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Isolation and characterisation of actinomycin D producing *Streptomyces* spp. from Sudanese soil

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Sudanese soil is an unexplored source of antibiotic-producing microorganisms. Here, we reported the screening of soil samples from Sudan for actinomycetes that have antibacterial activity. Two isolates, AH11.4 and AH47, displaying a broad antimicrobial spectrum were selected for further study. Morphological, physiological and biochemical studies indicated that the two isolates are *Streptomyces* spp. 16*S* rDNA sequencing confirmed that the closest matches were to other *Streptomyces*, but phylogenetic analysis demonstrated that the Sudanese strains were on a different node to previously identified strains. The antibiotic activity was isolated by preparative High-performance liquid chromatography (HPLC) and determined to be primarily actinomycin D on the basis of UV, 1H- and 13C-NMR, and MS analyses. One strain also produced actinomycin X2 and αB. These strains are distinct from the known producers of actinomycin, thus are new sources of these antibiotics.

Key words: Screening, antibiotic, antitumour, identification.

INTRODUCTION

Streptomyces are Gram-positive aerobic members of the order Actinomycetales within the class Actinobacteria and have a high DNA G-C content of 69 ± 78 mol% (Korn-Wendisch, 1992). They mainly occur in soil as spores, which germinate and produce substrate and aerial mycelia under favourable nutritional conditions. They have a significant effect on the microbial community in the soil environment, responsible for degradation and recycling of natural biopolymers, such as cellulose, lignin and chitin (Semedo et al., 2001). Actinomycetales are major producers of biologically active compounds: two-thirds of microbially derived antibiotics are obtained from actionmycetales, especially *Streptomyces* spp. Many important antibiotics have been isolated from soil actinomycetes; however, the microbiology of Sudanese soil has been relatively poorly investigated, thus may be a source of new bacterial species and interesting secondary metabolites. Due to the large degree of geographical variation, there are a wide variety of soil types found throughout Sudan, many of which are rich in flora and fauna and in microbial diversity.

The actinomycins are chromopeptide lactone antibiotics, an important class of natural products that despite being discovered more than 60 years ago,

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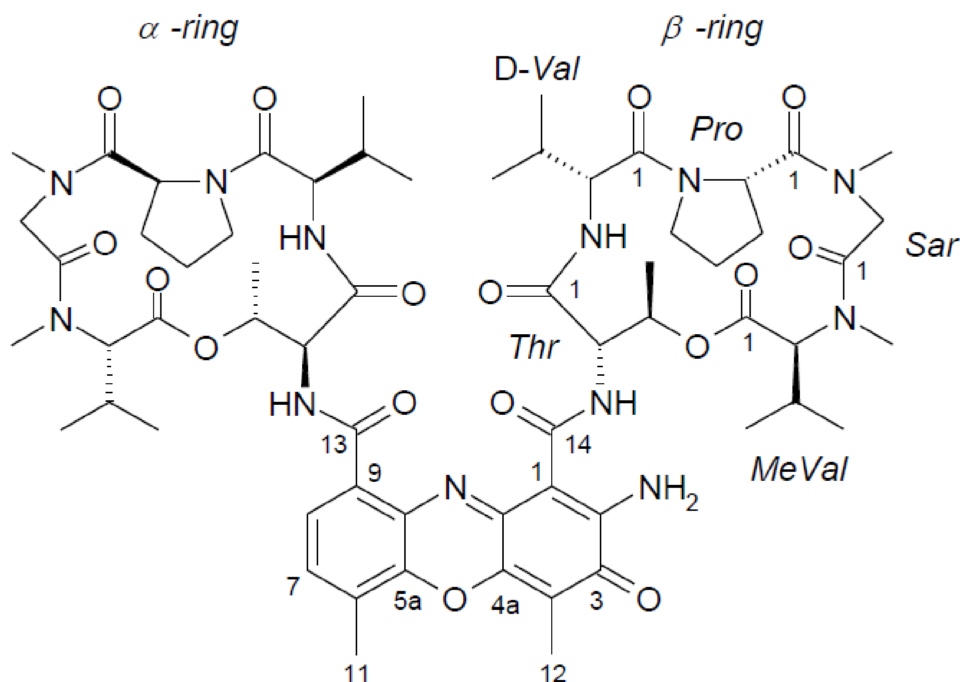

Figure 1. The structure of actinomycin D.

continue to be a focus of many research areas, especially in the biological and medicinal sciences. Among the actinomycins, actinomycin D has been studied most extensively and is used for treatment of malignant tumors, such as Wilms' tumor (Farber et al., 1960; Green, 1997), and childhood rhabdomyosarcoma (Womer, 1997). Actinomycin D (Figure 1) is produced by a range of *Streptomyces* species as part of a mixture of actinomycins, and by some strains of *Micromonospora* (Jones, 1997; Kurosawa et al., 2006; Praveen et al., 2008). This paper described the isolation of new *Streptomyces* spp. isolated from Sudanese soil. The identification of the strain and the isolation and determination of actinomycin D as the major active component, and other actinomycins, were described.

MATERIALS AND METHODS

Isolation, characterization and identification of strain AH47 and 11.4

Strains AH47 and AH11.4 were isolated after a screen of soil samples collected from different locations in the Sudan. Isolation of the strains was performed by soil dilution plate technique using starch-casein nitrate agar (SCNA) (starch 10.0 g, casein 0.3 g, KNO_3 2.0 g, NaCl 2.0 g, K_2HPO_4 2.0 g, $MgSO_4.7H_2O$ 0.05 g, $CaCO_3$ 0.02 g, $FeSO_4.7H_2O$ 0.1 g, Agar 18 g, H_2O 1.0 l), supplemented with 10 μg/ml cyclohexamide. The antimicrobial activity of the cell cultures was screened against a range of microbes. The pure isolates were maintained as lyophils and as spore suspensions at -80°C as described by Hopwood and co-workers (Kieser, 2000).

The bacteria were characterized morphologically and physiologically following the directions given by the International Streptomyces Project (ISP), Shirling and Gottlieb (1966, 1968a and 1968b;) and Bergey's Manual of Systematic Bacteriology. Microscopic characterization was carried out by cover slip culture method, and formation of aerial and substrate mycelium and spores was observed under a light microscope. Scanning electron microscopy was performed using a JEOLJSM 5410LV scanning electron microscope at 35 kV. A plug of agar containing the culture was removed and fixed with glutaraldehyde (2.5% v/v), washed with water and postfixed in osmium tetroxide (1% w/v) for 1 h. The sample was washed with water and dehydrated in ascending ethanol before drying in a critical drying point apparatus (Polaron E3000) and coated in gold. Cultural characteristics (growth, colouration of substrate mycelia, formation of soluble pigment) were tested in eight different media including nutrient agar, Bennett agar, yeast extract malt extract agar (ISP 2), oat meal agar (ISP 3), inorganic salt agar (ISP 4), glycerol-asparagine agar (ISP 5), peptone-yeast extract iron agar (ISP 6) and tyrosine agar (ISP 7) according to the procedures of ISP. Biochemical tests including H_2S production, nitrate reduction, milk peptonisation, starch, gelatine and casein hydrolysis, were performed as recommended by ISP. The effects of pH (5 to 9), temperature (25 to 45°C) and salinity (NaCl concentrations 2 to 7%), plus growth in the presence of the inhibitory compounds phenol (0.1%), crystal violet (0.05%) sodium azide (0.01%) and lysozyme (0.005%) were examined. Tests of antibiotic sensitivity against streptomycin (10 mg l^{-1}), kanamycin (25 mg l^{-1}), vancomycin (5 mg l^{-1}), and ampicillin (20 mg l^{-1}) were also performed. Utilization of carbon sources such as glucose, galactose, arabinose, fructose, raffinose, sucrose, xylose, raminose, cellulose and manitol, by the strains was tested.

The isolates were grown in nutrient broth for the preparation of genomic DNA which was extracted according to the methods described by Nikodinovic et al. (2003). Polymerase chain reaction (PCR) amplification and sequencing of 16*S* rRNA genes was carried out as described previously (Stackebrandt et al., 1997) using a

Peltier thermal cycler. The reaction mixture included the universal primers 27f (5'-CCG TCG ACG AGC TCA GAG TTT GAT CCT GGC TCA G- 3') and 1392r (5'-CCC GGG TAC CAA GCT TAA GGA GGT GAT CCA GCC GCA-3'). To improve the denaturation of the genomic DNA, 5 µl DMSO was added to the reaction mixture. Amplification of the 16*S* rRNA gene was performed according to the following temperature profile: 95°C for 2 min, followed by 30 cycles consisting of denaturing (40 s), primer annealing at 50°C (40 s) and extension at 70°C (1 min). A final extension step at 70°C was included (10 min). Amplified DNA was detected by electrophoresis on a 1% agarose gel and visualized by UV fluorescence after ethidium bromide staining. Amplified fragments were purified using Qiaquick PCR cleanup kit (Qiagen) according to the manufacturer's instructions, and sequenced commercially by MWG. The resulting sequences were subjected to Chimera check (Ribosomal Database Project II) before database searching. Sequences of the other *Streptomyces* spp. were obtained from GenBank and the sequences were aligned by CLUSTAL (Larkin et al., 2007). Phylogenetic analysis was conducted by the neighbour-joining method using the TREECON program.

Chemical composition of the cell wall

Cell walls were purified and analysed using the method of Lechevalier and Lechevalier (1980). The procedures of Becker et al. (1964) and Lechevalier and Lechevalier (1980) were used for analysis of whole-cell chemical composition.

Extraction, purification and isolation of actinomycin D

The production of antibiotic by the isolates was examined in different media, and tryptone soya broth (TSB) gave maximum growth and antibiotic activity. Baffled Erlenmeyer flasks (250 ml), containing 50 ml of medium, were inoculated from a spore suspension, and incubated on a rotary shaker (200 rpm) at 30°C for 48 h. The cultured broth (1 l) was centrifuged at 6000 rpm for 15 min to remove the biomass. Activities against test organisms (*Staphylo-coccus aureus*, *Bacillus subtilis*, *Streptococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Aspergillus niger*, *A. flavus*) were monitored during the isolation, using the antibiotic disk method. Mycelium and supernatant were extracted with ethyl acetate and the antimicrobial activities were observed in the organic phase. Extracts from both supernatant and mycelium were combined and concentrated under vacuum to give a red-orange powder.

The crude organic extract was separated by solid phase extraction (SPE) on a Hypersil C18 column (1 g), and eluted with a stepwise gradient of methanol (20 to 100%). Fractions containing highest antibiotic activity were purified further by HPLC (Varian Prostar system) using an isocratic elution (80% methanol-water) on a Zorbax StableBond column (10 × 250 mm, 5 µm). Peak purity was assessed by analytical HPLC with a gradient elution of acetonitrile using a Thermo Hypersil C18 column (4.6 × 150 mm, 5 µm). Purified compounds were analysed by ESI MS, 1H and 13C NMR with reference to the solvent CDCl3 (δH 7.26, δC 77.0). NMR data for actinomycin D were assigned by 2D NMR analysis and comparison with literature data.

Actinomycin production levels using different carbon sources were assessed as follows: Baffled Erlenmeyer flasks (250 ml) containing 50 ml of TSB medium (without glucose), plus the carbon source of interest (0.5 g) were inoculated using spores from a plate of AH11.4, and incubated on a rotary shaker (200 rpm) at 30°C for 144 h. The mycelium and supernatant were separated by centrifugation at 13000 rpm for 15 min, and the mycelium extracted with ethanol-acetone (1:4, 25 ml) for 14 h. Both supernatant and organic extract were analysed by HPLC (gradient elution from 10% acetonitrile-H_2O to 100% acetonitrile over 20 min, flow rate 0.8 ml/min, using a Varian Microsorb-MV 100-5 C8 250 × 4.6 mm column) to determine actinomycin D concentration, by comparison of peak areas at 440 nm with an actinomycin D standard (Sigma-Aldrich). The values were combined to give overall production levels. Experiments were conducted in duplicate and the values given are averages of the two results.

RESULTS AND DISCUSSION

Cultural and physiological characteristics and identification of the isolates

The biodiversity of microorganisms within Sudanese soils has not been investigated, which are potential new resources for novel antibiotic discovery. During screening of bacteria from Sudanese soils for bioactive natural products, different bacteria were isolated and tested for their antibiotic production against a range of target organisms. Two isolates, designated strains AH47 and AH11.4, which appeared to be actinomycetes, displayed a broad antimicrobial spectrum and were selected for further analysis.

The cultural and physiological characteristics of the isolates on various media are listed in Tables 1 and 2, respectively, when cultured on various media. As shown in Table 1, the strains produced well-developed grey spores on most media tested. They showed good growth on most media except ISP2 media and Bennett's agar. ISP medium 3 and 4 were the best media for growth, spore formation and soluble pigment production. The permissive temperature range for the growth and sporulation of the strains was 25 to 45°C, with an optimum at 37°C; the pH for growth ranged from 6.0 to 8.0 with an optimum at 7 (Table 2). The spore chains were white grey to dark grey; scanning electron microscopy revealed that both strains' spore chains are open loops, hooks and primitive spirals (retinaculum apertum, Figure 2). The comparison of these observed cultural characteristics with those of the known actinomycete species described in Bergey's manual of Systematic bacteriology strongly suggested that strains belongs to the genus *Streptomyces*. The utilization of various carbon sources by the strains is shown in Table 3, and it was observed that *Streptomyces* spp. AH47 utilized all carbon sources with exception of cellulose, whereas strain AH11.4 utilized all carbon sources tested. The cell wall of all the tow strains contained L-diaminopimelic acid and glycine. The whole-cell hydrolysates contained galactose.

Comparison (BLAST) of the 16S rDNA sequences of the two isolates with each other revealed 100% identity across 886 bases, suggesting that the strains are very closely related. The 16S rDNA from strain AH47 (1277 bp) was compared with sequences in GeneBank, revealing the closest match to be *Streptomyces* thermocarboxydus 173998 (97%). Phylogenetic analysis of the strains with the most closely related *Streptomyces* spp. and those that are known to produce actinomycins is shown in Figure 3.

Table 1. Cultural characteristics of strains AH 47, AH 11.4 and AH 4.4 on different media.

Medium	Micro organism							
	AH 47				AH 11.4/AH 4.4			
	Growth	**Spore colour**	**Vegetative Mycelia**	**Soluble pigment**	**Growth**	**Spore colour**	**Vegetative Mycelia**	**Soluble pigment**
ISP2	Poor	Grey, white edges	Cream	None	Poor	None	None	None
ISP3	Abundant	Grey	Cream	Green-yellow	Abundant	Grey, white edges	Yellow-green	Orange
ISP4	Abundant	Dark grey	Yellow	Yellow	Abundant	Dark grey	Orange	Yellow
ISP5	Poor	None	None	None	Moderate	Cream	None	None
ISP6	Abundant	None	None	None	Moderate	None	None	None
ISP7	Abundant	None	None	None	Moderate	None	None	None
Nutrient agar	Poor	None	None	None	Moderate	None	None	None
Bennett agar	Poor	None	None	None	Poor	None	None	None

Table 2. Physiological and biochemical properties of strains AH 47 and AH 11.4.

Property	Strain	
	AH 47	**AH 11.4**
Melanin formation (ISP6 and ISP7 media)	+	+
Starch hydrolysis (tryptone soya agar medium)	+	+
Casein hydrolysis (Casein agar medium)	+	+
Unease production (Nitrate Peptone Broth medium)	+	+
Gelatin hydrolysis (Nutrient gelatine medium)	-	-
Soluble pigment production (ISP media)	-	+ yellow
H_2S production (Triple sugar iron agar medium)	-	-
pH range of growth (ISP medium 4)	6-8	7-9
Temperature range of growth (ISP medium 4)	25-45°C	25-45°C
Antibiotic resistance		
Ampicillin (20 mg/l)	+	+
Kanamycin (25 mg/l)	-	-
Vancomycin (5 mg/l)	-	-
Steptomycin (10 mg/l)	-	-
NaCl tolerance		
NaCl 2% (ISP medium 4)	+	+
NaCl 4% (ISP medium 4)	+	+
NaCl 7% (ISP medium 4)	-	-
Growth on inhibitory compounds		
Phenol 0.1% (ISP medium 4)	+	+
Lysozyme 0.005% (ISP medium 4)	+	+
Sodium azide 0.01% (ISP medium 4)	-	-
Crystal violet 0.05% (ISP medium 4)	-	-

It is evident from Table 5 and Figure 3 that strains AH 47 and AH 11.4 forms a distinct phylogenic line and can be distinguished from the type strains of its most immediate phylogenetic neighbours of the genus Streptomyces by using a combination of phenotypic properties in the literature. Additional phenotypic properties are cited in the

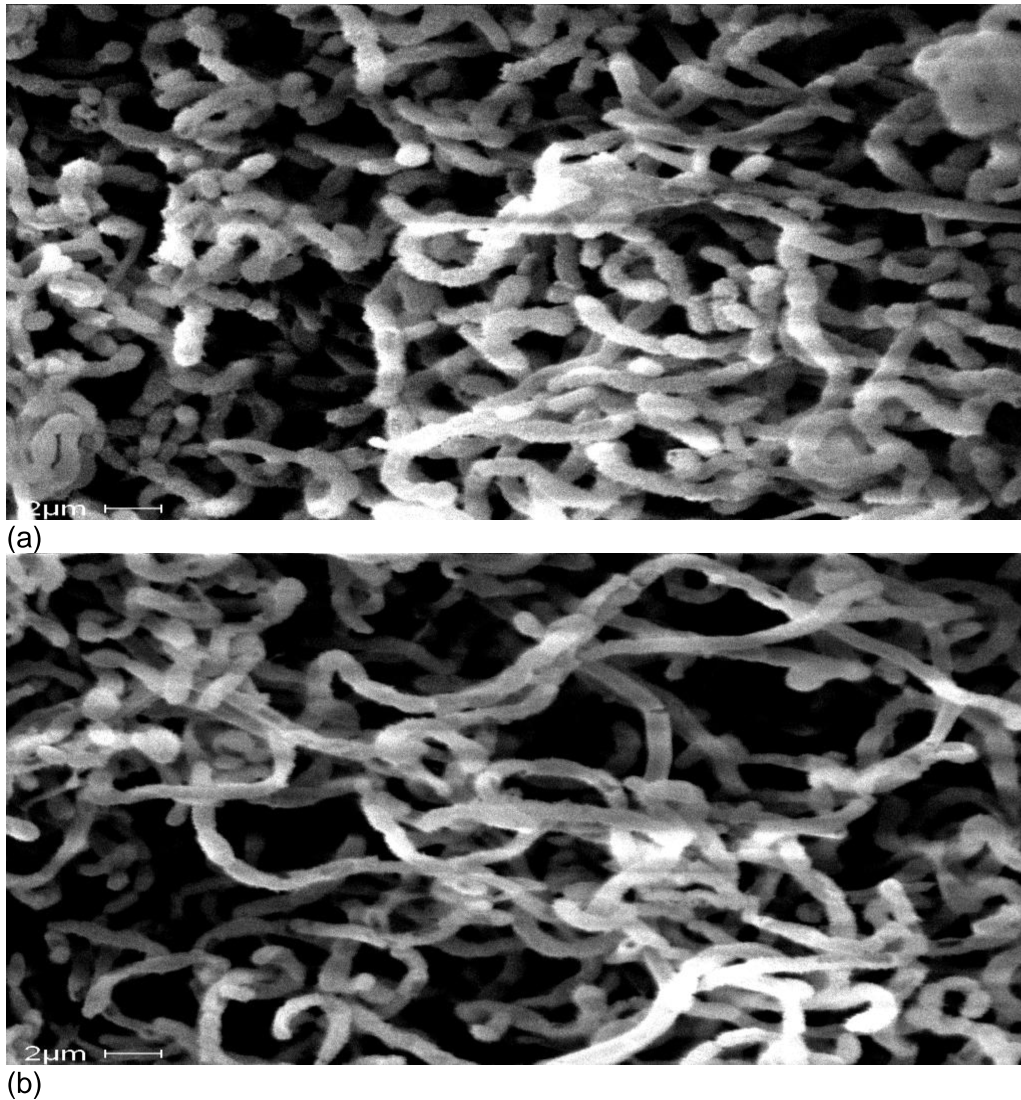

Figure 2. Scanning electron micrographs of *Streptomyces* spp. AH11.4 (A) and AH47 (B).

Table 3. Growth of strains AH 47 and AH 11.4 on different carbon sources.

Carbon source	Growth of strain	
	47	11.4
D-glucose	+++	++
Arabinose	++	+
Raffinose	+	+
Sucrose	++	+
Xylose	++	+++
Rhamnose	++	+
Manitol	++	++
D-Fructose	+++	++
Cellulose	-	++
Galactose	+	+++

(-) No growth, (+) moderate growth, (++) good growth, (+++) excellent growth.

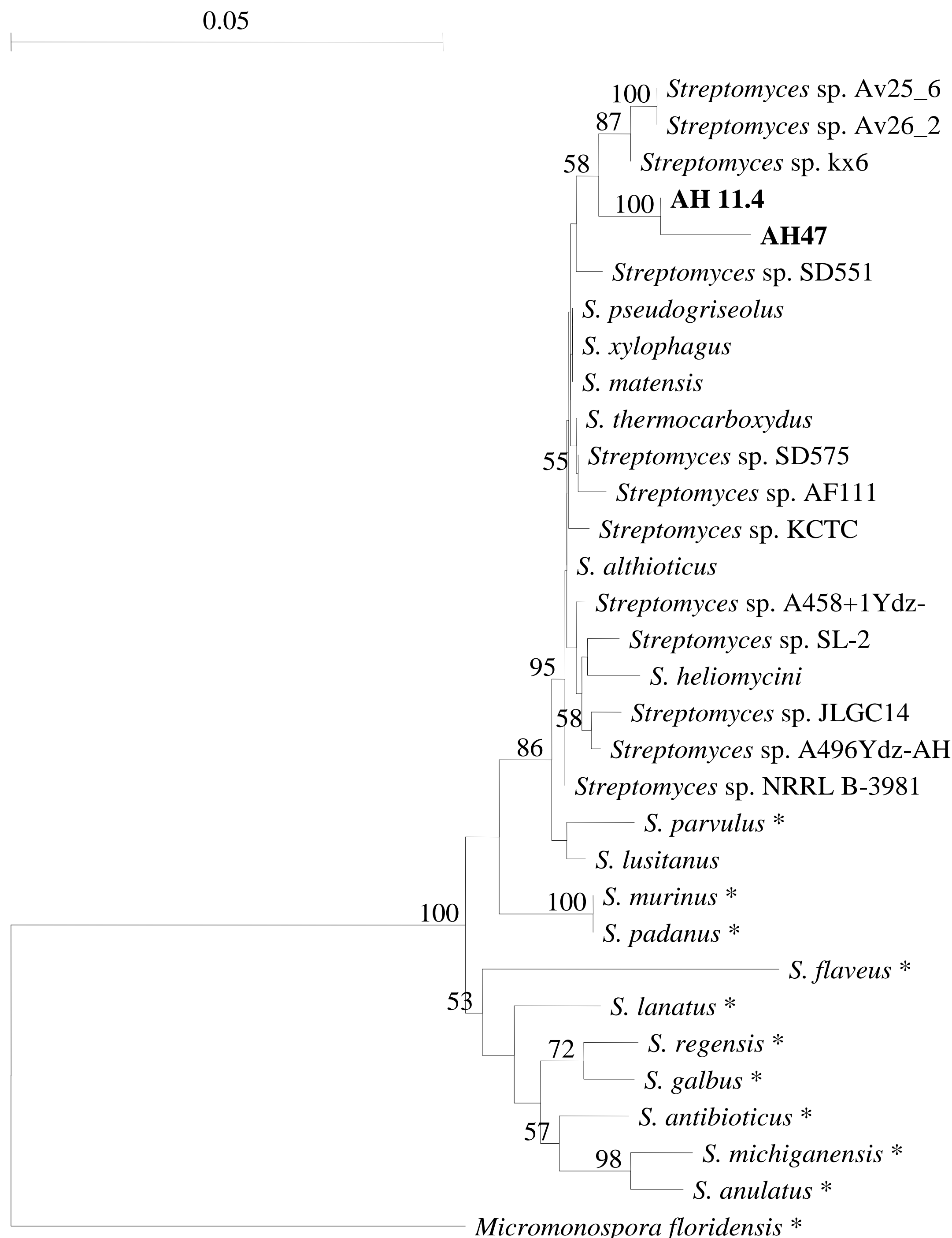

Figure 3. A neighbour-joining tree based on 16S rDNA sequences with *Micromonospor floridensis* as the outgroup. Bootstrap values greater than 50% are highlighted at the nodes (100 replications). The scale bar represents 5 substitutions per 100 bases and asterisks denote the known actinomycin producers.

In this regard, the morphological features and degradation of organic compounds were very significant in distinguishing strain AH 47 and AH 11.4 from its phylogenetic neighbours (Table 4). It was reported in previous studies that differential morphological and pigmentation features are especially significant for the delineation of members of phylogenetically related *Streptomyces* spp (Shirling and Gottlieb 1969 and 1972, Kim et al., 2000; Manfio et al., 2003). While the two bacteria are phylogenetically and ultrastructurally indistinct, some physiological differences were observed, for example growth on cellulose and behaviour on ISP media, thus it is probable

Table 4. Actinomycin D production in strain AH11.4 when grown in TSB containing different carbohydrates.

Carbohydrate source	Acinomycin D production (mg/L)
Arabinose	185
Fructose	192
Glucose	110
Glycerol	99
Maltose	225
Mannitol	223
Raffinose	100
Starch	305
Sucrose	83
Xylose	16

Table 5. Characteristics that separate strains AH 47 and AH 11.4 from the type strains of phylogenetically closely related *Streptomyces s pecies*

Characteristic	1	2	3	4	5	6	7	8
Morphology								
Aerial spore mass	Grey	White	Yellow to grey	Grey	Yellow-pink	Grey	Red to grey	White
Substrate Mycelium	Yellowish brown	Greyish	Yellow to brown	Light grayish	Yellow to brown		Blackish red	Yellow to brown
Diffusible Pigment	None	Yellow	None	None	ND	Brown	None	None
Production of								
Synnemata	+	-	-	+	-	-	-	+
Sclerotium	-	+	-	+	+	-	+	-
Degradation of								
Tyrosine	-	-	-	+	+	NT	-	-
Aesculin	-	-	d	-	-	-	+	-
Casein	+	+	-	NT	+	+	-	+
Aesculin	-	-	-	NT	-	d	-	-
Xanthine	+	-	+	NT	-	-	-	-
Growth at/in								
7% NaCl	-	-	NT	+	-	-	+	-
45°C	+	-	d	-	+	d	-	-

Strains: 1, *S sp.* Av25-6; 2, *S. sp.* Av26-2; 3, S.Kx6; 4, S.SD 551; 5, *S. pseudogriseolus*; 6, *S. xylophagus*; 7, *S. matensis*; 8, *S. themocarbodoxydus*. Data for the type strains of the species indicated were taken from Shirling and Gottlieb (1968a, b, 1969, 1972), Williams et al. (1983) and Kim et al. (1999). +, Positive; –, negative; NT, not tested; d, doubtful.

that they are different strains of the same, apparently novel, species. The 16S rDNA sequences of the isolates were submitted to GenBank (accession numbers GU013556 and GU012557). The cell wall of the tow strains contained L-diaminopimelic acid and glycine. The whole-cell hydrolysates contained galactose.

Production, purification and identification of antibiotic

The isolates produced a red/orange coloured active complex, and the filtrate and mycelia cake were extracted with ethyl acetate to give a combined organic extract. Pre-

Preliminary HPLC-DAD analysis of this extract identified an absorbance spectrum characteristic of actinomycins. This tentative identification was supported by the observation of strong ions in the ESI (+)MS spectra of the extracts corresponding to the presence of actinomycin D (*m/z* 1255, [M+H]+). Purification of the active compound was carried out using reverse phase SPE and HPLC. 2D nuclear magnetic resonance (NMR) analysis [COSY, heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC)] was conducted, which was used to assign the 1H and 13C NMR resonances of actinomycin D, as shown in Table 5.

These assignments are in agreement with those given by Lackner (1971) and Booth et al. (1976). The production of the actinomycin D in the cultures was 44 and 52 mg l^{-1} for strain AH47 and 11.4, respectively. Strain AH47 also yielded two minor compounds, which eluted from the HPLC column at retention times very close to actinomycin D (15.36 and 16.53 min) and had similar absorbance maxima to actinomycin D (220, 242 and 443 nm). While insufficient material was obtained for a thorough NMR analysis, on the basis of mass spectral data, we suggest that these might be actinomycin X2 (*m/z* 1269) and X0β (*m/z* 1271).

It is well established that actinomycin production is affected by carbohydrate source (Katz et al., 1958), thus actinomycin D production by strain AH11.4 was tested by including different carbon sources in the production medium (TSB) at a concentration of 1% (w/v). Table 4 illustrates that the antibiotic production by the isolate was strongly influenced by carbon source. Glucose, usually an excellent carbon source for growth, interferes with the biosynthesis of many antibiotics, including actinomycin (Gallo and Katz, 1972), and accordingly only moderate antibiotic production was observed with this carbon source in the current study (110 mg/l).

The use of starch as a carbon source yielded much higher levels of actinomycin D (305 mg/l), which compared favourably to other values reported in the literature for shake-flask culture, though much higher levels have been reported using a bioreactor (Souza et al., 2002). There is continuing interest in the actinomycins and their microbial producers; there are at least 20 species of *Streptomyces* capable of producing actinomycins.

The strains isolated in this study are not related to those that are known to produce actinomycins (Figure 3), thus are a new source of these important antibiotics. Further up-scaling of the processes is in progress, as the isolates seem to have enough commercial potential.

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