TAXONOMY AND ANTIMICROBIAL ACTIVITY OF *STREPTOSPORANGIUM* STRAIN SG163 ISOLATED FROM A SAHARAN SOIL

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(Received February 2017– Accepted September 2018)

ABSTRACT

K. Bouti, H. Boudjella, N. Bouras, A. Zitouni, F. Mathieu, N. Sabaou. 2019. Taxonomy and antimicrobial activity of *Streptosporangium* strain sg163 isolated from a saharan soil. *Lebanese Science Journal*. 20(1): 35-54.

In a continuing search for new antimicrobial products from actinobacteria collected in Algerian Saharan soils, an isolate of actinobacteria, designated Sg163, was selected for its interesting antimicrobial activity. The isolate was identified to the genus Streptosporangium by phenotypic and molecular criteria. The cultural and physiological characterizations as well as phylogenetic analysis indicated that the isolate was different from known members of the genus Streptosporangium. Analysis of the 16S rRNA sequence showed 97.09 to 98.27 % similarity with those of Streptosporangium type strains. The strain Sg163 produced antifungal and antibacterial activities on several culture media. The highest antimicrobial activities were obtained in ISP2 medium. Three active products C1, C2 and C3 with both antifungal and antibacterial activities were isolated and purified by chromatographic methods with C2, as the major compound. The data of the infrared spectroscopy and the chemical revelations, suggested that the active molecules were glycosylated aromatic compounds.

http://dx.doi.org/10.22453/LSJ-020.1.035-054

Keywords: Actinobacteria, *Streptosporangium*, Taxonomy, antimicrobial activity.

INTRODUCTION

The search for new antimicrobial agents is still required because of the increasing number of antibiotic-resistant strains of pathogenic microorganisms (Solecka *et al*., 2012; Demain and Sanchez, 2009; Fischbach and Walsh, 2009). Microbial natural products continue to represent an important route to the discovery of new antimicrobial compounds (Genilloud, 2014). Among microorganisms, actinobacteria, which are also named actinomycetes, represent a diverse and valuable resource of beneficial natural products (Kurtböke, 2012). The genus *Streptomyces* is the dominant actinobacteria in soils and the source of a broad range of bioactive compounds. However, other rare actinobacteria genera such as *Streptosporangium*, *Saccharothrix*, *Actinomadura*, *Micromonospora*, *Nocardiopsis* and *Nocardia* have proved to be important sources of industrially useful antibiotics (Kurtböke, 2012; Tiwari and Gupta, 2012; 2013; Genilloud *et al*., 2011). In this context, the research in our laboratory was focused on the isolation of new antimicrobial compounds produced by soil actinobacteria others than *Streptomyces*. Several Saharan soil samples collected in arid ecosystems were explored, and many novel taxa and new bioactive molecules were obtained (Boubetra *et al*., 2013; Meklat *et al*., 2013; Boudjella *et al*., 2007; Badji *et al*., 2006; Zitouni *et al*., 2004 a, b).

As part of this screening program, we were interested by members of the genus *Streptosporangium*, which are known to produce several secondary metabolites (Inahashi *et al*., 2011; Boudjella *et al*., 2010; Platas *et al*, 1999; Cooper *et al*., 1990; Hurley *et al*., 1979). During our initial survey, the antimicrobial activity of an isolate drew our attention as it produced antimicrobial activities against fungi and bacteria on several culture media. In the present paper, we reported the methods used for isolation and identification of this isolate as well as the purification and the partial characterization of the produced antibiotics.

MATERIALS AND METHODS

Strain isolation

Actinobacterium Sg163 was isolated by the dilution agar plating method from a Saharan soil sample collected in Adrar (south Algeria, latitude 27° 53’ N, longitude 0° 17’ W, Height 263 m). The dry soil sample was pretreated at 120 °C for 1 h (Nonomura and Ohara, 1969a), serially diluted and spread onto chitin vitamin agar medium (Hayakawa and Nonomura, 1987) supplemented with cycloheximide (50 µg/ml). The plates were incubated at 30 °C for 14 days.

Taxonomic studies of the strain

Taxonomic studies of isolate Sg163 were performed according to the standards for *Streptosporangium* (Quintana and Goodfellow, 2012; Holt *et al*., 1994).

The morphology of strain Sg163 was observed using a Zeiss light microscope. The cultural characteristics were studied using 14-day-old cultures grown on International *Streptomyces* Project (ISP) media (Shirling and Gottlieb, 1966), including yeast extract-malt extract agar (ISP2), oatmeal agar (ISP3) and inorganic salts-starch agar (ISP4), and also on oatmeal-yeast extract-glycerol agar (OYG) (Quintana and Goodfellow, 2012). The colors of aerial mycelium, substrate mycelium and the diffusible pigment were determined with the ISCC-NBS centroid color charts (National Bureau of Standards, 1964).

To obtain biomass for chemotaxonomic analyses, cultures were grown in ISP2 broth for 8 days at 30°C. The cultures were then harvested by centrifugation and washed three times with distilled water. Diagnostic cell-wall amino acids, sugars from whole-cell hydrolysates and membrane phospholipids were analyzed using the methods of Becker *et al*. (1964), Lechevalier and Lechevalier (1970) and Minnikin *et al*. (1977), respectively.

The physiological study consisted of 75 tests including the degradation of 24 carbohydrate compounds evaluated on C1 medium (Nonomura and Ohara, 1969b), the degradation of 12 organic compounds: Tween 80 (Sierra, 1957), adenine, guanine, xanthine, hypoxanthine, casein, tyrosine, testosterone (Goodfellow, 1971), gelatin, starch, esculin and arbutin (Marchal *et al*., 1987), the decarboxylation of 9 organic acids (Gordon *et al*., 1974) and the production of melanoid pigments on ISP6 and ISP7 media (Shirling and Gottlieb, 1966) and nitrate reductase (Marchal *et al*., 1987). The growth was examined on glucose-yeast extract agar (GYEA) medium supplemented with 11 different antibiotics (Athalye *et al*., 1985) and at pH 5, 7 and 9, and at 42 and 50°C. Growth in the presence of other inhibitory compounds (w/v), including sodium azide, 0.001%, sodium chloride, 1, 2, 3, 5 and 7%, potassium tellurite, 0.01% and 0.05%, phenol, 1.5% and lysozyme, 0.005% was also tested in the same medium (Gordon and Barnett, 1977; Athalye *et al*., 1981).

For DNA preparation, PCR amplification and sequence determination, isolate Sg163 was grown at 30°C for 7 days in a 500 ml shake flask, containing 100 ml of ISP2 medium. DNA was extracted using the procedure described by Liu *et al*. (2000).

PCR amplification of the 16S rRNA of strain Sg163 was performed using two primers: 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3'). Each 50 µl amplification reaction (Invitrogen kit) contained 1X PCR buffer (10 mM/lTris-HCl, 50 mM/l KCl, pH 9.0 at 25 °C), 1.5 mM/l $MgC1_2$, 200 µM/l of each dNTP, 1 µM/l of each primer, 1.25 U of *Taq* DNA

polymerase and 1 µl (500 ng) of the purified DNA. The amplification was performed on a STRATAGENE RoboCycler Gradient 96, according to the following profile: denaturation of the target DNA at 98°C for 3 min, after which *Taq* polymerase was added, followed by 30 amplification cycles at 94°C for 1 min, primer annealing at 52°C for 1 min, and primer extension at 72°C for 5 min. At the end of the cycling, the reaction mixture was held at 72°C for 5 min and then cooled to 4°C. Reaction products were electrophoresed on a 1% agarose gel and checked with ethidium bromide under UV fluorescence.

The PCR product was sequenced by MilleGen Company (Toulouse, France) with an automatic sequencer and the same primers as above. The obtained 16S rRNA sequence was compared for similarity level with the reference species of bacteria contained in genomic database banks, using the sequences present in public sequence databases as well as the EzTaxon-e server (http://eztaxon-e.ezbiocloud. net/ (Kim et al., 2012).

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura *et al*., 2011). The 16S rRNA sequence of strain Sg163 was aligned using the CLUSTAL W program (Thompson *et al*., 1994) against corresponding nucleotide sequences of representatives of the genus *Streptosporangium* retrieved from EzTaxon. Phylogenetic tree was inferred by the neighbor joining method (Saitou and Nei, 1987). Evolutionary distance matrices were generated as described by Jukes and Cantor (1969). Tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1 000 resamplings of the neighbor joining dataset.

Antimicrobial activity

The antimicrobial activity spectrum of strain Sg163 was examined during the primary screening for potential antimicrobial activities by the conventional cross-streak method (Williston *et al*., 1947) on ISP2 medium (Shirling and Gottlieb, 1966). Strain Sg163 was inoculated in a straight line on different agar plates of 90 mm diameter and incubated at 30°C for 14 days to allow it to secrete antibiotics into the medium. After incubation, target organisms were seeded in crossed streaks to Sg163 culture. The plates were then incubated for 48 h for fungi and 24 h for yeasts and bacteria. The antimicrobial activity was evaluated by measuring the distance of inhibition between target microorganisms and actinomycete colony margins. Tests were conducted in triplicate.

The test organisms included fungi (*Aspergillus carbonarius* M333, *A. westerdijkiae* NRRL 3174, *Botrytis cinerea*, *Fusarium culmorum* 3288, *F. graminearum* 5883, *F. sporotrichinoides* 13440, *F. verticilloides* 6442, *Geotrichum candidum*, *Umbelopsis ramanniana* NRRL 1829, *Penicillium citrinum* P1843, *P. verrucosum* P1850, *Pythium irregular* PY120, and *Sclerotium sclerotiorum* S358), yeasts (*Candida albicans* C200, *Candida albicans* C224, *Kluyveromyces lactis* K258, *Rhodotorula*

mucilaginosa R321, and *Saccharomyces cerevisiae* ATCC 4226), Gram-positive bacteria (*Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* CIP 82110, *Staphylococcus aureus* CIP 7625 and *Micrococcus luteus* ATCC 9314) and Gram-negative bacteria (*Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* CIP 8291). Most of these test organisms are phytopathogenic, or human pathogenic or toxigenic.

Effect of culture media on the production of antimicrobial activities

To determine the optimal culture medium for antibiotic production, five complex culture media pre-inoculated with Sg163 were investigated. The selected media were: ISP2 (Shirling and Gottlieb, 1966) containing glucose, yeast extract and malt extract, Bennett medium (Warren *et al*., 1955) composed of glucose, meat extract, yeast extract and peptone, ISP4 (Shirling and Gottlieb, 1966) containing starch and inorganic salts, the medium of Gilpin et al. (1995) designated M4 and containing glycerol, yeast extract, malt extract and peptone of soya, and the medium of Omura *et al*. (1989) designated M5 and containing glucose, meat extract, dry yeast and peptone.

The media were prepared in 500-ml Erlenmeyer flasks containing 100 ml of medium. The pH was adjusted to 7.3 prior to sterilization. For the seed culture, isolate Sg163 was grown on ISP2 agar for 7 days at 30°C. The mycelium was scraped and inoculated into 250-ml Erlenmeyer flasks containing 50 ml of each medium. After 4 days of growth on a rotary shaker (250 rpm) at 30°C, aliquots (5%, v/v) of the seed culture were transferred into 500-ml Erlenmeyer flasks containing 100 ml of the medium. The cultures were incubated on a rotary shaker (250 rpm) at 30°C. The time course fermentation was carried out for 13 days. The antimicrobial activity in the culture broths was tested by the agar well diffusion method using *Umbelopsis ramanniana* NRRL 1829, *Saccharomyces cerevisiae* ATCC 4226 and *Micrococcus luteus* ATCC 9314 as target organisms. A volume of 20 ml of nutrient agar was inoculated with 100 µl of microbial suspension containing 10^8 CFU/ml of the target organism and then poured onto a Petri dish and allowed to solidify at room temperature for 30 min. Wells of 10 mm diameter were aseptically bored into the culture medium and 200 µL of each culture filtrate were added to each well. The antimicrobial activity production, the growth (dry weight of mycelium) and the pH were measured every 24 h.

Extraction and purification of antimicrobial products

For the production of antibiotics, fermentations were carried out in 500-ml Erlenmeyer flasks containing 100 ml of ISP2 broth (250 rpm, 30°C, 7 days). The antimicrobial activity was obtained from 5 l of culture broth and purified by various separation steps including solvent extraction, preparative silica gel plates and high performance liquid chromatography (HPLC). The antimicrobial compounds were extracted on the day of optimal production rate. The culture broth was centrifuged (3000 *g*, 15 min) to remove the biomass. The cell-free supernatant was extracted with an equal

volume of *n*-butanol. This solvent was selected in a preliminary test, where 4 solvents were tested. Each volume of 60 ml of the cell free supernatant was extracted with an equal volume of the following organic solvents: *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol. The organic and aqueous phases were recuperated separately and concentrated to dryness (Laborota 4000 rotavapor). The organic extracts were recuperated in methanol, and the aqueous ones in water. Both organic and aqueous extracts were tested for their antimicrobial activity by the paper disk diffusion method against *Umbelopsis ramanniana*, *Saccharomyces cerevisiae* and *Micrococcus luteus*. The highest antimicrobial activity was obtained with *n*-butanol. Furthermore, no anti-yeast activity was detected in the organic extracts. It remains in the aqueous phases. Subsequently, we were interested by the butanolic extract, which contained both antifungal and antibacterial activities. The remaining culture filtrate was extracted with *n*-butanol for subsequent purification.

Preparative chromatography with silica gel plates (Merck Art. 5735, Kiesselgel 60F 254) was used for the partial purification of antimicrobial products. A dry crude extract, dissolved in methanol, was spotted and developed in the solvent system *n*-butanol-acetic acid-water (B.A.W.) (3:1:1, v/v). The developed TLC plates were air dried overnight to remove all traces of solvents. The separated compounds were visualized under UV at 254 nm (absorbance) and at 365 nm (fluorescence) and the active spots detected by bioautography (Betina, 1973) on silica gel plates seeded with *Umbelopsis ramanniana* or *Micrococcus luteus*. In this method, the plates were placed on a glass support in a polyethylene box (22 × 24 cm). A sheet of filter paper was placed at the bottom of the box and impregnated with about 30 ml of sterile water to maintain a moist atmosphere and thereby delay the desiccation of the agar during incubation. The device was then sterilized for 1 h under UV at 254 nm. A volume of fused 50 ml of semi-solid ISP2 (containing 7 g/l agar) was inoculated with 2 ml of a target organism suspension. This medium was uniformly distributed with a sterile pipette on the silicagel plates. After solidification of the agar, plates were placed at 4 °C for 2 h to allow the diffusion of antibiotic activity in the medium and then incubated at 30 °C. The retention factor (Rf) of the active spot was measured after 24 to 48 h.

The final purification was carried out with a Waters HPLC system equipped with a C18 column (Uptisphere UP 5 ODB, 250 mm × 7.8 mm, Interchim), a mobile phase with a continuous gradient (linear curve) solvent system from 20 to 75% v/v methanol in water, a flow rate of 1.5 ml/min and UV detection at 220 nm. The pure compounds were collected separately, concentrated and bioassayed against the indicator organisms *Umbelopsis ramanniana* and *Micrococcus luteus*.

Chemical characterization of antimicrobial activity

The pure antimicrobial compounds were revealed on silica gel TLC plates with several chemical agents including ninhydrin, sulfuric acid-naphtoresorcinol, nitro-4-anilin, ferrous iron chloride, formaldehyde-sulfuric acid and vanillin-sulfuric acid

(Merck). The UV-vis absorption spectra of the active molecules were determined with a Shimadzu UV 260 spectrophotometer. For the major compound, infra red spectrum was obtained with Shimadzu IR 470 spectrometer and the mass spectrum was recorded on ion-trap mass spectrometer (Finnigan MAT, San Jose, California, USA), equipped with a nanospray ion ESI source (negative ion mode).

RESULTS AND DISCUSSION

Strain identification

Isolate Sg163 produced aerial mycelium with spherical sporangia. The sporangia were commonly 6-10 µm in diameter. Colonies of Sg163 grown on various media at 30°C for 14 days were observed to be 5-10 mm in diameter, circular and smooth. It grew well on ISP2 and OYG and weak on ISP3 and ISP4 media. The mycelium was stable and not fragmented. The aerial mycelium was pinkish white in color on most of the media used. The substrate mycelium was yellowish brown and produced a yellow diffusible pigment on all used media.

Cell-wall hydrolysate of isolate Sg163 contained *meso*-diaminopimelic acid but not glycine. The whole-cell hydrolysates contained madurose as the characteristic sugar. From these results, strain Sg163 was considered to have a type III B cell wall (Lechevalier and Lechevalier, 1970). The phospholipids consisted of phosphatidylethanolamine and an unknown compound containing glucosamine. This pattern corresponds to the phospholipid type PIV (Lechevalier *et al*., 1977).

Based on the morphological and chemical characteristics, isolate Sg163 was identified as a member of the genus *Streptosporangium*.

The results from the physiological study indicated that strain Sg163 was positive for the utilization of casein, gelatin, starch, tyrosine, arabinose, cellobiose, dextrin, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, rhamnose and trehalose. The strain was negative for the utilization of adenine, arbutin, esculin, testosterone, hypoxanthine, adonitol, dulcitol, erythritol, glycerol, inositol, melibiose, raffinose, saccharose and sorbitol. B vitamins were not required for growth. Melanoid pigments were not produced on ISP6 and ISP7 media. Sodium acetate and pyruvate were degraded. The other sodium salts (butyrate, citrate, oxalate, propionate, succinate and tartrate) were not degraded. Strain Sg163 was resistant to chloramphenicol (25 mg/l), cycloserine (10 mg/l), erythromycin (10 mg/l), gentamicin (5 mg/l), oxytetracycline (25 mg/l), penicilline (25 mg/l), rifampicin (5 mg/l), streptomycin (10 mg/l), vancomycin (5 mg/l) and potassium tellurite (0.005% and 0.01%), but was sensitive to kanamycin (25 mg/l), novobiocin (10 mg/l), crystal violet (0.05%), lysozyme (0.005%), phenol (1.5%) and sodium azide (0.001%). The optimum growth was at 30°C and at pH 7.3. The strain was not able to grow at pH 5 and 9, and at 42 and 50°C.

The nearly complete 16S rRNA gene sequence (1446 nucleotides) of strain Sg163 was determined and deposited in GenBank under the accession number EF369475. Alignment of this sequence with reported 16S rRNA gene sequences in EzTaxon confirmed the identification of strain Sg163 to the genus *Streptosporangium*. The phylogenetic tree (Figure 1) showed the position of strain Sg163 within the type species of *Streptosporangium*. The similarity values between strain Sg163 and the type strains in the genus *Streptosporangium* ranged between 97.1% and 98.3%. The species showing the highest similarity values to strain Sg163 were *S. canum* HBUM 170018^T, *S. roseum* DSM 43021^T, *S. album* DSM 43023^T and *S. vulgare* DSM 43802^T (between 98.1 and 98.3%). Table 1 summarizes the differential characteristics of strain Sg163 and the related strains.

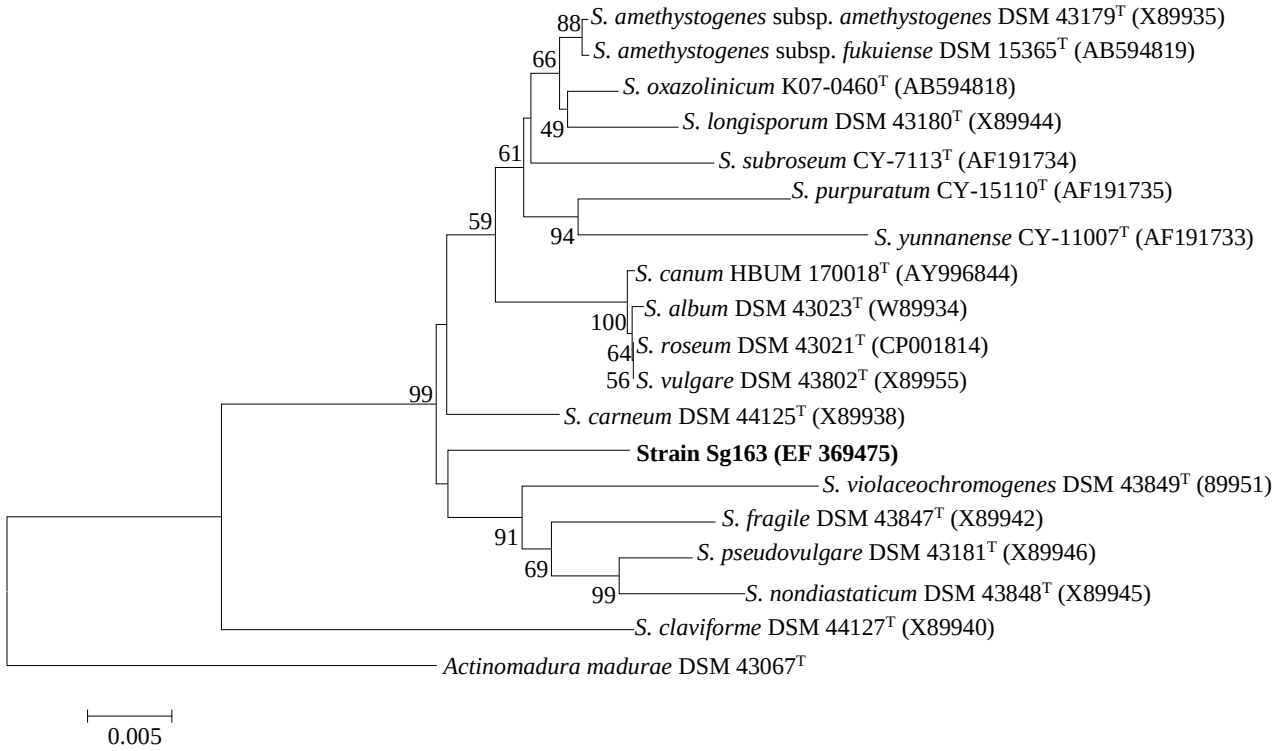

Figure 1. Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationship between strain Sg163 and members of the genus *Streptosporangium*.

Numbers at nodes indicate bootstrap percentages (based on 1000 resampled datasets); only values greater than 50% are given. Accession numbers are given in parentheses. The sequence of *Actinomadura madurae* DSM 43067^T was used as outgroup. Bar, 0.005 substitutions per nucleotide position.

On the basis of morphological and chemical characteristics, strain Sg163 was classified in the genus *Streptosporangium*. The results of comparative studies of physiology and phylogenetic analysis indicated that the isolate was different from known members of *Streptosporangium*. The high similarity value (98.3%) between the strain Sg163 and the type species of *Streptosporangium* is not surprising. Much higher 16S rRNA similarities have been found between different species of validly described S*treptosporangium*, such as the type strains of *S. roseum* and *S. vulgare* (100%), *S. album* and *S. vulgare* (99.8%), *S. album* and *S. roseum* (99.8%), *S. nondiastaticum* and *S. pseudovulgare* (98.8%), *S. longisporum* and *S. amethystogenes* (98.8%) and *S. carneum* and *S. longisporum* (98.6%). Furthermore, strain Sg163 could be distinguished from the related strains *S. album* (Quintana and Goodfellow, 2012) and *S. canum* (Zhang *et al*., 2009) by phenotypic properties. Eleven differences from closely related *Streptosporangium* species were recorded and consisted of the use of casein, starch, tyrosine, galactose, inositol, raffinose, sorbitol and xylose, the reduction of nitrate, and the decarboxylation of sodium citrate and sodium oxalate. These results strongly suggest that strain Sg163 may represent a new species of *Streptosporangium*.

Table 1. Differential characteristics of *Streptosporangium* sp. Sg163 (1) and the related strains *S. canum* HBUM 170018^T (2), *S. roseum* DSM 43021^T (3), *S. album* DSM 43023^T (4) and *S. vulgare* DSM 43802^T (5).

Tests/characteristics	Strains				
	1	2	3	4	5
Degradation activity					
Casein	+	–	+	–	+
Starch	+	–	+	–	+
Tyrosin	+	–	+	–	+
Galactose	+	–	–	+	+
Inositol	–	+	+	–	d
Raffinose	–	–	–	+	–
Sorbitol	–	–	–	+	–
Xylose	+	+	+	–	+
Nitrate reduction	+	+	+	–	–
Decarboxylation of sodium salts					
Citrate	–	+	–	+	–
Oxalate	–	+	nd	–	nd
Growth at					
42°C	–	nd	–	–	–
50°C	–	nd	–	–	–
Sporangium (µm)	6-10	3-6	6-10	6-10	6-10
Sporangiophores (µm)	10	nd	10	10	10
Spores	Spherical-oval	Rod	Spherical-oval	Spherical-oval	Spherical-oval

Tests/characteristics	Strains				
	1	2	3	4	5
Color of spore mass	White-pinkish	Whitish grey	Pink	White	Pink
Color of substrate mycelium	Yellowish brown	Brown	Red-orange	Yellowish brown	Yellowish brown to brown
Diffusible pigments	Pale yellow-brown	–	Red brown to purple brown	Pale yellow-brown	–

+, positive test; -, negative test; d, 11-89% of strains are positive; nd, not determined.
Data taken from Quintana and Goodfellow (2012); Zhang et al. (2009); Holt et al. (1994) and Whitham et al. (1993).

Antimicrobial activity

The antimicrobial activities of the strain Sg163 against various target microorganisms are shown in Table 2. The results were expressed as mean ± standard deviation (SD) from triplicate experiments. The strain exhibited a strong activity against the phytopathogenic and toxigenic fungi tested and two human pathogenic strains of *Candida albicans*. The antibacterial activity was moderate against Gram-positive bacteria and negative against Gram-negative bacteria.

Table 2. Antimicrobial activity of *Streptosporangium* sp. Sg163 by the cross-streak method.

Test organisms	Distance of inhibition (mm)[a]
Filamentous fungi	
Aspergillus carbonarius M333	12.0 ± 0.9
Aspergillus westerdijkiae NRRL 3174	10.3 ± 0.7
Botrytis cinerea B354	16.1 ± 0.5
Fusarium culmorum F3288	13.0 ± 0.3
Fusarium graminearum F5883	15.0 ± 0.7
Fusarium sporotrichioides F13440	11.1 ± 0.7
Fusarium verticilloides F6442	15.3 ± 1.1
Geotrichum candidum G669	12.0 ± 0.4
Umbelopsis ramanniana NRRL 1829	25.1 ± 1.0
Penicillium citrinum P1843	14.0 ± 0.5
Penicillium verrucosum P1850	30.1 ± 1.2
Pythium irregulare PY120	10.0 ± 0.7

Test organisms	Distance of inhibition (mm)[a]
Sclerotium sclerotiorum S358	8.0 ± 0.2
Yeasts	
Candida albicans C200	10.1 ± 0.1
Candida albicans C224	19.2 ± 0.2
Kluyveromyces lactis K258	13.1 ± 0.2
Rhodotorula mucilaginosa R321	8.0 ± 0.3
Saccharomyces cerevisiae ATCC 4226	34.2 ± 1.1
Gram-positive bacteria	
Bacillus subtilis ATCC 6633	6.0 ± 0.4
Listeria monocytogenes CIP 82110	2.0 ± 0.7
Micrococcus luteus ATCC 9314	10.1 ± 0.7
Staphylococcus aureus CIP 7625	4.0 ± 0.5
Gram-negative bacteria[b]	0

(*a) Values are mean ± SD of three independent experiments*.
(b) No activity obtained against the Gram-negative bacteria: Escherichia coli ATCC 10536, Pseudomonas aeruginosa ATCC 27853 and Klebsiella pneumoniae CIP 8291.

Consequently, *Umbelopsis ramanniana* and *Micrococcus luteus*, the most sensitive strains, were selected as the indicator microorganisms for determining the antimicrobial activity in shake culture media.

Fermentation studies

In liquid media, antifungal and antibacterial activities were observed on all media tested (Figure 2). The maximal antimicrobial activity was observed in ISP2 medium.

During the time course of growth and antibiotic production in ISP2 broth, the antifungal and antibacterial activities were detected on day 3 and 4 of fermentation respectively (Figure 3) and reached the maxima on day 7. The antifungal activity was stronger than the antibacterial activity. A strong activity against the yeast was detected on day 4 with a maximum recorded on day 8. These activities were correlated with cell growth. Little variations in pH were recorded and the maximum (pH 8.6) was reached on day 8 of fermentation.

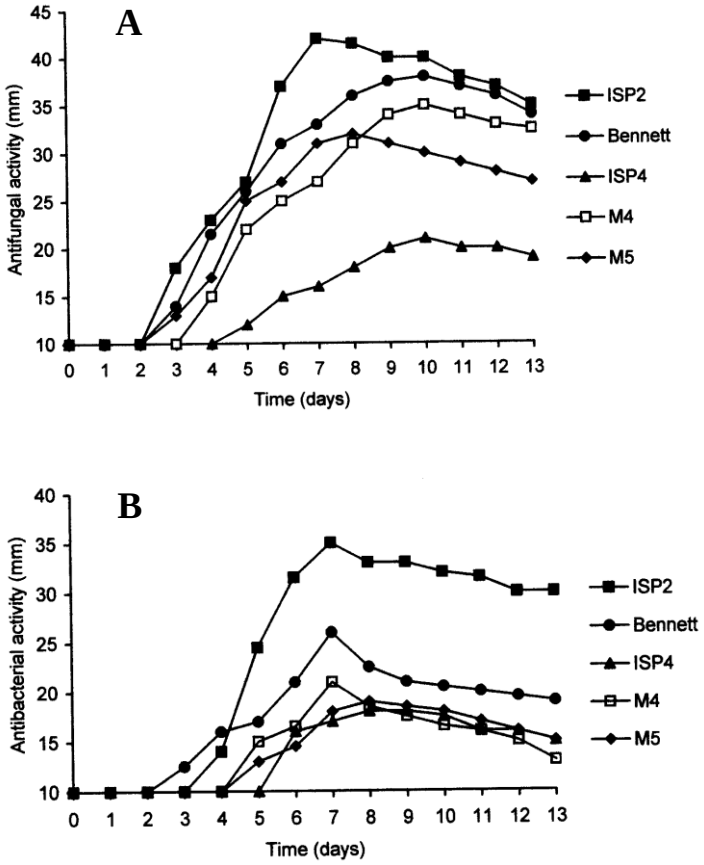

Figure 2. Effect of various culture media on production of antimicrobial activities against *Umbelopsis ramanniana* (A) and *Micrococcus luteus* (B) in 500 ml flasks.

Values include the diameter of wells (10 mm). Media: ISP2, yeast extract – malt extract – glucose ; Bennett, meat extract – yeast extract – peptone – glucose ; ISP4, inorganic salts – starch ; M4, yeast extract – malt extract – glycerol – peptone; M5, dry yeast – meat extract – peptone – glucose.

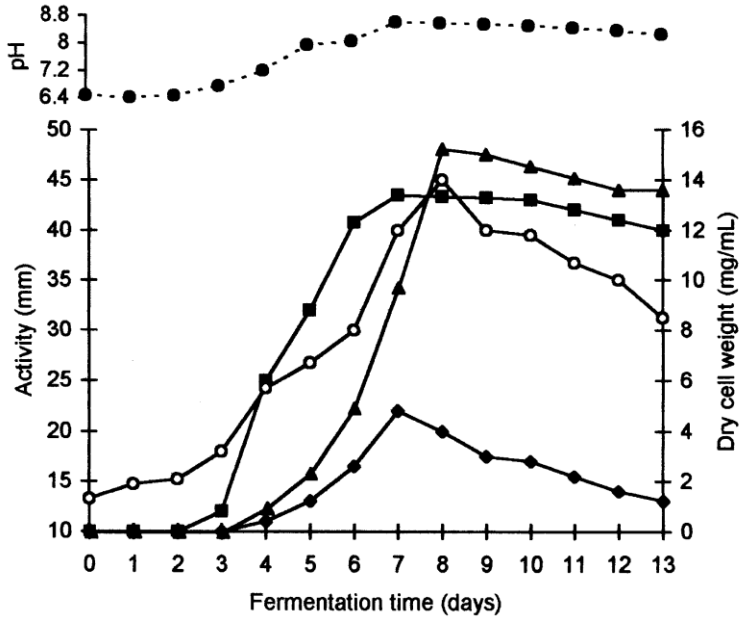

Figure 3. Time course of pH, growth and antibiotic production by *Streptosporangium* sp. Sg163 in 500 ml flasks.

■*, Umbelopsis ramanniana;* ♦*, Micrococcus luteus;* ▲*, Saccharomyces cerevisiae. Values include the diameter of wells (10 mm);* ○*, dry cell weight;* ●*, pH.*

Extraction and purification of antibiotics

The cell-free supernatant of 5 l shake culture was extracted by *n*-butanol. Recalling that the *n*-butanol was selected as solvent extraction in a preliminary test where four solvents with different polarities were tested. The organic and aqueous phases were bioassayed against *Umbelopsis ramanniana*, *Micrococcus luteus* and *Saccharomyces cerevisiae* and by paper disk method. The activity against the yeast was detected only in the aqueous phase. This work focused on the organic phase, which exhibited activities against filamentous fungi and bacteria. On analytic TLC plates, one spot with both antifungal and antibacterial activity was detected by bioautography at Rf 0.71. The pooled active band was purified by HPLC. The separated fractions were tested against *Umbelopsis ramanniana* and *Micrococcus luteus*. Three active peaks designated C1, C2 and C3 were obtained at the retention times 36.65, 37.70 and 39.49 min, respectively (Figure 4). Each showed both antifungal and antibacterial activity, with C2 as the major compound. The antifungal activity was stronger than the antibacterial one.

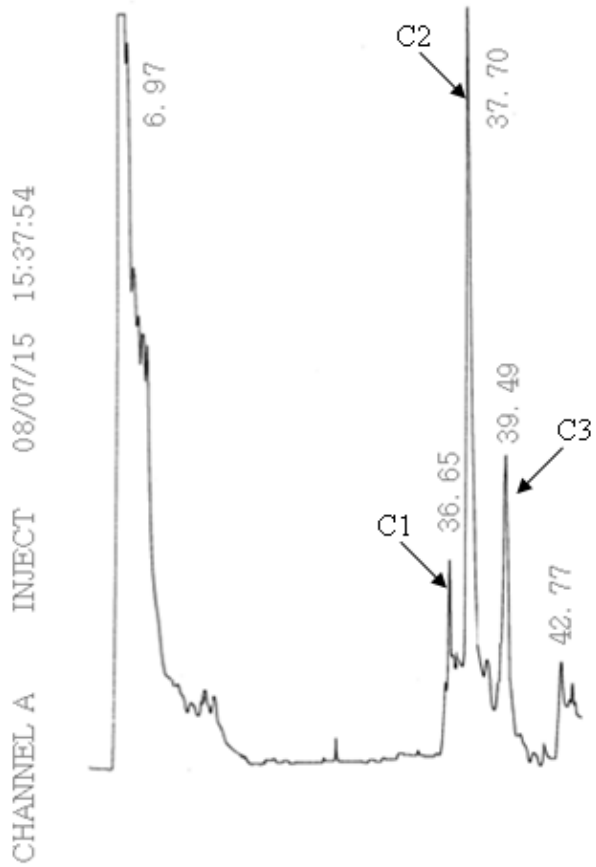

Figure 4. HPLC profile of the first injection showing the active peaks corresponding to the antibiotics C1, C2 and C3 secreted by the strain *Streptosporangium* Sg163.

Column C18; gradient system 20 to 75% v/v methanol in water; detection at 220 nm.

Partial characterization of antimicrobial activities

The pure antimicrobial compounds C1, C2 and C3 presented the same Rf on silica gel TLC plates developed in B.A.W. solvents system. They were revealed positively by the chemical reagents, naphtoresorcinol-sulfuric acid (sugar revelator), formaldehyde-sulfuric acid (visualization reagent of polycyclic aromatic compounds), nitro-4-anilin (revelator of phenols) and ferrous iron chloride (revelator of phenols and hydroxamic acids). Ninhydrin test was negative suggesting the absence of free amines. These results showed for the three molecules the presence of one or more sugars, phenolic moieties and probably other aromatic moieties. These results also suggested that the three molecules were closely related. The UV-vis spectra in methanol (data not

shown) exhibited absorption maxima at 220 and 351 nm for C1, 221 and 351 nm for C2 and 199 and 353 nm for C3.

The results of chromogenic reactions and UV-vis absorption, and the migration at the same Rf on TLC plates suggested that the three produced compounds C1, C2 and C3 are similar in their structure.

The infrared spectrum of the major compound C2 showed OH groups at 3450 cm^{-1}, 1400-1410 and 1040-1130 cm^{-1}, CH_3, CH_2 and/or CH groups at 2850-2950 cm^{-1}, 1450-1460 cm^{-1}, and 1375 cm^{-1}. Bands at 1520 and 1625 cm^{-1} indicated aromatic cycles and at 1640 to 1670 cm^{-1}, ketone group. The nano-electrospray-ionisation mass spectrum (Figure 5) indicated a molecular weight of 492 for the C2 compound.

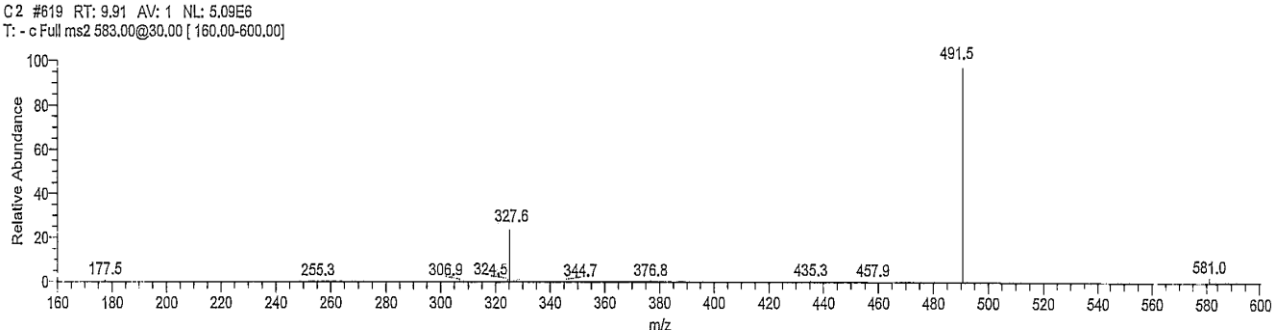

Figure 5. Mass spectrum of C2 compound produced by *Streptosporangium* sp. Sg163.

The results of chemical revelations and infrared spectrum suggested that the major compound, C2, has a glycosylated aromatic structure. Among antibiotics secreted by strains of *Streptosporangium*, only sibiromycin and sinefungins are glycosylated aromatics. However, sibiromycin differs from compound C2 by its activity, which is only antibacterial, its UV-vis (λ max = 230 and 310 nm) and infrared spectra (Hurley et al., 1979). Sinefungins have only antifungal activity and molecular weights between 363 and 422 (Cooper *et al*., 1990). Numerous glycosylated aromatic antibiotics active against both fungi and bacteria are secreted by actinobacteria (Dictionary of Natural Products, 2017; HeteroCycles, 2017; Buckingham, 1997; Bycroft, 1988; Umezawa, 1988; Berdy *et al*., 1987). Their comparison with the compound C2 indicated that they are different by colors and/or UV-vis and infrared spectra and molecular weights. NMR analysis is necessary for the structure elucidation of the compound C2.

Furthermore, the UV-vis spectra indicated that the three active molecules are non-polyenic antifungal compounds because of the absence of the characteristic three-absorption bands between 260 and 405 nm. The polyene antifungals are potentially toxic and therefore, only few molecules are used in medicine. This drug toxicity along

with the development of resistance highlights the need for the advent of new effective and less toxic antifungal agents.

CONCLUSION

In this study, an actinobacterial strain of the genus *Streptosporangium* showing an interesting antimicrobial activity during the primary screening for antibiotic activities, was identified by morphological, chemical, physiological and molecular methods. The comparative studies revealed that the strain was different from known members of *Streptosporangium*. DNA-DNA hybridation with the most related species is required to confirm the originality of the strain Sg163.

Strain Sg163 produced three antibiotics with both antifungal and antibacterial activity. Their partial characterization suggested that they were glycosylated aromatics, a large group of molecules with several chemical structures. Comparison of characteristics of our antibiotics with those cited in the literature did not allow us to identify them. Investigations on the determination of the structures are currently in progress.

ACKNOWLEDGEMENTS

This work was gratefully supported by the 'Comite´d'Evaluation et de Prospective de Cooperation Inter-universitaire Franco-Algérienne' (CMEP N° 02 MDU 564), French Embassy in Algiers.

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