Taxonomy and chemical characterization of antibiotics of *Streptosporangium* Sg 10 isolated from a Saharan soil

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KEYWORDS
Actinomycetes;
Antibiotics;
Antimicrobial activity;
Streptosporangium;
Taxonomy

Summary

A new actinomycete strain designated Sg 10, producing antimicrobial substances was isolated from an Algerian soil. Morphological and chemical studies indicated that strain Sg 10 belonged to the genus *Streptosporangium*. The comparison of its physiological characteristics with those of known species of *Streptosporangium* showed significant differences with the nearest species *Streptosporangium carneum*. Analysis of the 16S rDNA sequence of strain Sg 10 showed a similarity level ranging between 96.3% and 97.8% within *Streptosporangium* species, with *S. carneum* the most closely related. However, the phylogenetic analysis indicated that strain Sg 10 represent a distinct phyletic line suggesting a new genomic species. The antimicrobial activity of strain Sg 10 showed an antibacterial activity against Gram-positive bacteria as well as an antifungal one. Four active products were isolated from the culture broth using various separation procedures. On the basis of UV-VIS spectrometry, infrared spectroscopy and chemical revelations, the antibiotics were classified in the group of glycosylated aromatics.

Introduction

The search for new antibiotics continues to be of utmost importance in research programs around the world because of the increase of resistant

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pathogens and toxicity of some used antibiotics. Among microorganisms, actinomycetes are one of the most investigated groups particularly members of the genus *Streptomyces* from which, a large number of antibiotics was obtained and studied (Okami and Hotta, 1988). The vast majority of actinomycetes have originated from soil (Davies and Williams, 1970) and their isolation method deal almost exclusively with those suitable for *Streptomyces* species which grow rapidly on soil dilution plates. However, in recent years, the rate of discovery of new antibiotics in the genus *Streptomyces* was declining and isolation of other actinomycete genera, appeared to be necessary to assess the health hazard and to find novel strains producing commercially valuable antibiotics. With the discovery of new antibiotics from strains of *Actinomadura*, *Micromonospora*, *Saccharothrix*, and *Streptosporangium*, increased emphasis was placed on developing methods for the isolation and identification of non-streptomycete actinomycetes (Shearer, 1997).

In our laboratory, during a screening program for search of potent antimicrobial products, interest was focused on antibiotic producing rare actinomycetes. Selective methods were used to isolate new strains producing new antibiotics (Sabaou et al., 1998; Lamari et al., 2002; Zitouni et al., 2004a, b). It has been also found that *Streptosporangium* strains can produce valuable substances of biotechnological interest (Cooper et al., 1990; Lazzarini et al., 2000; Pfefferle et al., 2000). These promising results emphasize the need to continue the researches in this way.

In the present work, we describe the isolation of a *Streptosporangium* strain, designated Sg 10, from a Saharan soil sample, and its identification by conventional and molecular methods as well as the production, the isolation and the partial characterization of produced antibiotics.

Materials and methods

Strain isolation

The strain Sg 10 was isolated in our laboratory by the dilution agar plating method, from a Saharan soil sample collected at Beni-Abbes, a South-West location of Algeria. One gram of the soil sample was aseptically added to 9 ml sterile distilled water. The suspension was vortexed and diluted. Aliquots (0.2 ml) of each dilution were spread on the surface of chitin-B vitamins medium (Hayakawa and Nonomura, 1987) consisting of (per liter of distilled water): 2 g of chitin, 0.35 g K_2HPO_4, 0.15 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.3 g NaCl, 0.02 g $CaCO_3$, 10 mg $FeSO_4 \cdot 7H_2O$, 1 mg $ZnSO_4 \cdot 7H_2O$, 1 mg $MnCl_2 \cdot 4H_2O$ and 18 g agar. The pH was adjusted to 7.2 prior to autoclaving. The B vitamins including thiamine-HCl, riboflavin, niacin, pyrodoxin-HCl, inositol, Ca-pantothenate, p-aminobenzoic acid (0.5 mg/l for each) and biotin (0.25 mg/l) were added to the autoclaved medium. Kanamycin and cycloheximide were sterilized and added to the medium as selective agents to a final concentration of 25 and 50 mg/l, respectively. The plates were incubated at 30 °C for 3 weeks.

Morphological and cultural characteristics

Taxonomic studies of isolate Sg 10 were performed based on morphological, molecular, chemical and physiological analyses using the methods described by Shirling and Gottlieb (1966), Nonomura (1989) and Holt et al. (1994).

The morphological and cultural characteristics of the organism were determined by naked eyes examination of 14-day-old cultures grown on various International *Streptomyces* Project (ISP) media (Shirling and Gottlieb, 1966): yeast extract–malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salts–starch agar (ISP 4) and on oatmeal–yeast extract-glycerol (OYG) (Nonomura, 1989). The micromorphology and sporulation were observed by light and electron microscopy. Colors of aerial and substrate mycelia were determined with the ISCC-NBS centroïd color charts (US National Bureau of Standard, 1976).

For electron microscopic observation, a culture of strain Sg 10 grown on ISP 2 medium for 14 days, was exposed to 1% osmium tetroxyde vapor for 3 days and then washed with distilled water, dehydrated with ethyl alcohol and dried. The sample was then coated with gold and observed with a Cambridge Stereoscan 240 electron microscope.

Chemotaxonomic analysis

Biomass for chemotaxonomic analysis was obtained from a culture grown in shake ISP 2 medium (Shirling and Gottlieb, 1966) and incubated at 30 °C for 14 days. Diaminopimelic acid (DAP) isomers, whole-cell sugar pattern and phospholipids were analyzed according to the methods of Becker et al. (1964), Lechevalier and Lechevalier (1970) and Minnikin et al. (1977), respectively.

Physiological characteristics

A total of 75 tests were considered for this study, including the utilization of 24 carbohydrate compounds evaluated on C1 medium (Nonomura and Ohara, 1969), the degradation of 12 organic compounds: adenine, guanin, xanthin, hypoxanthin, milk casein, tyrosin, testosterone (Goodfellow, 1971), tween 80 (Sierra, 1957), gelatin, starch, esculin and arbutin (Marchal et al., 1987), the decarboxylation of 9 organic acids (Gordon et al., 1974), the production of melanoid pigments on ISP 6 and ISP 7 media (Shirling and Gottlieb, 1966) and nitrate reductase (Marchal et al., 1987). The strain was also examined for its ability to grow on glucose-yeast extract agar (GYEA) medium supplemented with 11 different antibiotics (Athalye et al., 1985) and inhibitory compounds including (w/v): sodium azide, 0.001%; sodium chloride, 1%, 2%, 3%, 5% and 7%; potassium tellurite, 0.01% and 0.05%; phenol, 0.005% and lysozyme, 0.005% (Gordon and Barnett, 1977; Athalye et al., 1981) and to grow at pH 5, pH 9 and at 42 °C.

DNA preparation, PCR amplification and sequence analysis

The strain Sg 10 was grown at 30 °C for 5 days in shake flasks, containing 100 ml of ISP 2 medium (4 g/l yeast extract, 10 g/l malt extract and 4 g/l glucose). Mycelium was obtained by centrifugation and washed twice with bi-distilled water. Approximately, 200 mg of mycelium were used for genomic DNA extraction as follows: the sample was dispersed in 800 μl of the lysis solution (100 mM Tris-HCl, pH 7.4, 20 mM EDTA; 250 mM NaCl; 2% SDS; 1 mg/ml; lysozyme; qsp 100 ml H_20), added with 5 μl of RNAase (50 mg/ml) and incubated at 37 °C for 60 min. Then 10 μl of proteinase K solution (20 mg/ml) were added, and the lysis solution was reincubated at 65 °C for 30 min.The lysate was extracted two times with an equal volume of phenol, centrifuged and then re-extracted with chloroform (v/v) to remove residual phenol. DNA was precipitated by adding NaCl (at a final concentration of 150 mM) and 2 volumes of 95% cool ethanol. After centrifugation, the DNA was cleaned with 50 μl of ethanol 70%, centrifuged, and then re-suspended in 50 μl of TE buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA, pH 8.0). The DNA purity and quantity were checked by spectrophotometer at 260 and 280 nm.

PCR amplification of the 16S rDNA of Sg10 strain was performed using two primers: 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′). The 16S rDNA was amplified by PCR using Promega kit. The final volume of reaction mixture of 50 μl contained 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 9.0 at 25 °C), 1.5 mM $MgCl_2$, 200 mM of each dNTP, 1 mM of each primer, 1.25 U of Taq DNA polymerase and 500 ng of template DNA. The amplification was performed on a Touchgene (Techne) thermal cycler according to the following profile: an initial denaturation step at 98 °C for 3 min, after which Taq polymerase was added, followed by 30 amplification cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min, and a final extension step of 72 °C for 10 min. The PCR product was detected by agarose gel electrophoresis and was visualized by UV fluorescence after ethidium bromide staining.

The PCR products obtained were submitted to GenomExpress for sequence determination. The same primers as above and an automated sequencer were used for this purpose. The sequence determined was compared for similarity level with the reference species of bacteria contained in genomic database banks, using the "NCBI Blast" available at the *ncbi-nlm-nih.gov* Web site.

Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using softwares included in MEGA version 3.0 (Kumar et al., 2004) package. The 16S rDNA sequence of the strain Sg 10 was aligned using the CLUSTAL W program (Thompson et al., 1994) against corresponding nucleotide sequences of representatives of the genus *Streptosporangium* retrieved from GenBank. Evolutionary distance matrices were generated as described by Jukes and Cantor (1969) and a phylogenetic tree was inferred by the Neighbor joining method (Saitou and Nei, 1987). Tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings of the neighbor joining data set.

Antimicrobial activity

Antimicrobial activity was evaluated on ISP 2 medium, by the streak method against various microorganisms. The strain Sg 10 was inoculated in straight line on plates of 90 mm diameter and incubated at 30 °C for 14 days. Target microorganisms were seeded in crossed streaks to actinomycete culture. The antimicrobial activity was evaluated by measuring the distance of inhibition

between target microorganisms and actinomycete colony margins. The target microorganisms included Gram-positive (*Bacillus subtilis* ATCC 6633, *Micrococcus luteus* ATCC 9314) and Gram-negative bacteria (*Agrobacterium tumefaciens* No. 2410, *Agrobacterium tumefaciens* biovar 3 No. 964, *Erwinia amylovora* No. 1994, *Erwinia carotovora* subsp. *carotovora* No. 1336, *Xanthomonas campestris* pathovar *campestris* No. 1119, *Escherichia coli* ATCC 10536, *Pseudomonas fluorescens* and *Serratia marcescens*), yeasts (*Candida albicans* No. 200, *Candida albicans* No. 224, *Kluyveromyces lactis, Rhodotorula mucilaginosa* and *Saccharomyces cerevisiae* ATCC 4226) and fungi (*Botrytis cinerea, Fusarium culmorum, Geotrichum candidum, Mucor ramannianus* NRRL 1829, *Penicillium* sp., *Pythium irregulare* and *Sclerotium sclerotiorum*). The target microorganisms without accession number resulted from our laboratory collection.

Analyses of antimicrobial product formation in shake flask cultures

Fermentations were carried out in ISP 2 broth (composition mentioned above). The pH was adjusted to 7.2 before autoclaving. A seed culture was prepared with the same medium and used to inoculate 500 ml-Erlenmeyer flasks containing 100 ml of medium. The cultures were incubated on a rotary shaker (250 rpm) at 30 °C for 17 days. The antimicrobial activities were daily assayed against *Micrococcus luteus* and *Mucor ramannianus* by the agar diffusion method (wells technique). Each well of 10 mm in diameter was filled with 0.2 ml of supernatant.

For the purification of antibiotics, repeated fermentations were carried out to obtain a total of 5 l of culture broth.

Isolation and purification of antimicrobial products

The culture broth was centrifuged to remove the biomass. The cell-free supernatant was extracted with an equal volume of organic solvent. Five extraction solvents were tested for effectiveness, including *n*-hexane, ethyl acetate, dichloromethane, benzene and *n*-butanol. Each organic extract was concentrated to dryness. The resulting dry extract was recuperated in 1 ml of methanol and bioassayed against *Micrococcus luteus* and *Mucor ramannianus,* by paper disk diffusion method.

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 (BAW) (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). The TLC plates were placed in a plastic bioassay dish (23 cm × 23 cm × 2.2 cm, Fisher Scientific Labosi) and overlayed with 150 ml of ISP 2 medium (containing 7 g/l agar) seeded with *Micrococcus luteus* or *Mucor ramannianus* as target organisms and incubated at 30 °C. Clear areas due to the inhibition of the growth of target organisms indicated the location of antibiotic compounds. The retention factor (Rf) of the active spots were measured.

The final purification was carried out by reverse phase HPLC using a C18 (5 μm) column (300 × 7.8 mm, Interchim). The elution was at a flow rate of 2 ml/min with a discontinuous gradient solvent system of 20%, 40%, 60%, 80% and 100% methanol in water. The detection was by UV at 220 nm.The peaks were collected separately, concentrated and bioassayed against the indicator organisms *Micrococcus luteus* and *Mucor ramannianus*.

Partial characterization of products

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

The absorption spectrum of the active molecules in methanol was determined with a Shimadzu UV 260 spectrophotometer.

Infra red spectrum was obtained by dispersing 2 mg of the analyzed molecule in potassium bromide with Shimadzu IR 470 spectrometer.

Mass spectrum was recorded on LCQ ion-trap mass spectrometer (Finnigan MAT, San Jose, CA), equipped with a nanospray ion ESI source (negative ion mode).

Results

Taxonomy

Colonies of Sg 10 strain grown on various media at 30 °C for 14 days were observed to be 5–10 mm in diameter, circular and smooth. Good growth was

observed on ISP 2 and OYG and moderate on ISP 3 and ISP 4 media. The mycelium is stable and not fragmented. The aerial mycelium (AM) is pink-white and the substrate mycelium orange to yellowish brown. The AM produces spherical sporangia with 4–8 μm in diameter, each one containing a single chain of numerous and non-motile spores (Fig. 1). The sporangiospores are spherical or oval and 1–2 μm in diameter. No soluble pigment was produced on all media used.

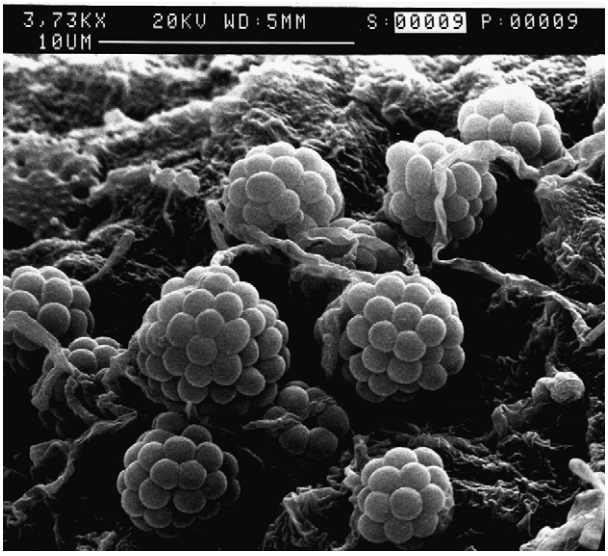

Figure 1. Scanning electron micrograph of aerial mycelium of strain Sg10 showing morphology of sporangia and spores.

The chemotaxonomic study showed the presence of meso-diaminopimelic acid isomer in the cell-wall but not glycin, and madurose as characteristic sugar in the whole-cell hydrolysates, as well as glucose, galactose, ribose and traces of mannose. This corresponds to chemotype III B according to Lechevalier and Lechevalier (1970). The phospholipid profile was of type PIV characterized by phosphatidylethanolamine and phospholipids containing glucosamine (Lechevalier et al., 1977).

Through 16S rDNA sequence analysis, an amplified fragment of 1446 bp was obtained and compared with sequences of the reference species of bacteria contained in genomic database banks. The similarity level ranged from 96.3% to 97.8% with *Streptosporangium carneum* DSM 44125^T having the closest match. The phylogenetic tree obtained by applying the neighbor joining method is illustrated in Fig. 2.

Table 1 shows the results of physiological tests of strain Sg 10 in comparison with those of *S. carneum*.

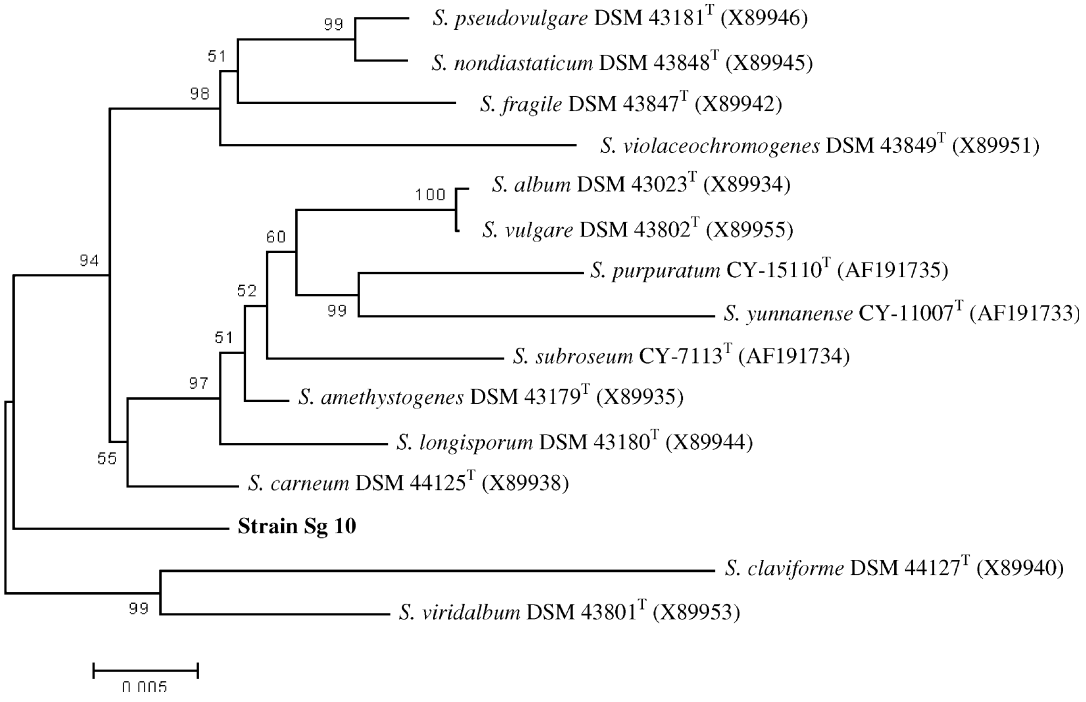

Figure 2. Neighbor-joining tree, based on 16S rDNA sequences showing the relations between strain Sg 10 and type species of the genus *Streptosporangium*. The numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1000 resampled data sets; only values over 50% are given. Bar, 0.005 nt substitution per nt position.

Table 1. Physiological characteristics of the strain Sg 10

Tests	Strains	
	Sg 10	*S. carneum* DSM 44125^{T}*
Degradation activity		
Adenine	−	−
Arbutin	+	+
Casein	+	+
Esculin	+	−
Gelatin	+	−
Starch	+	−
Testosterone	−	+
Tween 80	−	ND
Tyrosin	+	+
Xanthin	−	−
Adonitol	−	−
Arabinose	+	−
Cellobiose	+	+
Dextrin	+	−
Dulcitol	−	−
Erythritol	−	−
Fructose	+	+
Galactose	+	+
Glucose	+	+
Glycerol	−	−
Hypoxanthine	−	−
Inositol	−	−
Lactose	+	+
Maltose	+	−
Mannitol	+	−
Mannose	+	+
Melibiose	−	−
α-methyl-D-glucoside	−	−
Raffinose	−	−
Rhamnose	+	−
Ribose	−	−
Saccharose	+	−
Sorbitol	−	−
Trehalose	+	+
Xylose	+	−
Production of melanoid pigment	−	−
Nitrate reduction	−	−
Decarboxylation of sodium		
Acetate	+	+
Benzoate	−	−
Butyrate	−	−
Citrate	−	−
Oxalate	−	−
Propionate	+	−
Pyruvate	+	+
Succinate	+	−
Tartrate	−	−
Resistance to antibiotics		
Chloramphenicol (25 mg/l)	−	ND
Cycloserine (10 mg/l)	−	ND
Erythromycin (10 mg/l)	−	ND
Gentamicin (5 mg/l)	−	+
Kanamycin (25 mg/l)	+	ND
Novobiocin (10 mg/l)	−	ND
Oxytetracycline (25 mg/l)	−	ND
Penicilline (25 mg/l)	−	+
Rifampicin (5 mg/l)	+	−
Streptomycin (10 mg/l)	−	+
Vancomycin (5 mg/l)	−	−
Growth in the presence of inhibitory compounds		
Crystal violet (0.05% w/v)	−	ND
Lysozyme (0.005% w/v)	−	−
Phenol (1.5% w/v)	−	ND
Potassium tellurite (0.005% and 0.01% w/v)	+	ND
Sodium azide (0.001% w/v)	−	ND
Sodium chloride (2% w/v)	+	+
Growth at		
42 °C	−	−
pH 5	−	ND
pH 9	−	ND

+ growth; − no growth; ND: not determined.
*Data from Mertz and Yao (1990).

Antimicrobial activity

The antimicrobial activity of the strain Sg 10 against various target microorganisms is shown in Table 2. The strain exhibited a good activity against tested Gram-positive bacteria and no activity against Gram-negative bacteria, except for *Pseudomonas fluorescens* (weak activity). An antiyeast activity was also recorded against *Sacharomyces cerevisiae* but the growth of *C. albicans* was not affected. The antifungal activity was moderate.

Analyses of antimicrobial product formation in shake flask cultures

During the time course of fermentation, antibiotic production, and pH parameters were monitored as shown in Fig. 3. The antimicrobial activity was detected the 4th day of fermentation and reached a maximum after 11 days against both the fungus and the bacterium. The antibacterial activity was stronger than the antifungal one. Little variations in pH were recorded.

Table 2. Antimicrobial activity of strain Sg 10

Test organisms	Distance of inhibition (mm)
Gram-positive bacteria	
Bacillus subtilis ATCC 6633	36
Micrococcus luteus ATCC 9314	41
Gram-negative bacteria	
Pseudomonas fluorescens	3
Yeasts	
Saccharomyces cerevisiae ATCC 4226	6
Filamentous fungi	
Fusarium culmorum	1
Mucor ramannianus NRRL 1829	12
Pythium irregulare	9
Sclerotium sclerotiorum	3

The following microorganisms were not inhibited by the strain Sg 10: *Agrobacterium tumefaciens* No. 2410, *A. tumefaciens* biovar 3 No. 964, *Erwinia amylovora* No. 1994, *E. carotovora* subsp. *carotovora* No. 1336, *Xanthomonas campestris* pathovar *campestris* No. 1119, *Escherchia coli* ATCC 10536, *Serratia marcescens*, *Candida albicans* No. 200, *C. albicans* No. 224, *Kluyveromyces lactis*, *Rhodotorula mucilaginosa*, *Botrytis cinerea*, *Geotrichum candidum* and *Penicillium* sp.

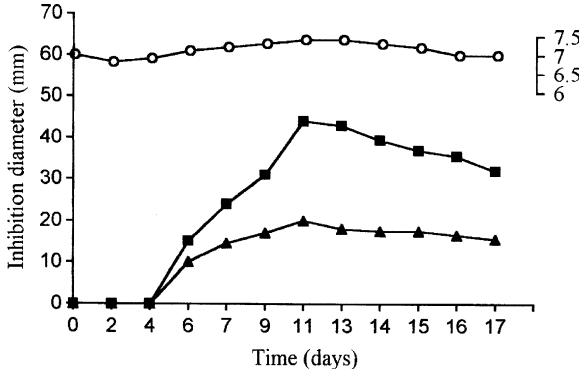

Figure 3. Time course of antibiotic production in ISP 2 medium. (■) antibacterial activity, (▲) antifungal activity, (○) pH.

Isolation and purification of antimicrobial products

Various separation methods including solvent extraction, preparative silica gel plates and HPLC were realized for the purification of antibiotics.

Five organic solvents were tested for extraction. The results (data not shown) indicated that *n*-butanol is the most appropriate for antibiotic extraction. The cell-free supernatant of 5 l shake culture was then extracted by *n*-butanol. The organic phase was concentrated to dryness, recuperated in methanol and chromatographed by TLC. On analytic TLC plates developed in BAW solvent system, four active spots were detected by bioautography at 0.74, 0.68, 0.61 and 0.36 Rf values and designated A, B, C and D, respectively. B and D antibiotics showed respectively strong and moderate antibacterial and antifungal activities, while A and C antibiotics were antibacterial only. The preparative silica gel plates were then used to spot the crude extract in bands in order to collect each of the four antibiotics in appreciable quantity. The active bands were recuperated and desorbed in methanol, concentrated to dryness and purified by HPLC.

Partial characterization of antimicrobial products

The UV-VIS spectrum of the crude extract showed maxima at 206 and 260 nm. The absence of the three characteristic maxima of polyenes indicated that the antibiotics are not polyenic.

Antibiotics A–D are strongly soluble in *n*-butanol and methanol. They are revealed positively by the chemical reagents, naphtoresorcinol (sugars reagent), nitro-4-anilin (phenol reagent) and ferric chloride (alcohols, ethered oils and steroids reagent). Ninhydrin test was negative suggesting the absence of free amines. These results suggested that the four antibiotics contained one or several sugars, phenolic moieties and probably other aromatic moieties. Antibiotics A–D have in common, similarities concerning solubility and chromogenic reactions.

Antibiotic B exhibited the most interesting activity. Its UV-VIS spectrum (Fig. 4) in methanol showed maxima at 201, 222 and 261 nm.The infrared spectrum of antibiotic B (Fig. 5) showed hydroxyl units (bands at 3450 cm^{-1}, 1400–1410, 1040 and 1130 cm^{-1}), CH_3, CH_2 and/or CH groups (bands between 2850 and 2950, between 1450 and 1460 as well as at 1375 cm^{-1}) and aromatic cycles (large zone between 1520 and 1670 cm^{-1}). The bands at 1190, 1220 and in 1010 cm^{-1} indicated the presence of ether bonds as well as hydroxyl groups. The bands at 830 cm^{-1} and at 690–670 cm^{-1} revealed the presence of benzenic cycles, di- and monosubstituted, respectively. The mass spectrum of antibiotic B (Fig. 6) showed a base peak at m/z 326.8 representing the deprotonated molecule [M-H]; thus the molecular weight of antibiotic B is $M = 328$.

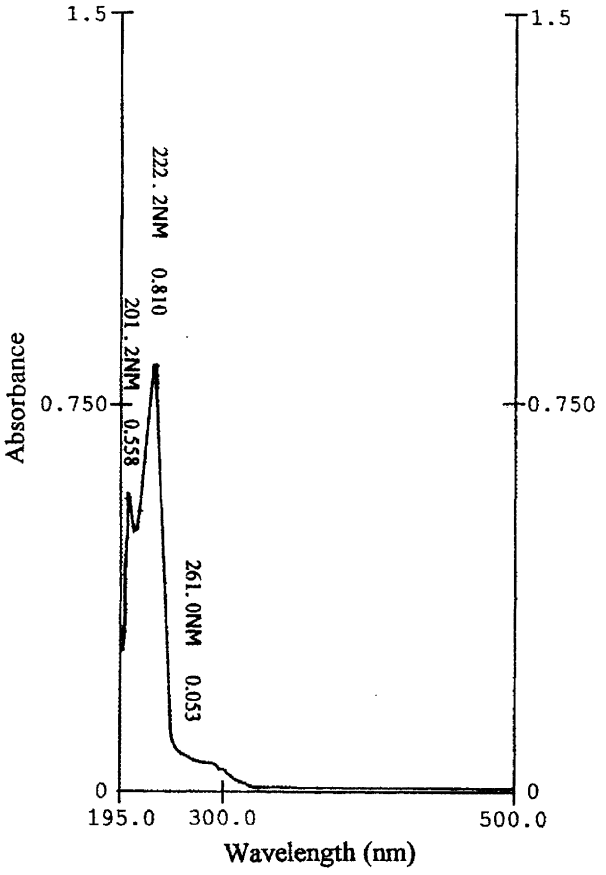

Figure 4. UV-VIS spectrum of antibiotic B (in methanol).

Discussion

On the basis of its morphological and chemical properties, the strain Sg 10 was classified in the genus *Streptosporangium*. The distinction of *Streptosporangium* species is mainly based on the color of aerial and substrate mycelia and of soluble pigment, the size of sporangia, the shape of spores and some physiological characters such as growth at 42 °C, the degradation of starch, gelatin, inositol and rhamnose, the reduction of nitrates, and the production of iodinin (Nonomura, 1989). Some additional tests relative to the use of adonitol, arabinose, glycerol, galactose and mannitol are also considered (Holt et al., 1994).

Comparison of cultural and physiological characteristics of the strain Sg 10 with those of *Streptosporangium* known species indicated that *Streptosporangium carneum* was the nearest species. The two strains have the same aerial and substrate mycelia colors, sporangia size, spore shape and some physiological characters like degradation of adonitol, galactose, glycerol and inositol, growth at 42 °C, iodinin production and reduction of nitrate. However, significant differences in physiological characteristics (17 differences) were recorded between strain Sg 10 and *S. carneum* (Table 1). The differences consisted of the degradation of arabinose, maltose, mannitol,

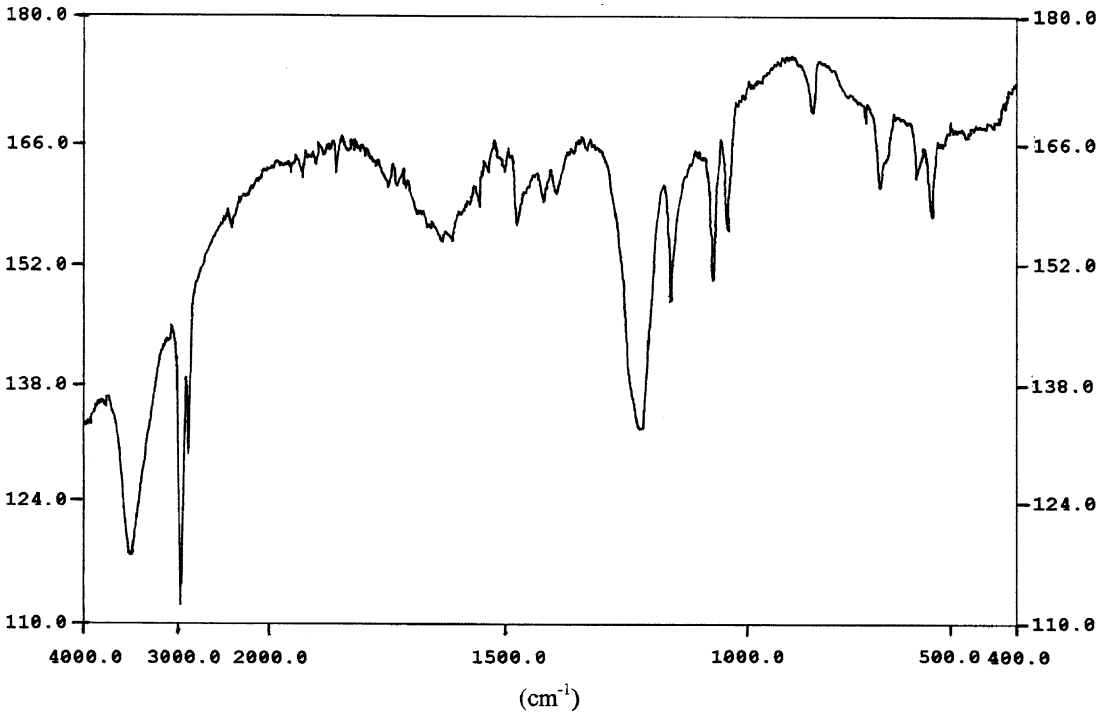

Figure 5. Infrared spectrum of antibiotic B (in KBr).

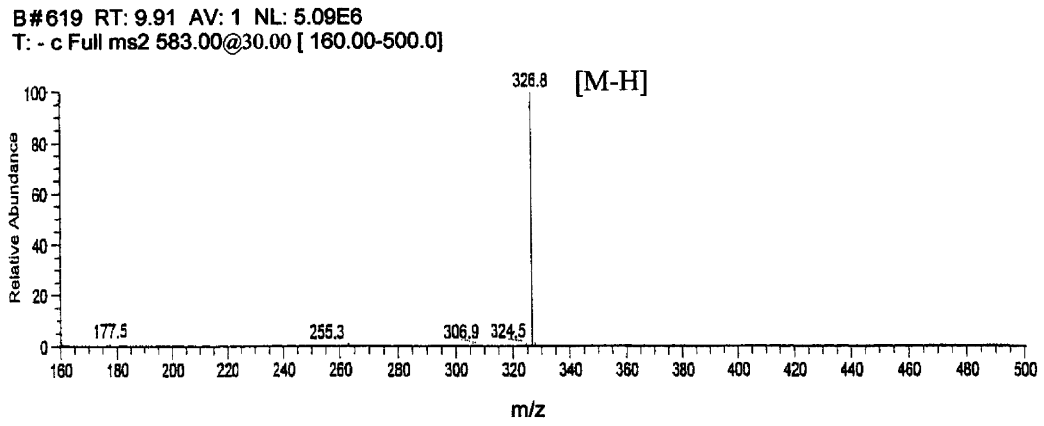

Figure 6. Nano-ESI-mass spectrum of antibiotic B.

rhamnose, saccharose, xylose, dextrin, esculin, gelatin, starch and testosterone and, the decarboxylation of sodium propionate and succinate, and the resistance to gentamicin, penicilline, streptomycin and rifampicin.

Furthermore, the whole-cell hydrolysate of *S. carneum* contained, in addition to madurose and mannose, the arabinose (Mertz and Yao, 1990), which was not detected in strain Sg 10.

The 16S rDNA sequence of strain Sg 10 was compared with those of other *Streptosporangium* species. The similarity level ranged from 96.3% to 97.8% with *S. carneum* DSM 44125^T the most closely related species. However, it is clear from phylogenetic analysis that strain Sg 10 did not cluster with neither *S. carneum* nor any of *Streptosporangium* species and represented a distinct phyletic line suggesting a new genomic species.

The data relative to chemical revelations and to infrared spectrum suggested that the main antibiotic B belongs to the group of the glycosylated aromatics, which are little produced by *Streptosporangium* species. The comparison of our antibiotic with 16 glycosylated aromatic antibiotics described by Umezawa (1988), Berdy et al. (1987) and Bycroft (1988) and secreted by different species of *Streptomyces* revealed that five of them have the same biological activity, but they are of yellow color and absorb between 300 and 400 nm.

Thirty three antibiotics are known to be produced by *Streptosporangium* as reported by Umezawa (1988), Berdy et al. (1987), Cooper et al. (1990), Hida et al. (1995) and Takizawa et al. (1995). The comparison of the antibiotic B with these 33 molecules showed that they are different by their nature (glycosylated aromatic), by their UV-VIS spectra and by their molecular weights.

Among antibiotics secreted by members of *Streptosporangium*, only sibiromycin and sinefungins are glycosylated aromatics. Sibiromycin, secreted by *S. sibiricum* (Hurley et al., 1979) differs from our antibiotics by its molecular weight ($M = 475$), its UV-VIS (λ max $= 230$ and 310 nm) and its infrared spectrum. *S. carneum* have been reported (Michel and Yao, 1994) to produce a lipoglycopeptide antibiotic complex with an activity against Gram-positive bacteria, a maximum UV absorption at 282 nm and high molecular weights (between 1676 and 1718).

From the present work, the results suggest that the strain Sg 10 is probably a novel species of *Streptosporangium*. The produced antimicrobial substances are glycosylated aromatics, different from those cited in the literature. The investigations of search on these molecules are in progress.

Acknowledgements

This work was gratefully supported by the "Comité d'Evaluation et de Prospective de Cooperation Inter-universitaire Franco-Algérienne" (CMEP No. 02 MDU 564), French Embassy in Algiers.

References

Athalye, M., Lacey, J., Goodfellow, M., 1981. Selective isolation and enumeration of actinomycetes using rifampicin. Appl. Bacteriol. 51, 289–297.

Athalye, M., Goodfellow, M., Lacey, J., White, R.P., 1985. Numerical classification of *Actinomadura* and *Nocardiopsis*. Int. J. Syst. Bacteriol. 35, 86–98.

Becker, B., Lechevalier, M.P., Gordon, R.E., Lechevalier, H.A., 1964. Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol. 12, 421–423.

Berdy, J., Aszalos, A., Mc Nitt, K.L., 1987. CRC Handbook of Antibiotic Compounds, vol. XIII. Microbial Metabolites, Parts 1–3. CRC Press, Boca Raton, FL, USA.

Betina, V., 1973. Bioautography in paper and thin layer chromatography and its scope in the antibiotic field. J. Chromatogr. 78, 41–51.

Bycroft, B.W., 1988. Dictionary of Antibiotics and Related Substances. Chapman & Hall, London.

Cooper, R., Das, P., Federbush, C., Mierzwa, R., Patel, M., Pramanik, B., Truumees, I., 1990. Characterization of peptidyl-nucleoside antifungal antibiotics from fermentation broth. J. Ind. Microbiol. 5, 1–8.

Davies, F.L., Williams, S.T., 1970. Studies on the ecology of actinomycetes in soil. I. The occurrence and distribution of actinomycetes in a pine forest soil. Soil Biol. Biochem. 2, 239–246.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Goodfellow, M., 1971. Numerical taxonomy of some nocardioform bacteria. J. Gen. Microbiol. 69, 33–90.

Gordon, R.E., Barnett, D.A., 1977. Resistance to rifampicin and lysozyme of strains of some species of *Mycobacterium* and *Nocardia* as a taxonomic tool. Int. J. Syst. Bacteriol. 27, 176–178.

Gordon, R.E., Barnett, D.A., Handarhan, J.E., Pang, C.H.-N., 1974. *Nocardia coeliaca*, *Nocardia autotrophica*, and the nocardin strains. Int. J. Syst. Bacteriol. 24, 54–63.

Hayakawa, M., Nonomura, H., 1987. Humic acid-vitamin agar, a new medium for the selective isolation of soil actinomycetes. J. Ferm. Technol. 65, 501–509.

Hida, T., Hayachi, K., Yukishige, K., Kawamura, N., Harada, S., 1995. Synthesis and biological activities of TAN-1511 analogues. J. Antibiot. 48, 589–603.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T., Williams, S.T., 1994. Bergey's Manual of Determinative Bacteriology. Williams & Wilkins, Baltimore, USA.

Hurley, L.H., Lasswell, W.L., Malhotra, R.K., Das, N.V., 1979. Pyrrolo[1,4]benzodiazepine antibiotics. Biosynthesis of the antitumor antibiotic sibiromycin by *Streptosporangium sibiricum*. Biochemistry 18, 4225–4229.

Jukes, T.H., Cantor, C.R., 1969. Evolution of protein molecules. In: Munro, H.N. (Ed.), Mammalian Protein Metabolism, vol. 3. Academic Press, New York, pp. 21–132.

Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: Integrated software for Molecular Evolutionary Genetic Analysis and sequence alignment. Briefings Bioinformatics 5, 150–163.

Lamari, L., Zitouni, A., Boudjella, H., Badji, B., Sabaou, N., Lebrihi, A., Lefebvre, G., Seguin, E., Tillequin, F., 2002. New dithiolopyrrolone antibiotics from *Saccharothrix* sp. SA 233. I. Taxonomy, fermentation, isolation and biological activities. J. Antibiot. 55, 696–701.

Lazzarini, A., Cavaletti, L., Toppo, G., Marinelli, F., 2000. Rare genera of actinomycetes as potential producers of new antibiotics. Ant. Leeuw. 78, 399–405.

Lechevalier, M.P., Lechevalier, H.A., 1970. Chemical composition as a criterion in the classification of aerobic actinomycetes. Int. J. Syst. Bacteriol. 20, 435–443.

Lechevalier, M.P., De Bievre, C., Lechevalier, H.A., 1977. Chemotaxonomy of aerobic actinomycetes: phospholipid composition. Biochem. Syst. Ecol. 5, 249–260.

Marchal, N., Bourdon, J.L., Richard, Cl., 1987. Les milieux de culture pour l'isolement et l'identification biochimique des bactéries. Doin, Paris.

Mertz, F.P., Yao, R.C., 1990. *Streptosporangium carneum* sp. nov. isolated from soil. Int. J. Syst. Bacteriol. 40, 247–253.

Michel, K.H., Yao, R.C., 1994. New *Streptosporangium carneum* cultures-used for producing antibiotic A 84575 cpds. used for treating bacterial infections in mammals. US Patent 5,286,648.

Minnikin, D.E., Patel, P.V., Alshamaony, L., Goodfellow, M., 1977. Polar lipid composition in the classification of *Nocardia* and related bacteria. Int. J. Syst. Bacteriol. 27, 104–117.

Nonomura, H., 1989. Genus *Streptosporangium* Couch. In: Williams, S.T., Sharpe, M.E., Holt, J.G. (Eds.), Bergey's Manual of Systematic Bacteriology, vol. 4. Williams & Wilkins Co., Baltimore, MD, pp. 2545–2551.

Nonomura, H., Ohara, Y., 1969. Distribution of actinomycetes in soil. VII. A culture method effective for both preferential isolation and enumeration of *Microbispora* and *Streptosporangium* strains in soil (Part 2). J. Ferm. Technol. 47, 701–709.

Okami, B., Hotta, A.K., 1988. Search and discovery of new antibiotics. In: Goodfellow, M., Williams, S.T., Mordarski, M. (Eds.), Actinomycetes in Biotechnology. Pergamon Press, Oxford, pp. 33–67.

Pfefferle, C., Theobald, U., Gürtler, H., Fielder, H.-P., 2000. Improved secondary metabolite production in the genus *Streptosporangium* by optimization of the fermentation conditions. J. Biotechnol. 80, 135–142.

Sabaou, N., Boudjella, H., Bennadji, A., Mostefaoui, A., Zitouni, A., Lamari, L., Bennadji, H., 1998. Les sols des oasis du Sahara algérien, source d'actinomycètes rares producteurs d'antibiotiques. Sécheresse 9, 147–153.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Shearer, M.C., 1997. Methods for the isolation of non streptomycete actinomycete. J. Ind. Microbiol. 28, 91–98.

Shirling, E.B., Gottlieb, D., 1966. Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16, 313–340.

Sierra, G., 1957. A simple method for the detection of lipolytic activity of microorganisms and some observations on the influence of the contact between cells and fatty substrates. J. Microbiol. Serol. 23, 15–22.

Takizawa, M., Hida, T., Horiguchi, T., Harada, S., Tanida, S., 1995. TAN-1511 A, B and C, microbial lipopeptides with G-CSF inducing activity. J. Antibiot. 48, 579–588.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighing, position specific gap penalties and weight matrix choice. Nucl. Acids Res. 22, 4673–4680.

Umezawa, H., 1988. Low molecular weight enzyme inhibitors and immunodifiers. In: Goodfellow, M., Williams, S.T., Mordarski, M. (Eds.), Actinomycetes in Biotechnology. Pergamon Press, Oxford, pp. 285–325.

Zitouni, A., Boudjella, H., Mathieu, F., Sabaou, N., Lebrihi, A., 2004a. Mutactimycin PR, a new anthracycline antibiotic from *Saccharothrix* sp. SA 103. I. Taxonomy, fermentation, isolation and biological activities. J. Antibiot. 57, 367–372.

Zitouni, A., Lamari, L., Boudjella, H., Badji, B., Sabaou, N., Gaouar, A., Mathieu, F., Lebrihi, A., Labeda, D.P., 2004b. *Saccharothrix algeriensis* sp. nov., isolated from Saharan soil. Int. J. Syst. Evol. Microbiol. 54, 1377–1381.