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Antimicrobial compounds produced by *Actinomadura* sp. AC104 isolated from an Algerian Saharan soil

B. Badji, A. Zitouni, F. Mathieu, A. Lebrihi, and N. Sabaou

Abstract: During a search for nonpolyenic antifungal antibiotics, an actinomycete designated AC104 was isolated from a Saharan soil sample by a dilution agar plating method using a chitin – vitamins B medium supplemented with rifampicin. Isolate AC104 presented the morphological and the chemical characteristics of the genus *Actinomadura*. On the basis of 76 physiological tests and 16S rDNA analysis, this isolate was determined to be quite different from the known species of *Actinomadura*. It is active against filamentous fungi and both Gram-positive and Gram-negative bacteria. The production of antibiotic substances was investigated using several culture media. The highest antimicrobial activities were obtained on ISP2 medium. The benzenic extract contained five bioactive spots detected on thin layer chromatography plates. Among these antibiotics, a complex called 104A, which showed the more interesting antifungal activity, was selected and purified by reverse-phase high-pressure liquid chromatography. This complex is composed of four compounds. Ultraviolet-visible, infrared, mass, and ¹H nuclear magnetic resonance spectroscopy studies showed that these molecules contain an aromatic ring substituted by aliphatic chains. These compounds differ from the known antibiotics produced by *Actinomadura* species.

Key words: *Actinomadura*, taxonomy, antimicrobial compounds, Saharan soil.

Résumé : Dans le cadre d'un programme de screening d'actinomycètes rares producteurs d'antifongiques non-polyéniques, nous avons isolé à partir d'un sol saharien, un actinomycète nommé AC104 sur milieu « chitine – vitamines B » additionné de rifampicine. Sur la base des caractéristiques morphologiques et chimiques, cet isolat a été rattaché au genre *Actinomadura*. L'étude physiologique (76 tests) et l'analyse de la séquence de l'ADNr 16S ont montré que l'isolat AC104 est assez différent des espèces connues d'*Actinomadura* et pourrait ainsi être original. Cet isolat présente une très forte activité antimicrobienne vis-à-vis de champignons filamenteux et de bactéries à Gram positif et à Gram négatif. La production d'antibiotiques a été testée sur plusieurs milieux de culture, le meilleur étant le milieu ISP2. Cinq zones actives ont été localisées par bioautographie. Parmi ces antibiotiques, le complexe nommé 104A qui a présenté la plus forte activité antifongique, a été purifié par HPLC en phase inverse. Ce complexe est composé par quatre molécules. Les études spectroscopiques, notamment l'UV-visible, l'infrarouge, la spectrométrie de masse et la RMN protonique, ont permis de constater que ces molécules contiennent un noyau aromatique para di-substitué par des chaînes aliphatiques. Ces composés diffèrent de ceux synthétisés par les espèces du genre *Actinomadura*.

Mots clés : *Actinomadura*, taxonomie, composés antimicrobiens, sols sahariens.

Introduction

The search for new and safe antimicrobial antibiotics with greater potency is a major challenge to the pharmaceutical

industry today, especially with the increase in opportunistic infections in the immunocompromised host. However, the search of antifungal compounds has been progressing slowly (Gupte et al. 2002). The reason for the slow progress compared to antibacterials is that, like mammalian cells, fungi are eukaryotic and have machinery for protein and nucleic acid synthesis similar to that of higher animals.

The history of new drug discovery shows that novel skeletons have come, in the majority of cases, from actinomycetes (Sanglier et al. 1996). However, the rate of discovery of new compounds has decreased since the ubiquitous species have been studied extensively. Several research studies are currently oriented towards isolation of rare actinomycetes from different ecosystems (Lazzarini et al. 2001). Therefore, selective isolation methods have been developed for taxa that are rarely isolated by conventional dilution plate methods (Hayakawa et al. 1995).

Among the rare actinomycetes, the genus *Actinomadura* is reported to be the most predominant (Miyadoh 1993) and

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the most important targets in screening programs for pharmacologically active compounds (Dairi et al. 1999; Zakharova et al. 2003).

The ecological distribution of actinomycetes from Saharan soils has been studied in Algeria and their biodiversity in those arid soils has been demonstrated (Sabaou et al. 1992, 1998).

In the present study, we describe the isolation and characterization of a new actinomycete isolate from an Algerian Saharan soil having antimicrobial activity against various fungi and bacteria. Different culture media were tested for optimal production of antimicrobial activities. The extraction and purification of four active molecules from the supernatant culture of this isolate and the preliminary characterization of these molecules are reported as well.

Materials and methods

Isolation of actinomycete

Actinomycete AC104 was isolated from a Saharan soil sample collected at a palm grove in Beni-Abbes, Algeria, by a dilution agar plating method using chitin – vitamins B agar (Hayakawa and Nonomura 1987) supplemented with rifampicin (5 µg/mL) and actidione (50 µg/mL).

Morphological and chemotaxonomic studies

The generic status of the isolate was determined from its morphological properties and the chemical composition of its cell components. The methods adopted by the International *Streptomyces* Project were used for morphological and cultural studies (Shirling and Gottlieb 1966). Detailed observation of mycelial morphologies was performed with the use of scanning electron microscopy (Stereoscan 260, Cambridge, England). Chemical analysis of the cell components was by the methods of Becker et al. (1964) and Lechevalier and Lechevalier (1970) for the determination of the isomeric form of diaminopimelic acid and the whole cell sugars pattern. Phospholipids and mycolic acids were analyzed using the procedures of Minnikin et al. (1977, 1980).

Physiological studies

Production of melanoid pigments was tested on peptone – yeast extract – iron agar (ISP6) and tyrosine agar (ISP7) (Shirling and Gottlieb 1966). Carbohydrate and organic acid assimilation as sole carbon source and utilization of testosterone were determined as described by Gordon et al. (1974) and Goodfellow et al. (1979). Decomposition of adenine, guanine, hypoxanthine, tyrosine, and xanthine were determined as previously described (Gordon et al. 1974), as were determinations of arbutin and aesculin decomposition, gelatin liquefaction, starch and Tween 80 hydrolysis, and nitrate reductase production (Marchal and Bourdon 1973). Lysozyme sensitivity was studied using the method of Gordon and Barnett (1977). Sensitivities to phenol, potassium tellurite, sodium azide, sodium chloride, and violet crystal were determined on glucose – yeast extract agar (Athalye et al. 1985). Growth at different temperatures and pH and in the presence of various antibiotics was tested in the same medium.

DNA preparation

Isolate AC104 was grown for 3–4 days at 28 °C with agitation in 500 mL flasks containing 100 mL of ISP2 medium. Biomass was harvested by centrifugation (7000g, 10 min) and washed twice with double-distilled water. Two hundred milligrams of mycelia was used for DNA extraction as follows: the sample was dispersed in 800 µL of the aqueous lysis solution (100 mmol/L Tris–HCl, pH 7; 20 mmol/L EDTA; 250 mmol/L NaCl; 2% *m/v* SDS; 1 mg/mL lysozyme), 5 µL of a 50 mg/mL RNase solution was added, and the suspension incubated at 37 °C for 60 min. Ten microlitres of a proteinase K solution (20 mg/mL) was added and the lysis solution was re-incubated at 65 °C for 30 min. The lysate was extracted with an equal volume of phenol and centrifuged (7000g, 10 min). The aqueous layer was re-extracted with phenol (50%–50% *v/v*), then by chloroform (50%–50% *v/v*). DNA was recovered from the aqueous phase by the addition of NaCl (150 mmol/L final concentration) and 2 volumes of cool 95% *v/v* ethanol prior to centrifugation. The precipitated DNA was cleaned with 50 µL of 70% *v/v* ethanol, centrifuged (7000g, 10 min), resuspended in 50 µL of TE buffer (10 mmol/L Tris–HCl, pH 7.4; 1 mmol/L EDTA, pH 8), and stored at –20 °C. The purity of DNA solutions was checked spectrophotometrically at 260 and 280 nm, and the quantities of DNA were measured at 260 nm.

16S rDNA sequence analysis

The 16S rDNA was amplified using the PCR method with *Taq* DNA polymerase and primers 27f (5'-AGAGTTTGA TCCTGGCTCAG-3') and 1492r (5'-GGTACCTTGTTACG ACTT-3'). The conditions for thermal cycling were as follows: denaturation of the target DNA at 98 °C for 3 min followed by 30 cycles at 94 °C for 1 min, primer annealing at 53 °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. PCR amplification was detected by agarose gel electrophoresis and was visualized by ultraviolet (UV) fluorescence after ethidium bromide staining.

The PCR product obtained was submitted to GenomExpress (Meylan, France) for sequence determination. The same primers as above and an automated sequencer were used for this purpose. The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST available at <http://www.ncbi.nih.gov/>.

Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using software included in MEGA version 3.0 (Kumar et al. 2004) package. The 16S rDNA sequence of AC104 was aligned using the CLUSTAL W program (Thompson et al. 1994) against corresponding nucleotide sequences of representatives of the genus *Actinomadura* 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 dataset.

Fig. 1. Scanning electron micrography of the aerial mycelium of *Actinomadura* sp. AC104 grown on ISP2 medium (yeast extract – malt extract – glucose) for 8 days at 28 °C.



Antimicrobial activity

Antifungal and antibacterial activities were tested by the bioassay method described subsequently against the pathogenic and nonpathogenic fungi (*Alternaria* sp., *Candida albicans*, *Fusarium oxysporum* f.sp. *albedinis*, *F. oxysporum* f.sp. *lini*, *Kluyveromyces lactis*, *Mucor ramannianus*, *Penicillium* sp., and *Saccharomyces cerevisiae*) and bacteria (*Agrobacterium tumefaciens*, *Bacillus coagulans*, *B. subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Staphylococcus aureus*). Isolate AC104 was grown on ISP2 medium for 8 days at 28 °C. Then, plates were covered by 10 mL of ISP2 containing 10^4 cfu/mL for fungi or 10^5 cfu/mL for bacteria. After 24–48 h of incubation at 28 °C, plates were examined for evidence of antimicrobial activity, represented by an inhibition zone of microbial growth around the actinomycete isolate.

Liquid media cultures

In the investigation of the culture medium that would enable optimal production of antimicrobial activities, the growth of the isolate was carried out in eight broth media: yeast extract – malt extract – glucose (ISP2), oatmeal (ISP3), inorganic salts – starch (ISP4), glycerol – asparagine (ISP5) (Shirling and Gottlieb 1966), meat extract – yeast extract – peptone – glucose (Bennett medium), yeast extract – glucose (GYEA; Athalye et al. 1985), starch – asparagine, and starch – casein (Kuster 1964). The pH of each medium was adjusted to 7.2 prior to autoclaving. Each flask containing 100 mL of medium was inoculated with 5% v/v of actinomycete culture grown in ISP2 medium for 72 h at 28 °C. The flasks were incubated at 28 °C for 10 days with shaking at 250 r/min. Five millilitre aliquots were collected regularly to estimate antibiotic activity by the agar well method against *Mucor ramannianus* and *Bacillus subtilis*. Wells (10 mm in diame-

ter) made in the ISP2 agar plates were filled with 0.2 mL of the supernatant samples.

Thermal and pH stability

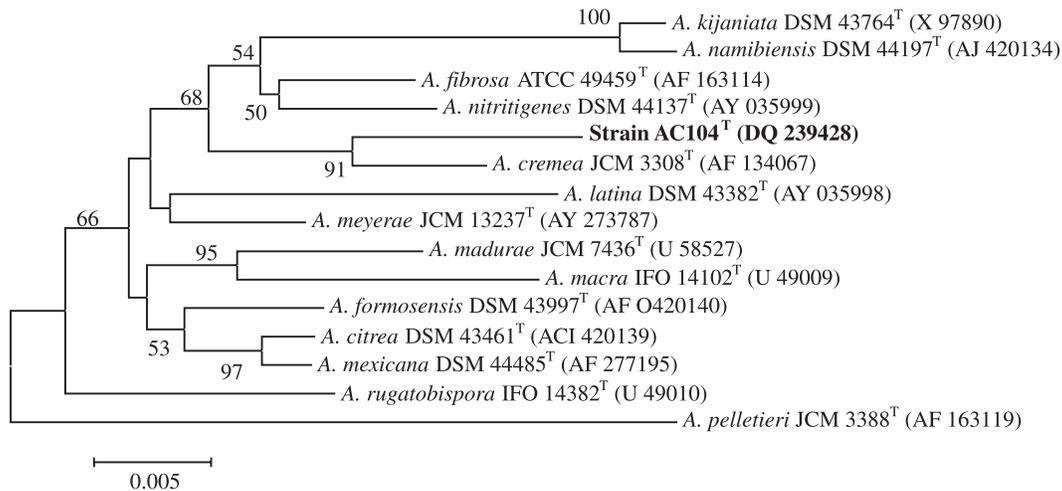
Antimicrobial activity of the crude extract was tested prior to final purification. Fractions of 5 mL of the crude extract were adjusted with NaOH or HCl to pH 3–11. After 24 h at 25 °C, the fractions were adjusted to pH 7. Other fractions of 5 mL (pH 7) were treated for 60 min at 60 °C, 45 min at 80 °C, and 30 min at 100 °C. The antimicrobial activities were tested by the agar well method against *M. ramannianus* and *B. subtilis*. Five millilitres of the crude extract (pH 7) obtained at room temperature (25 °C) was used as control treatment.

Fermentation, extraction, and purification of antibiotics

Isolate AC104 was precultured in a 250 mL flask containing 50 mL of liquid ISP2 medium and was incubated at 28 °C for 3 days. The preculture was transferred into a 2.5 L fermentor containing 1.5 L of the same medium. The fermentation was carried out at 28 °C for 7 days under aeration of 3 L/min at an agitation rate of 375 r/min. A range of extraction solvents was screened for effectiveness including *n*-hexane, benzene, dichloromethane, *n*-butanol, and ethyl acetate.

The 5 L of culture broth was centrifuged (3000g, 15 min) and the supernatant was extracted twice with the organic solvent, which gave the highest inhibition diameter. The extract was concentrated to dryness in vacuo. The crude extract was dissolved in methanol and applied to a thin-layer chromatography silica gel plate (Merck 60 F 254; Merck, Darmstadt, Germany), which was developed with an ethyl acetate – methanol mixture (100:15, v/v). The spots were detected by bioautography (Betina 1973) on silica gel plates seeded with *M. ramannianus* or *B. subtilis*. The active spots were also visualized under UV irradiation at 254 and 365 nm

Fig. 2. Phylogenetic tree based on 16S rDNA gene sequences showing relationships among *Actinomadura* sp. strain AC104 and the most-close type-strain species of *Actinomadura*. Numbers at nodes indicate percentages of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values above 50% are given. Bar: 0.005 substitutions per nucleotide position.



and revealed by chemical reagents. The fraction that showed more antifungal activity was purified by semi-preparative high-pressure liquid chromatography on reversed-phase material, under the following conditions: Uptisphere UP 15 WOD C18 column (300 mm × 7.8 mm Interchim); mobile phase, gradient elution system of methanol–water; flow rate, 2 mL/min; UV detection at 220 nm at room temperature.

Spectroscopic analysis

The spectroscopic studies were made with the pure antifungal compounds. UV-visible absorption spectra were recorded on a Lambda 20 spectrophotometer (Perkin-Elmer, Wellesley, Massachusetts, USA) and an infrared spectrum was obtained on a FT-IR 1760x spectrometer (Perkin-Elmer). Mass spectrum was recorded on LCQ ion-trap mass spectrometer (Finnigan MAT, San Jose, California, USA) equipped with a nanospray ion ESI source (negative ion mode). Proton magnetic resonance (^1H NMR) was recorded at 300 MHz using a Bruker AC-300 spectrometer (Bruker Instruments, Billerica, Massachusetts, USA).

Results and discussion

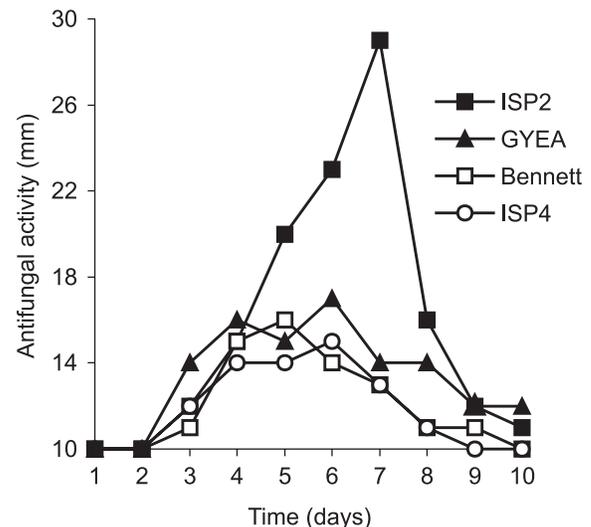
Cultural characteristics and identification

Isolate AC104 had a nonfragmented substrate mycelium. Scarce aerial mycelium were produced and these contained short chains of 3–10 spores having the form of hooks or irregular spirals with 1–2 coils. Electron microscopy observation revealed that the spores were rough walled (Fig. 1).

Colonies of isolate AC104 grew well on most organic media and were convex. The aerial mycelium was yellow and pink. The substrate mycelium was vivid yellow on ISP2 and GYEA media and pale beige on ISP3 and ISP4 media. Abundant yellow diffusible pigments were formed only on ISP2 and GYEA media.

Hydrolyzed whole cells contained the meso isomer of diaminopimelic acid but glycine was not found. Madurose (3-*O*-methyl-*D*-galactose) was the characteristic sugar component of the whole cell in addition to galactose, glucose, mannose, and ribose. Thus, isolate AC104 had a type III cell

Fig. 3. Effect of various culture media on the production of antifungal activity against *Mucor ramannianus* in 500 mL flasks. Values include the diameter of wells (10 mm). Media: ISP2, yeast extract – malt extract – glucose; GYEA, yeast extract – glucose; Bennett, meat extract – yeast extract – peptone – glucose; ISP4, inorganic salts – starch.



wall and a type B sugar pattern (Lechevalier and Lechevalier 1970). Nitrogenous phospholipids such as phosphatidylethanolamine and glucosamine-containing phospholipids were not detected. This pattern corresponded to phospholipid type PI (Lechevalier et al. 1977). Mycolic acids were not present. Based on the chemical and morphological characteristics, this isolate was identified as a member of the genus *Actinomadura* (Meyer 1989).

The physiological properties of AC104 are shown in Table 1. The isolate was able to hydrolyze a great number of compounds such as aesculin, arbutin, casein, gelatin, guanine, starch, testosterone, Tween 80, tyrosine, adonitol, arabinose, cellobiose, dextrin, fructose, galactose, glucose, lactose, mannitol, mannose, rhamnose, trehalose, xylose, sodium acetate,

Table 1. Physiological properties of *Actinomadura* sp. AC104.

Test	Result
Hydrolysis of:	
Adenine	-
Aesculin	+
Arbutin	+
Casein	+
Gelatin	+
Guanine	+
Hypoxanthine	-
Starch	+
Testosterone	+
Tween 80	+
Tyrosine	+
Xanthine	-
Utilization of glucides and derivatives	
Adonitol	+
Arabinose	+
Cellobiose	+
Dextrin	+
Dulcitol	-
Erythritol	-
Fructose	+
Galactose	+
Glucose	+
α -Methyl-D-glucoside	-
Glycerol	-
Inositol	-
Lactose	+
Maltose	-
Mannitol	+
Mannose	+
Melibiose	-
Melezitose	-
Raffinose	-
Rhamnose	+
Ribose	-
Sorbitol	-
Sucrose	-
Trehalose	+
D-Xylose	+
Production of melanoid pigments	-
Decarboxylation of sodium salts	
Acetate	+
Benzoate	-
Butyrate	+
Citrate	-
Oxalate	-
Propionate	-
Pyruvate	+
Succinate	+
Tartrate	-
Growth in presence of (g/L):	
Crystal violet (0.1)	-
NaCl (30)	+
NaCl (50)	-
Lysozyme (0.05)	-
Phenol (0.5)	-

Table 1 (concluded).

Test	Result
Potassium tellurite (0.1)	+
Sodium azide (0.05)	+
Sodium azide (0.1)	-
Nitrate reduction	+
Growth at:	
48 °C	+
pH 5	-
pH 9	+
Growth in the presence of antibiotics (mg/L)	
Ampicillin (75)	+
Benzyl-penicillin (25)	+
Chloramphenicol (25)	-
Cycloserine (25)	+
Erythromycin (10)	+
Gentamicin (5)	-
Kanamycin (25)	-
Novobiocin (10)	-
Oleandomycin (75)	+
Oxytetracycline (5)	-
Rifamycin (5)	+
Streptomycin (10)	-
Sulfanylamide (75)	+
Vancomycin (5)	-

Note: +, positive test; -, negative test.

Table 2. Antimicrobial activity of *Actinomadura* sp. AC104 against various microorganisms on ISP2 medium.

Bioassay organism	Inhibition zone (mm)
Gram-positive bacteria	
<i>Bacillus coagulans</i>	24
<i>Bacillus subtilis</i>	30
<i>Micrococcus luteus</i>	17
<i>Staphylococcus aureus</i>	29
Gram-negative bacteria	
<i>Agrobacterium tumefaciens</i>	00
<i>Escherichia coli</i>	20
<i>Klebsiella pneumoniae</i>	20
<i>Pseudomonas aeruginosa</i>	00
<i>Salmonella enterica</i>	30
Yeasts	
<i>Candida albicans</i>	08
<i>Kluyveromyces lactis</i>	04
<i>Saccharomyces cerevisiae</i>	04
Filamentous fungi	
<i>Alternaria</i> sp.	27
<i>Fusarium oxysporum</i> f.sp. <i>albedinis</i>	13
<i>F. oxysporum</i> f.sp. <i>lini</i>	16
<i>Mucor ramannianus</i>	24
<i>Penicillium</i> sp.	20

Note: ISP2 medium: yeast extract - malt extract - glucose.

Fig. 4. Effect of various culture media on production of antibacterial activity against *Bacillus subtilis* in 500 mL flasks. Values include the diameter of wells (10 mm). Media: ISP2, yeast extract – malt extract – glucose; GYEA, yeast extract – glucose; Bennett, meat extract – yeast extract – peptone – glucose; ISP4, inorganic salts – starch; ISP3, oatmeal; ISP5, glycerol–asparagine; SAS, starch–asparagine; SCA, starch–casein.

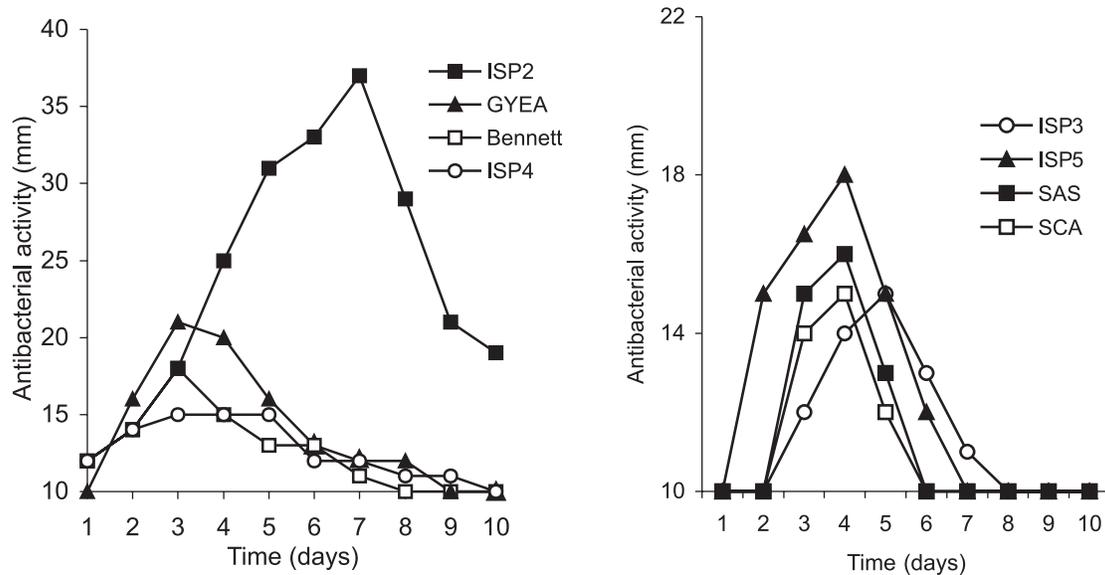
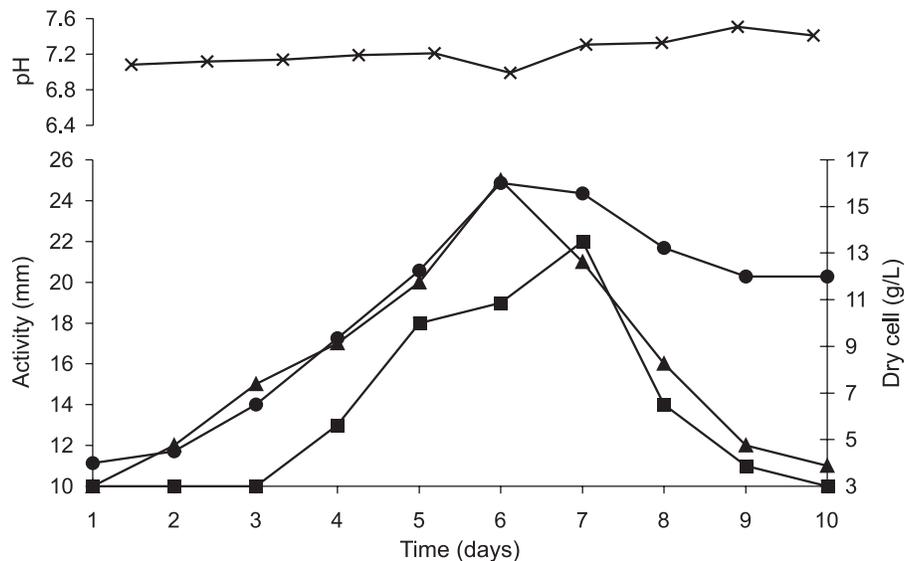


Fig. 5. Time course of antibiotic production by *Actinomadura* sp. AC104 in fermentor on ISP2 (yeast extract – malt extract – glucose) broth. ■, *Mucor ramannianus*; ▲, *Bacillus subtilis*. Values include the diameter of wells (10 mm); ●, dry cell (g/L); ×, pH.



sodium butyrate, sodium pyruvate, and sodium succinate. It was resistant to potassium tellurite (0.1 g/L), sodium azide (0.05 g/L), and to several antibiotics such as ampicillin (75 mg/L), benzylpenicillin (25 mg/L), cycloserine (25 mg/L), erythromycin (10 mg/L), oleandomycin (75 mg/L), rifamycin (5 mg/L), and sulfanilamide (75 mg/L). Permissive temperatures for growth ranged from 15 °C to 48 °C.

Phylogenetic analysis using the 16S rDNA sequence confirmed that isolate AC104 belonged to the genus *Actinomadura*. Its position in the 16 rDNA *Actinomadura*

tree is shown in Fig. 2. The similarity level was only 97.5% with *A. cremea* JCM 3308^T, the most closely related species. Much higher 16S rDNA similarities have been found between representatives of validly described *Actinomadura* species, such as the type strains of *A. citrea* and *A. mexicana* (99.3%), *A. madurae* and *A. macra* (99.2%), *A. kijaniata* and *A. namibiensis* (99.2%), and *A. fibrosa* and *A. macra* (98.3%).

Strain AC104 could also be distinguished from the *A. cremea* type strain (Preobrazenskaya et al. 1975) by phenotypic

Fig. 6. IR spectrum of 104A2 compound produced by *Actinomadura* sp. AC104.

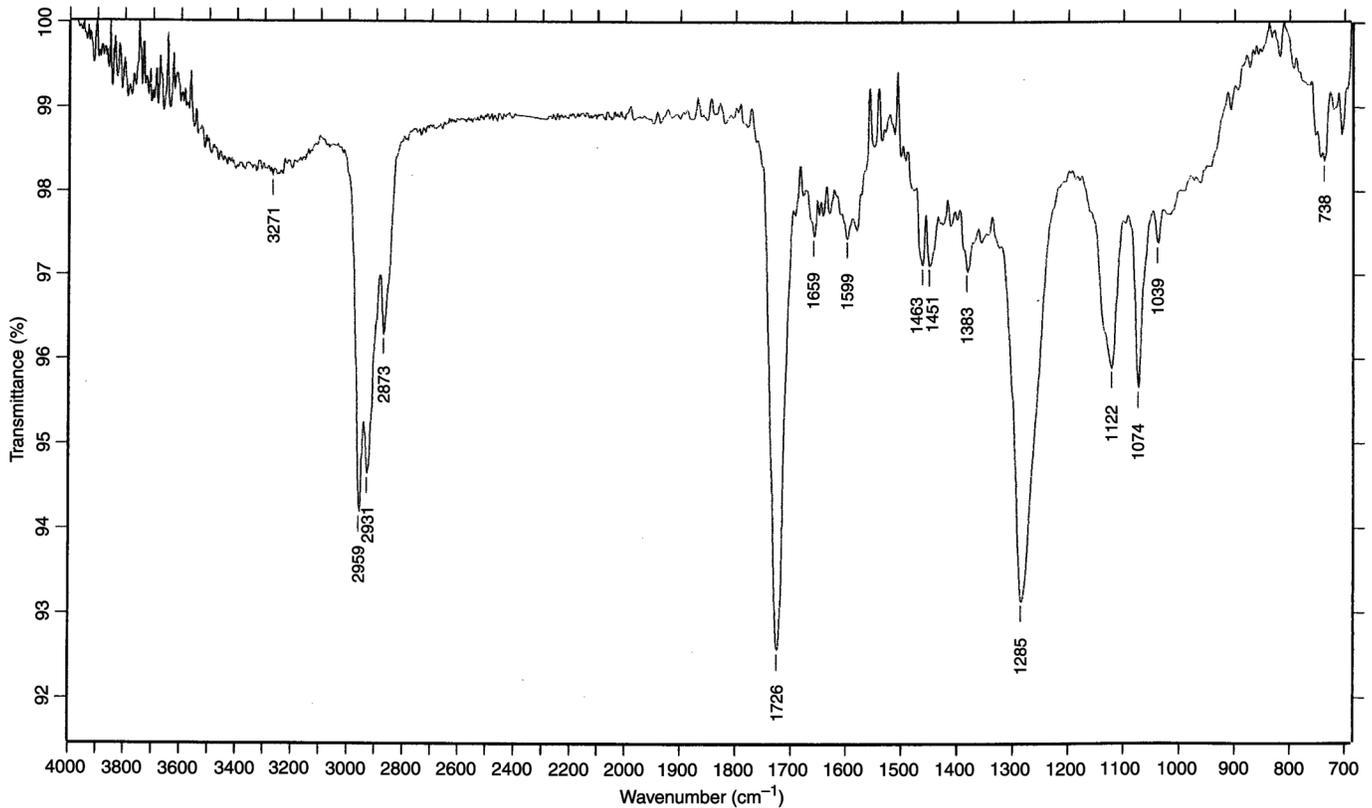


Fig. 7. Mass spectrum of 104A2 compound produced by *Actinomadura* sp. AC104.

104A2_021105152835 #9 RT: 0.10 AV: 1 NL: 1.53E7
 Γ: - c ms [100.00-1000.00]

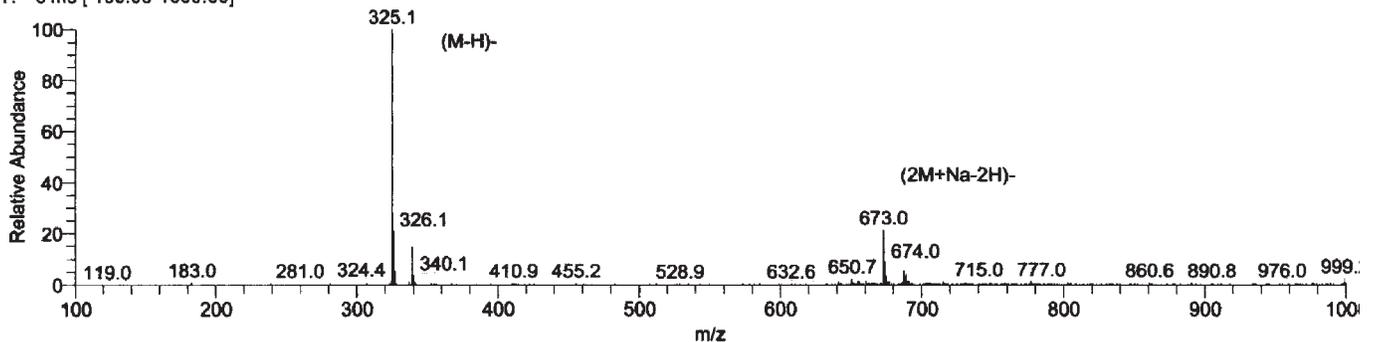


Table 3. Effect of temperature on the antimicrobial activities of the crude extract of *Actinomadura* sp. AC104.

	Activity (mm) at:			
	25 °C	60 °C (60 min)	80 °C (45 min)	100 °C (30 min)
Antibacterial ^a	31	20	15	–
Antifungal ^b	21	14	11	–

Note: The activity was measured by the well method. Values include the diameter of wells (10 mm). –, no activity.

^aActivity against *Bacillus subtilis*.

^bActivity against *Mucor ramannianus*.

properties such as the production of diffusible pigments and melanoid pigments; degradation of guanine, starch, cellobiose, lactose, maltose, mannose, sorbitol, sucrose, trehalose, tyrosine, sodium butyrate, sodium propionate, and sodium pyruvate; and the tolerance to potassium tellurite (0.1 g/L) and sodium azide (0.05 g/L).

These results strongly suggest that strain AC104 is a new species. The 16S rDNA sequence of strain AC104 has been deposited in GenBank under the accession No. DQ239428.

Antimicrobial activity

Isolate AC104 showed a broad-spectrum antimicrobial activity (Table 2). It was strongly active against fungi such as *Alternaria* sp., *M. ramannianus*, *Penicillium* sp., and against

Table 4. Effect of pH on the antimicrobial activities of the crude extract of *Actinomadura* sp. AC104.

	Activity (mm) at pH:									
	3	4	5	6	7	8	9	10	11	12
Antibacterial ^a	25	25	30	31	32	31	25	25	23	18
Antifungal ^b	18	18	21	22	23	20	16	–	–	–

Note: The activity was measured by the well method. Values include the diameter of wells (10 mm). –, no activity.

^aActivity against *Bacillus subtilis*.

^bActivity against *Mucor ramannianus*.

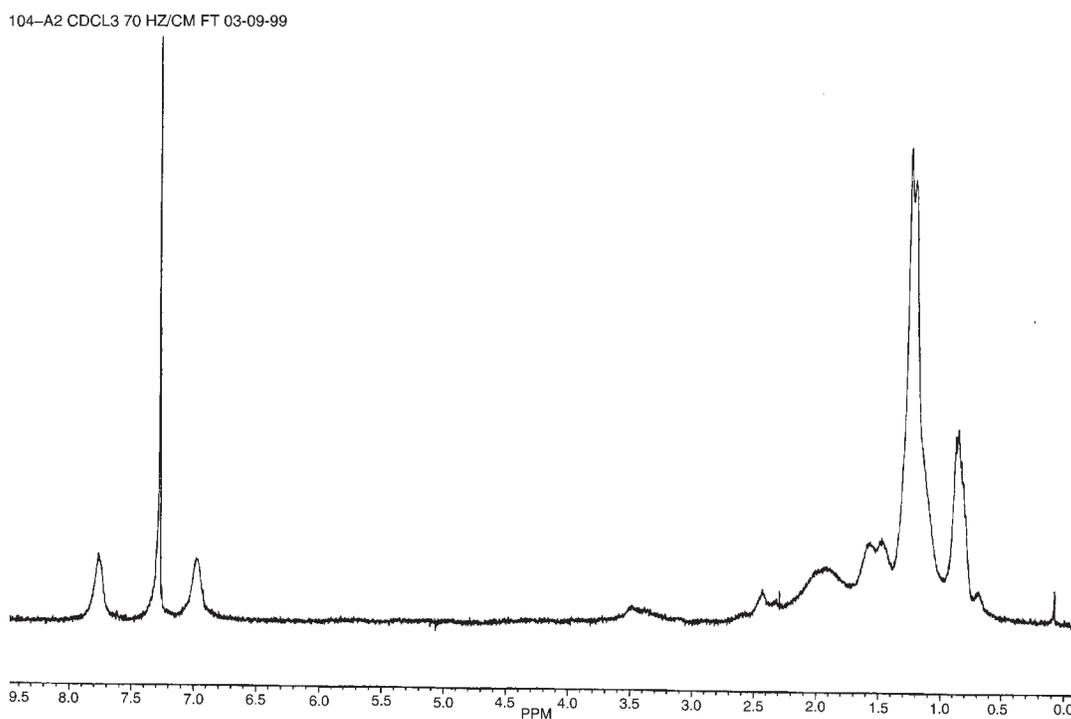
Table 5. Crude antibiotics produced by *Actinomadura* sp. AC104 on ISP2 medium and extracted with benzene.

Antibiotic	R_f^a	Color under UV (254 nm)	Activity (mm) ^b		
			<i>Bacillus subtilis</i>	<i>Saccharomyces cerevisiae</i>	<i>Mucor ramannianus</i>
104B	0.80	Green	17	–	32
104A	0.73	Olive green	19	13	36
104F	0.65	Vivid blue	18	–	15
104S	0.51	Pale blue	13	10	10
104C	0.17	Brown	16	–	14

Note: ISP2 medium, yeast extract – malt extract – glucose.

^aThe R_f of antibiotics was calculated after bioautography.

^bThe activity was measured by a paper disc method. Values include the diameter of disc (6 mm). – no activity.

Fig. 8. ¹H NMR of 104A2 compound produced by *Actinomadura* sp. AC104.

bacteria such as *B. subtilis*, *B. coagulans*, *Staphylococcus aureus*, *Salmonella enterica*, *Escherichia coli*, and *Klebsiella pneumoniae*. However, it was moderately active against *Fusarium oxysporum* f.sp. *albedinis* and *F. oxysporum lini* and weakly active against yeasts.

Fermentation studies

In liquid media, antifungal activities were observed only on ISP2, Bennett, GYEA, and ISP4 media (Fig. 3). However,

antibacterial activities were obtained on all media tested (Fig. 4). Among the media, which allowed a good antimicrobial production, ISP2 medium was found to be the best for both antifungal and antibacterial activities. It was thus chosen as the production medium for the antibiotic compounds. The best organic solvent for extraction was benzene (data not shown).

For the production of antibiotics, a fermentor was used. In ISP2 broth, antifungal activity was detected after 4 days of

culture and reached a maximum after 7 days (Fig. 5). The antibacterial activity began after 2 days of fermentation and reached a maximum at day 6. The pH varied between 6.8 and 7.8 during the incubation. The biomass increased during the first 5 days, reached the maximum at day 6, and decreased after day 7.

The antimicrobial activity of the filtrate was significantly affected by heating (Table 3) and extreme alkaline pH (Table 4).

Purification and characterization of the antimicrobial compounds

The butanolic extract showed no characteristic polyenic chromophore. On silica gel thin-layer chromatogram, the benzene extract was separated into five bioautographic spots with a different retention factor, which were all shown to be active against *B. subtilis* (Table 5). The spot, designated as 104A (retention factor = 0.73), was found to be significantly active against *M. ramannianus* and *Saccharomyces cerevisiae*. Chromogenic reactions were negative with FeCl₃, ninhydrine, naphtoresorcinol-H₂SO₄, anisaldehyde-H₂SO₄, vanilline-H₂SO₄, and formaldehyde-H₂SO₄. 104A appeared olive green at 254 nm and pale blue at 365 nm.

Spot 104A was selected and purified by high-pressure liquid chromatography. The active fractions were eluted with 80% methanol in water. 104A was composed by four components that were designated 104A1, 104A2, 104A3, and 104A4, which eluted at retention times of 10.0, 10.2, 11.0, and 11.2 min, respectively. These components were recovered separately and reinjected several times until complete purification was achieved. The pure antibiotics were whitish and presented a greasy appearance.

Spectroscopic studies were carried out for the four compounds. The UV-visible spectra in methanol (data not shown) for the four components exhibited the same maxima at 212 and 240 nm. The IR spectrum exhibited CH, CH₂, CH₃ at 2959/cm (strong), 2931/cm (strong), and 2873/cm (moderate), respectively, and C=O at 1726/cm (very strong), and C=C of aromatic ring at 1599/cm. Molecular weights were 312, 326, 340, and 354 for 104A1, 104A2, 104A3, and 104A4, respectively. ¹H NMR showed some common main signals in the 0.9–2.4 ppm regions indicating the presence of alkyl groupings. The signals at 6.9 ppm (doublet) and 7.7 ppm (doublet) indicate the presence of *p*-disubstituted benzenic ring. The difference among these four molecules was a value corresponding to CH₂ succession. Figs. 6–8 show the IR spectrum, mass spectrum, and ¹H NMR spectrum, respectively, of the major compound (104A2).

Our results suggest that all these antibiotics belong to the same chemical family containing a benzenic ring di-substituted by aliphatic chains. *Actinomadura* genus is reported to contain 57 species (Euzéby 2005), which produce antibiotics belonging to the polyether family (Nakamura and Isono 1983; Berdy et al. 1987), anthracyclin family (Berdy et al. 1987; Bycroft 1988), naphthoquinone family (Kakinuma et al. 1993), and macrolactam family (Hedge et al. 1992).

Our results further indicate that the four antibiotics produced by *Actinomadura* sp. AC104 differ from known antibiotics produced by *Actinomadura* species and also the bioactive molecules described in the *Dictionary of Antibiotics and Related Substances* (Bycroft 1988) and in the *Dictionary of Natural Products* (Buckingham 1997).

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