating aliquots of ten-fold serially diluted pre-treated soil samples on a chemically defined *Streptomyces* medium, ISPI, using the crowded-plate technique. Cultures on the crowded plate were bioautographed against *Staphylococcus aureus* (ATCC 25932).Nine of the bioactive strains (UY1 – UY9), which showed appreciable antagonism and clear inhibition zones were selected and grown as pure cultures in ISP1 medium. Antibiotic production and activity of the bioactive *Streptomyces* strains were carried out in submerged fermentation in the production medium, ISP2. Cells were harvested by centrifugation, processed into antibiotic-filtrates and sensitivity test on the test organisms by the modified agar-well diffusion technique, indicated high potency. Broth cultures of strains in ISP1 were subjected to plasmid analysis. Rapid screening for plasmid revealed bands of plasmid DNA. Plasmid DNA extracted by a miniprep method and plasmid profiling conducted on 1.0 % agarose gel electrophoresis, revealed probable identical single linear copies of high molecular weight plasmid DNA bands for the strains, aligning somewhat above the 10,000 base-pairs (bp), high molecular weight DNA biomarker. The role of plasmid in antibiotic production and activity was studied by plasmid curing with sub-inhibitory concentrations of acridine-orange and tested for activity and profile on agarose gel electrophoresis, which indicated the loss of antibiotic production and activity, disappearance and non-alignment of any plasmid bands on the gel electrophoresis. Transformation of the competent cured strains demonstrated antibiotics production and activity as well as the re-appearance of plasmid bands on agarose -gel electrophoresis like the native strains. The comparison between the native, cured and transformed *Streptomyces*isolates, showed that there was significant ($p< 0.05$) difference in antibiotic production and activity amongst these three forms of the *Streptomyces* strains. These possibly suggest and confirmed that the antibiotic production and activity of the isolated *Streptomyces* strains are plasmid –mediated.

***Keywords*:** Antibiotic Production, Activity-spectra, Plasmid Analysis, *Streptomyces* species.

INTRODUCTION

Before now, many researchers had searched for new antibiotics from a wide variety of microorganisms and many natural habitats. Such screening programmes usually focus on bacteria and fungi. Among the bacteria, members of the group actinomycetes, particularly those in the genus *Streptomyces,* as well as bacteria in the genera *Bacillus* and *Pseudomonas,* gained the most attention, since most of the earlier known bacterial antibiotics were discovered from these groups of microorganisms (Crueger, 1984; Singleton, 1997). The actionmycetes comprise a large group of Gram-positive filamentous bacteria primarily isolated from soil. Most members are spore-formers, exhibiting wide physiological and morphological diversities, of which the genus *Streptomyces* is primarily important in the elaboration of secondary metabolites, particularly antibiotics (Crandall and Hamil, 1986; Goodfellow *et al.,* 1988; Ekong, *et al.,* 2013).

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The filamentous soil bacteria belonging to the genus *Streptomyces* are widely recognized as industrially important microorganisms, because of their ability to produce many kinds of novel secondary metabolites including antibiotics (Crueger, 1984; Singleton, 1997; Korn-Wendisch and Kutzner, 1992). Of all known drugs, 70 % have been isolated from the Actinomycetes bacteria, out of which 75 % and 60 % are used in medicine and agriculture respectively (Crandall and Hamil, 1986; Goodfellow *et al.,* 1988). The genus *Streptomyces* was proposed by Waksman and Henrici, for aerobic and spore-forming Actinomycetes (Williams *et al.,* 1983a; Williams *et .al.,* 1989).

The taxon currently accommodates Gram-positive, spore-forming bacteria having a deoxyribonucleic acid (DNA) with a high guanosine (G)-plus cystocine (C) (G+C) content of 69-73 mol %, with an extensive branching substrates and aerial mycelia. The genus *Streptomyces* is the largest and most important antibiotics producer in the microbial world so far discovered. Bacterial species

in this genus account for the exponential increase in the number of antibiotics per year, totaling over forty-thousand, isolated and characterized in the past decades. Also, recent reports showed that this group of microorganisms still remains an important source of commercially and medically useful antibiotics. These antibiotics span the diverse classes including the aminoglycosides, β-lactams, macrolides, glycopeptides, peptides, nucleosides, polyenes, tetracyclines, chloramphenicol, etc. (Crandall and Hamil, 1986; Watve *et al.*, 2001; Sahin and Ugur, 2003; Ceylan *et al.*,2008; Tomita *et al.*,1987).

The biosynthesis of antibiotics by microorganisms has been reported to occur as a result of secondary metabolism (Bullock, 1965; Bulock, 1974; Demain, 1998; Bibb, 2005). Hence, antibiotics are generally regarded as secondary metabolites (Gottlieb and Shaw, 1967; Martin and Demain, 1980; Bibb, 2005). The biosynthesis of antibiotics usually occurs during the late growth phase of the producing microorganisms. This implies that the temporal nature of their formation is mainly genetical, but expression can be greatly influenced by nutritional and environmental manipulations; often triggered by exhaustion of nutrients, addition of inducers and/or by a decrease in the growth rate (Sermonti, 1969; Martin, 1978; Demain, 1998; Bibb, 1996; Bibb, 2005; Martin and Demain, 1980). These events generate signals that cause a cascade of regulatory events resulting in the biochemical (secondary metabolism), and morphological differentiation (morphogenesis) of the microbial antibiotics producers (Martin and Demain,1980; Bibb, 1996; Bibb, 2005; Ohnishi *et al.,* 2005). These signals are often low molecular weight inducers, notably butyrolactone that act by binding to and then inactivating a regulatory protein (repressor-protein/receptor-protein). The regulatory protein normally prevents secondary metabolism and morphogenesis during rapid growth phase and nutrient sufficiency (Sermonti, 1969; Gottlieb and Shaw, 1967; Martin, 1978; Demain, 1998; Bibb, 1996; Bibb, 2005).

To this end, antibiotics are formed by enzymatic pathways that occur through individual protein: free or complexes; or through parts of large multifunctional polypeptides carrying-out a multitude of enzymatic steps such as polypeptide synthases and peptide synthases (Demain, 1998; Martin and Demain, 1980). The genes encoding these antibiotic synthesizing enzymes like other secondary metabolites are frequently linked in clusters, and are usually chromosomal borne (Bibb, 1996; Martin and Demain, 1980; Bibb, 2005). For instance, the 30 biosynthetic *Str/sts* and b*lu* genes of streptomycin biosynthesis (and of related antibiotics such as 5-hydroxystreptomycin and bluensomycin) that were cloned from S*treptomyces griseus* and *Streptomyces glaucescens*, were all reported to be found in a chromosomal region of 30- 40 kb (Martin, 1978; Bibb, 1996; Bibb, 2005; Ohnishi, 2005). Conversely, only a few antibiotic biosynthesis genes have been shown to be plasmid borne, particularly those encoding methylenomycin-a synthesis in *Streptomyces coelicolor* and an antibiotic AS-48 in *Enterococcus faecalis* S-48 (Martinez *et al.,* 1990; Bibb, 1996; Bibb, 2005; Ohnishi, 2005). However, whether chromosomal or plasmid borne, antibiotics biosynthesis genes are usually clustered, especially in prokaryotes, but not necessarily as single operons (Martin and Demain, 1980).

Presently, there is little or no documented information on the isolation from Nigerian soil of *Streptomyces* species with antibiotic production potential. Also, there is a paucity of information on the role of either plasmids or chromosomes on antibiotic production from *Streptomyces* species. Thus, this research work is a continuing effort at exploiting the potentials of *Streptomyces* as a local source of antibiotics of primary economic value; and to ascertain if the antibiotic biosynthesis is chromosomally or plasmid encoded.

MATERIALS AND METHODS

Isolation of Antibiotic Producing *Streptomyces* species

Antibiotics producing *Streptomyces* cultures were isolated from $CaCO_3$ (10:1 w/w) pretreated soil samples by the crowded-plate technique (Sahin and Ugur, 2003; Solomon and Isreal, 1986) with modifications (Ekong *et.al*.,2013). Aliquots of ten-fold serially diluted pretreated soil samples, previously placed in a water-bath at 45 ^oC for 16 h, to separate the spores from vegetative cells were spread on agar plates of a chemically defined actinomycetes medium ISP1.The assay plates were incubated at 28 ^oC for 4 days, for growth and enumeration of potential bioactive cultures. The bioactive *Streptomyces* isolates were selected and confirmed by an overnight bioautography of the crowded-plate against *Staphylococcus aureus* seeded –plate (Meyers *et al.,* 1968; Ekong *et al.,* 2013). Thereafter, the potential antibiotic producing *Streptomyces* colonies with clear zones of inhibition, were isolated and subcultured on fresh plates of ISP 1, incubated at 28 ^oC for 4 days. The isolates were purified by repeated sub-culturing on ISP1 and maintained as culture slants at 4 ^oC.

Test Organisms

Test organisms used in the assay were stock cultures of bacteria and yeast obtained from the

Pharmaceutical Microbiologyand Biotechnology Unit Laboratory, Faculty of Pharmacy, University of Uyo, Nigeria. They were *Staphylococcus aureus* (ATCC 25923); *Escherichia coli* (ATCC 25922), *Candida albicans* and *Aspergillus niger*. Inocula of the bacterial and yeast cultures grown on nutrient and Sabouraud dextrose broths were standardized following the methods of Baron and Finegold (1990); Tilton and Howard, (1987) as reported with slight modifications (Ekong *et al.,* 2004). The turbidity of the broth cultures was aseptically adjusted by ten-fold serial dilutions, with an approximated cell density of 1x 10^8 cfu/ml. Cultures of the Gram positive bacterial and yeast were diluted to 1:1000; while that of the Gram-negative bacteria was diluted to 1:10,000 (Ekong *et al.,* 2004).

Fermentation and Antibiotic Production in Submerged Cultures

Antibiotic production by submerged cultures of the isolated bioactive *Streptomyces* species was assayed in the sterile secondary fermentation medium of the following composition: NaCl 1.0 g/l; NH_4Cl 1.0 g/l; KCl 1.0 g/l; K_2HPO_4 1.0 g/l; $MgSO_4$. $7H_2O$; 2.0 g/l; CaCl. $2H_2O$ 0.04 g/l; glucose 2.0 g/l; yeast-extract 3.0 g/l; distilled water I litre, following the method of Sahin and Ugur (2003), with modifications (Ekong, 2013). Fermentation was conducted in submerged culture in 250 ml Erlenmeyer flasks, each containing 50 ml of the sterile secondary fermentation medium. The flasks were inoculated with 1.0 ml suspension of the bioactive *Streptomyces* cultures and incubated at 28 ^oC for 5 days in an orbital-shaker incubator (Gallenkamp, England) operating at 105 rpm. After fermentation, harvesting and processing of cells for antibiotic production were carried out as previously described (Ekong *et. al*., 2013). On the fifth day, cells were harvested by centrifugation at 4000 x g for 30 min, using an RC5-B centrifuge (Ivan Sorval, Inc., Norwalk, USA). The supernatant fractions obtained, were aseptically filtered using an oven-sterilised filtration apparatus (Ekong *et. al.,* 2004), comprising 100 ml Erlenmeyer flask and a funnel containing an acrodisc filter –paper of 0.48 µm averages pore diameter (Gelman sciences, Ann Arbor, Michigan, USA). The filtrates obtained were referred to as the crude-antibiotic solutions and were stored at 4 ^oC for subsequent assay. Antibiotic activity spectra against the test organisms were carried -out by the agar-well diffusion technique (Ekong *et. al*., 2013). Activity index was calculated as the percentage change in antibiotic activity of the crude antibiotic substances compared with the standard antibiotics (Ekong *et al*. 2015a).

Rapid Screening for Plasmids

The *Streptomyces* isolates were rapidly screened for plasmids following the methods of Anderson and McKay (1983) and Rezaee *et. al.* (2005), with some modifications using the Pure Yield™ Plasmid Minipreps System technique, (Promegar, USA) (Ekong, 2013). A small number of cells of the *Streptomyces* isolates were inoculated into a microcentrifuge tube containing 300 µl of primary isolation medium, ISP 1 broth and incubated at 28 ^oC for 3-5 days. After growth, the cells were pelleted in a microfuge operating at 10,000 rpm for 2 minutes. The cells were then suspended by vortexing in 2 µl of gel-loading mix (0.25 % bromophenol-blue and 30 % glycerol). Then 40 µl each of chloroform and phenol (saturated with 1.0 M Tris- HCl, pH 8.0) was added. The mixture was vortexed at full speed for 1 minute, followed by centrifugation at 12,000 rpm for 10 minutes. Thereafter, 10 µl of the aqueous fraction was subjected to electrophoresis on 1.0 % agarose mini-gel (5.2 x 6.0 cm), with Tris-acetic acid-EDTA (TAE) buffer (40 µM Tris-acetate, pH 8.0, containing 2 mM (Na – EDTA) at 100 V for 30 minutes. The gel was stained with ethidium-bromide (0.5 µg/ml), and DNA bands were visualized under a UV-transilluminator.

Plasmid Extraction

Plasmid DNAs of the *Streptomyces* cultures were extracted using the Pure Yield™ Plasmid Miniprep System (Promegar, USA), following the protocol provided by the manufacturer (Ekong, 2013). The isolates were cultured overnight in tryptone Soy broth (TSB). The cells were harvested in a 1.5 ml microcentrifuge tube by centrifuging for 5 minutes at 14,000 rpm. The supernatants were discarded and the cells resuspended in 600 µl of Tris-EDTA (TE)–buffer (pH 6.0). Thereafter, 100 µl cell lysis buffer (Blue) was added, and mixed by inverting the tubes six (6) times. After, 350 µl of cold (4-8 ^oC) neutralization solution (NS) was added and mixed thoroughly by inverting the tube. These were centrifuged at 14,000 rpm in a microcentrifuge for 3 minutes. The supernatants (900 µl) were transferred to a PureYield™ minicolumn without disturbing the cells debris pellet. The mini columns were placed into a collection tube, and centrifuged at 14, 000 rpm in a microcentrifuge for 15 seconds. The flow- through was discarded, and the minicolumn placed into the same collection tube. To these mini columns were added 200 µl of endotoxin removal wash (ERW), and centrifuged at 14,000 rpm in a microcentrifuge for 15 seconds. Again, 400 µl of column wash solution (CWC), was added to the mini column, centrifuged at 14,000 rpm in a microcentrifuge for 30 seconds. Thereafter, the minicolumns were

transferred to a clean 1.5 ml microcentrifuge tube, and 30 µl of elution buffer (EB) was added directly to the minicolumn matrix. This was left to stand at room temperature for one minute, after which it was centrifuged at 14,000 rpm for 15 seconds to elute the plasmid DNA. Subsequently, the microcentrifuge tubes were capped, and the eluted plasmid DNA stored at – 20 °C.

Plasmid profile

The profile of the isolated plasmid DNA was carried out by agarose-gel electrophoresis on 1 % agarose –with TAE buffer (40mM the of Tris-acetate, pH8.0, containing of 2 mM Na_2 - EDTA) at 100V for 30 minute as previously described (Rezaee *et al.,* 2005). The gel was prepared by boiling agarose powder 1.0 g in 100 ml of TAE buffer. On cooling, 0.5 µg/ml of ethidium-bromide was added to stain the molten gel. The stained agarose-gel was poured into a casting tray and a comb was inserted over its rim to form wells on removal after solidification. Aliquots of the isolated plasma DNA were mixed with bromophenol blue and then loaded into the wells. A 10,000 base-pairs (bp) DNA molecular weight biomarker was also loaded into one of the wells as standards. The gel was then electrophoresed in a horizontal tank at a constant voltage of 100 V for 30 minutes. The plasmid DNA bands were visualized by fluorescence of bound ethidium-bromide under a UV-transilluminator, the alignment of the DNA bands were matched with that of the standard biomarker, and photograph taken with a digital camera.

Plasmid Curing

Plasmid curing of the *Streptomyces* isolates was performed, following the methods described by Razaee *et. al.* (2009); Edward-Raja and Selvan (2009), with slight modifications (Ekong, 2013). The curing of the antibiotic-producing plasmids was done using sub-inhibitory concentrations of acridine orange (5-600 µg/ml). To 20 ml medium M broth in 250 ml Erlenmeyer flasks containing different concentrations of the curing agent, were added 2.0 ml broth cultures of the antibiotic-producing *Streptomyces* isolates. The flask containing the cells without acridine-orange served as positive control, while those with the curing agent but without the cells served as negative control. All flasks were incubated at 28 °C for 3 – 5 days for visible growth. Flasks with the highest concentrations of acridine orange (in which growth was manifested), were selected. These were then serially diluted with sterile distilled water, aliquots spread-plated on the primary isolation medium ISP1, and incubated at 28 °C for 5 days. Colonies were counted, and replica-plated on primary isolation medium ISP1. Thereafter, the colonies were inoculated into 50 ml secondary fermentation medium, ISP2, in 250 ml Erlenmeyer flasks, incubated of 28 °C for 5 days in orbital shaker, operating at 120 rpm. After fermentation, the cultures were harvested by centrifugation, filtered and assayed for antibiotic production and activity potential on seeded NA plates of *Staphylococcus aureus* as previously described. The absence or presence of less inhibition zones on the seeded NA by activity-index analysis, indicated plasmid mediated and enhanced antibiotic substances elaboration; hence, plasmid cured. While the presence of inhibition zone on the seeded NA indicated chromosomal-mediated antibiotic-substances production; hence, plasmid not cured (Akinjoguna and Enabulele, 2010).

Transformation of Cured Isolates

Transformation of the non-antibiotic producing plasmid-cured *Streptomyces* isolates was carried-out, following the standard transformation procedure of Sambrook and Russel (2001), with modifications (Ekong, 2013). Competent cells of the plasmid-cured non-antibiotic producing *Streptomyces* isolates, were prepared using calcium chloride, and were transformed with 10,000 bp plasmid obtained from the native isolates as transforming agent, in ISP1 agar plates. The transformed *Streptomyces* strains were subsequently sub-cultured in ISP1 broth for easy plasmid isolation and profiling on 1.0 % agarose-gel electrophoresis as previously described above. The transformed strains were also subjected to growth in ISP 2 broth for evaluation of antibiotic production and activity assay against the indictor test organisms by the modified agar-well diffusion assay as previously described above. Residual antibiotic production and activity by the transformed strains were evaluated by activity-index as previously described elsewhere in this work.

RESULTS

Plasmid Profile, Antibiotic Production and Activity Spectra of Isolated *Streptomyces* Species

The result of the gel-electrophoresis of plasmid DNA of the antibiotic producing *Streptomyces* cultures is given in Figure 1. The result showed that the plasmid DNA from all the isolates aligned somewhat above the 10,000 bp DNA molecular weight biomarker ladder (positive control) in lane 9. This indicates that the isolated plasmids DNA were seemingly identical and have high molecular weights. The isolated plasmids of the *Streptomyces* species were of larger sizes of the range of more than 10,000 bp DNA. This was confirmed by comparing with DNA marker of known size, and by the slow movement of plasmid DNA on the

agarose gel. The results of antibiotic production and activity spectra indicated significant ($p<0.05$) production and activity comparable with the standard antibiotics (Table 1). The result showed that all the *Streptomyces* antibiotics substances were antibacterial, while antibiotic substances from UY4, UY5, UY8 and UY9 elicited both antibacterial and antifungal activities.

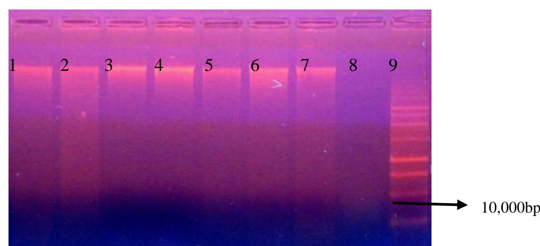

Figure 1: Gel electrophoresis of plasmid DNA of isolates. Lane 1, AA_1; Lane 2, AA_4; Lane 3, AA_5; Lane 4, AA_6, Lane 5, AA_7; Lane 6, AA_8; Lane 7, AA_9 ;Lane 8, negative control; Lane 9, 10,000 bp DNA ladder

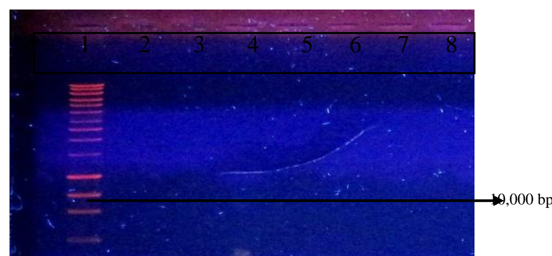

Figure 2: Gel electrophoresis of cured plasmid of isolates. Lane 1, 10,000 bp DNA ladder; Lane 2, AA_1; Lane 3, AA_4; Lane 4, AA_5, Lane 5, AA_6; Lane 6, AA_7; Lane 7, AA_8; Lane 8, AA_9

Plasmid Curing of Isolated *Streptomyces* Species

The result of the agarose gel electrophoresis of the cured plasmids of the antibiotic producing *Streptomyces* isolates is presented in Figure 2. The result shows that none of the cured *Streptomyces* isolates showed alignment to any base-pair of the standard 10,000 bp DNA biomarker in lane 1. This indicates the absence or removal of the plasmids which hitherto were responsible for the high-molecular weight DNA bands. Consequently, the result of antibiotics sensitivity assay by the plasmid-cured isolates, indicates the none production and absence of activity on the assay test organisms measured by inhibition zone diameter (Table 2). This denotes the lack of potency by the antibiotic substances, produced by the cured isolates, confirming plasmid removal, implying that antibiotic production potential and activity was plasmid-borne.

Transformation of Cured Isolates

The result of the transformed plasmid cured, *Streptomyces* isolates indicated significant levels ($P<0.05$) of antibiotics production and activity, comparable by the activity index to the parental strains (Table 3). The result evidently showed that antibiotic-production and activity by the isolated *Streptomyces* species could be plasmid-borne.

DISCUSSION

The isolated *Streptomyces* species showed clear evidence of antibiotic production and activity tested against the test organisms with high potencies significantly ($p< 0.05$) compared with that of the standard antibiotics (Table1): This result further lend credence to the widely reported fact that *Streptomyces* species have provided approximately two-third (about 70%) of the many important natural bioactive compounds of high medical and commercial values; and as well will continue to be routinely screened for new bioactive substances as widely reported by several authors and previously reviewed (Ekong, *et al.,* 2013).

Bacterial plasmids are exrtrachromosomal molecules of deoxyribonucleic acid (DNA), that are capable of autonomous replication, implying that they encode products necessary for their own replication and do not rely on chromosomally encoded functions (Woodfall *et.al.,* 1994). Accordingly, plasmids which vary in size from less than one megadalton (MDa) to several hundred megadaltons, usually exist as covalently closed circular (CCC) molecules, even though some linear plasmids have been reported. Although, all plasmids, encode functions for their own replication, some confer no other detectable phenotypic functions on their host (cryptic-plasmids); while most plasmids (phenotypic) confer additional phenotypic functions or roles, related to antibiotic resistance, virulence, use of metabolic substrates, antibiotics and other secondary metabolites biosynthesis. As mentioned above, plasmid DNA is usually present in most bacterial cells in the forms of CCC molecules, where each of the two DNA strands are circular and are twisted around each other to achieve the double helical structure, which renders CCCDNA highly stable. However, the nicking of both strands of a plasmid by such factors as exposure to UV light, (especially if ethidium bromide is intercalated into the molecules) and freeze thawing; the circular form is lost completely and the plasmid opens into its linear (L) form (Woodfall *et al.,* 1994). These

three structural forms of plasmids DNA may cause problems when plasmids are analyzed by agarose gel electrophoresis, as they each migrate at different rates. Thus, a single plasmid can therefore, appear as three separate bands on a gel (Woodfull *et al.,* 1994). However, the above assertion is countered in this study, as the plasmid DNA from all the *Streptomyces* isolates following extraction and profiling on agarose-gel electrophoresis showed only one or single broad-band alignment somewhat above the 10,000 bp, but not led to the separate bands on a well in the gel (Figure 1). This depicts that the *Streptomyces* strains plasmids were of the linear forms and single high molecular weight DNA molecules, given their slow elution on the agarose-gel.

Table 1: Antibiotic production and activity of native *Streptomyces* strains

Test organisms	*Streptomyces* isolates/inhibitory activity, IZD (mm)[a]										
	UY1	UY2	UY3	UY4	UY5	UY6	UY7	UY8	UY9	CHL	KTZ
Staphylococcus aureus	20.0 (60.0)	20.0 (60.6)	21.0 (63.6)	25.0 (75.8)	23.0 (69.7)	24.0 (72.7)	24.0 (72.7)	24.0 (72.7)	25.0 75.8)	33.0	NT
Escherichia coli	20.0 (50.0)	26.0 (65.0)	20.0 (50.0)	22.0 (55.0)	24.0 (60.0)	38.0 (95.0)	30.0 (75.0)	26.0 (65.0)	32.0 (80.0)	40.0	NT
Candida albicans	-	-	-	24.0 (66.7)	26.0 (72.2)	-	-	28.0 (77.8)	30.0 (83.3)	NT	36.0
Aspergillus niger	-	-	-	20.0 (71.4)	22.0 (78.6)	-	-	24.0 (85.7)	26.0 (72.6)	NT	28.0

– = No activity; NT = Not tested; CHL = Chloramphenicol; KTZ = ketoconazole.
a = mean of duplicate assay; figures in parenthesis indicate activity index.

Table 2: Antibiotic production and activity of plasmid-cured native *Streptomyces* isolates

Test organisms	Cured *Streptomyces* Isolates/Inhibitory Activity IZD (mm)[a]										
	UY1	UY2	UY3	UY4	UY5	UY6	UY7	UY8	UY9	CHL	KTZ
Staphylococcus aureus	-	-	-	-	-	-	-	-	-	33.0	NT
Escherichia coli	-	-	-	-	-	-	-	-	-	40.0	NT
Candida albicans	-	-	-	-	-	-	-	-	-	NT	36.0
Aspergillus niger	-	-	-	-	-	-	-	-	-	NT	28.0

– = No activity; NT = Not tested

Table 3: Antibiotic production and activity of transformed *Streptomyces* isolates

Test organisms	**Cured *Streptomyces* Isolates/Inhibitory Activity IZD (mm)[b]**										
	UY1	**UY2**	**UY3**	**UY4**	**UY5**	**UY6**	**UY7**	**UY8**	**UY9**	**CHL**	**KTZ**
Staphylococcus aureus	**5.0 (15.2)**	**5.0 (15.2)**	**6.0 (18.2)**	**10.0 (30.3)**	**7.0 (21.2)**	**7.0 (21.2)**	**9.0 (27.3)**	**9.0 (27.3)**	**9.0 (27.3)**	**33.0**	**NT**
Escherichia coli	**5.0 (12.5)**	**6.0 (15.0)**	**5.0 (12.5)**	**7.0 (17.5)**	**6.0 (15.0)**	**16.0 (40.0)**	**11.0 (27.5)**	**12.0 (30.0)**	**10.0 (25.0)**	**40.0**	**NT**
Candida albicans	**-**	**-**	**-**	**80 (22.0)**	**11.0 (30.6)**	**-**	**-**	**12.0 (33.3)**	**14.0 (38.9)**	**NT**	**36.0**
Aspergillus niger	**-**	**-**	**-**	**5.0 (17.9)**	**7.0 (25.0)**	**-**	**-**	**10.0 (35.7)**	**13.0 (46.4)**	**NT**	**28.0**

– = *No activity; NT = Not tested*; b = % change in activity indicated by intergers in parenthesis.

Nevertheless, even though the *Streptomyces* strains possessed profiles appearing to be similar in both number of plasmids harbored and molecular mass of these plasmids; however, it is not true that all those plasmids are identical. This may be so, in that the antibiotic substances produced by these strains are differing in antimicrobial activity against the indicator organisms. Accordingly, all the antibiotic substances exhibited antibacterial activity, while only UY4, UY5, UY8 and UY9, elicited antifungal activity. The apparent differences in plasmids appearing to be similar in both number of harbored plasmids and molecular masses or the plasmids have been reported in other bacterial species (Ruiz Barba *et al*., 1991; Jamuna *et.al.,* 2010)

To determine the possible linkage of antibiotic production activity with plasmids DNA in the *Streptomyces* isolates, plasmid-curing experiments were conducted as plasmid linked functions are generally ascertained by curing experiments with curing agents such as ethidium – bromide, acriflavine, acridine orange, novobiocin, etc. In our study, sub-inhibitory concentrations of acridine orange was used to cure all the *Streptomyces*

isolates of the 10,000 bp single copy plasmids after repeated passages. The use of acridine- orange as a potent curing agent has been previously reported by others (Razaee *et al.,* 2005; Sheikh *et al.,* 2003; Yah *et al.,* 2007; Akortha and Filgona, 2009; Akinjoguna and Enabulele, 2010). The antibiotic production and activity of antibiotic substances from the cured strains of the isolates showed loss of antimicrobial activity against the test organisms by the absence of zones of inhibition in the agar well diffusion assay (Table 2). Similarly, the profile of the cured *Streptomyces* isolates on 1.0 % agarose gel electrophoresis indicated absence and non-alignment of plasmid bands as against the parental strains (Fig. 2), confirming the loss of antibiotic production and activity in these cured strains. Consequently, plasmid curing of the isolated *Streptomyces* strains showed that all the colonies failed to produce antibiotics, possibly suggesting that the isolated *Streptomyces* strains antibiotic-substances biosynthetic genes are plasmid encoded. From these results, it can be inferred that the genetic determinants for antibiotics production and activity in the isolated *Streptomyces* strains was encoded by the 10,000 bp plasmids. Similar absence of both metabolic and physiological activities in cured plasmid have been reported for other bacterial species (Anderson and Mckay, 1983; Rezaee *et al.,* 2005; Edward-Raja and Selvan, 2009; Akinjoguna and Enabulele, 2010; Sheikh *et al.,* 2003; Yeh *et al.,* 2007; Akortha and Filgona, 2009).

To confirm that the contribution for antibiotic production and activity was from the 10,000 bp plasmid DNA, transformation experiment was carried out. Antimicrobial activity of antibiotic substances from the transformed *Streptomyces* isolates indicated a rejuvenated inhibitory activity against the test organisms, though significantly ($p<0.05$) lower than that of the parental strains (Tables 3). Similarly, the profile of the transformed *Streptomyces* strains on agarose gel electrophoresis indicated plasmid bands related to plasmid bands of the respective native strains (Figure not shown). The seemingly revived antibiotic production and activity measured by the passively active inhibitions by the transformed cultures, lend further credence and confirmed the removal of the plasmid DNA in the cured strains, hence loss of antibiotic production and activity. Thus, it is evidently confirmed that antibiotic production and activity by the isolated *Streptomyces* strains was plasmid-mediated. These findings are in line with the only few antibiotic biosynthesis genes reported to have been plasmid-borne, particularly those encoding methylenomycin-a synthesis in *Streptomyes coelicolor*, and the antibiotic AS-48, in *Enterococcus faecalis* S-48 (Martinez *et al.,* 1990; Bibb, 1996; Bibb, 2005).

CONCLUSION

Based on the findings in the study, it could be evidently concluded that antibiotic production and activity by the *Streptomyces* isolates was plasmid mediated. The plasmids were seemingly identical single copy, linear, broad-band stable high molecular weight 10,000 bp extra-chromosomal DNA molecules constitutively encoding for the role of antibiotic production and activity in the *Streptomyces* isolates.

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