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TAXONOMIC STUDY AND PARTIAL CHARACTERIZATION OF ANTIMICROBIAL COMPOUNDS FROM A MODERATELY HALOPHILIC STRAIN OF THE GENUS *ACTINOALLOTEICHUS*

Farida Boudjelal¹; Abdelghani Zitouni¹; Florence Mathieu²; Ahmed Lebrihi²; Nasserine Sabaou^{1*}

¹Laboratoire de Recherche sur les Produits Bioactifs et la Valorisation de la Biomasse, Ecole Normale Supérieure de Kouba, BP 92, 16050 Kouba, Alger, Algérie; ² Université de Toulouse, Laboratoire de Génie Chimique UMR 5503 (CNRS/INPT/UPS), ENSAT/INP de Toulouse, 1 avenue de l'Agrobiopôle, Castanet-Tolosan Cedex, France.

ABSTRACT

A moderately halophilic actinomycete strain designated AH97 was isolated from a saline Saharan soil, and selected for its antimicrobial activities against bacteria and fungi. The AH97 strain was identified by morphological, chemotaxonomic and phylogenetic analyses to the genus *Actinoalloteichus*. Analysis of the 16S rDNA sequence of strain AH97 showed a similarity level ranging between 95.8% and 98.4% within *Actinoalloteichus* species, with *A. hymeniacidonis* the most closely related. The comparison of the physiological characteristics of AH97 with those of known species of *Actinoalloteichus* showed significant differences. Strain AH97 showed an antibacterial and antifungal activity against broad spectrum of microorganisms known to be human and plant pathogens. The bioactive compounds were extracted from the filtrate culture with *n*-butanol and purified using thin layer chromatography and high pressure liquid chromatography procedures. Two active products were isolated, one hydrophilic fraction (F1) and another hydrophobic (F2). Ultraviolet-visible, infrared, mass and ¹H and ¹³C nuclear magnetic resonance spectroscopy studies suggested that these molecules were the dioctyl phthalate (F2) and an aminoglycosidic compound (F1).

Key words: *Actinoalloteichus*, taxonomy, biological activities, structure, dioctyl phthalate

INTRODUCTION

Actinomycetes are the most significant group of microorganisms considered as an important producer of antibiotics and other important bioactive substances (4). The filamentous *Actinomycetales* species produces over 10000 bioactive compounds. The most frequent producers, the

Streptomyces species, produce 7600 compounds (74% of all *Actinomycetales*), while other actinomycetes represent 26%, altogether 2500 compounds (2). These organisms produce perhaps the most diverse and most unique, unprecedented, sometimes very complicated compounds exhibiting excellent antibacterial potency and usually low toxicity (2).

In the course of screening for new bioactive compounds,

*Corresponding Author. Mailing address: Laboratoire de Recherche sur les Produits Bioactifs et la Valorisation de la Biomasse, Ecole Normale Supérieure de Kouba, BP 92, 16050 Kouba, Alger. ; Tel.: (213) 21-29-75.11. Fax: (213) 21-28-20-67. ; E-mail: sabaou@yahoo.fr

several research studies are currently oriented towards isolation of rare actinomycetes from different ecosystems (13). The isolation of these microorganisms that produce bioactive compounds is of great interest in the development of new molecules to fight against many pathogens especially with the emergence of antibiotic multi-resistance pathogenic bacteria.

Halophilic and salt-tolerant actinomycetes are extremophilic microorganisms, which are used by several research programs around the world to isolate new natural compounds (4).

In fact, our laboratory takes a particular interest in extreme environment, like saline soils and their microflora. The ecological distribution of actinomycetes from Saharan soils has been studied in Algeria and their biodiversity in those arid soils has been demonstrated (23).

During a screening programme of the search for new antibiotics from non-*Streptomyces* genera, new molecules were obtained (12, 32, 33). As part of this program, we identified a new halophilic actinomycete (strain AH97) having interesting antimicrobial activity, which assigned to the genus *Actinoalloteichus*.

In present study, we describe the polyphasic taxonomic characterization of actinomycete strain AH97 isolated from an Algerian Saharan soil having antimicrobial activity against various bacteria and fungi. The production, extraction, purification and partial characterization of active molecules of this strain are reported as well.

MATERIALS AND METHODS

Origin of actinomycete

The strain AH97 was isolated from a Saharan soil collected in Tamanrasset (Hoggar, south of Algeria; latitude, 22° 49' N; longitude, 5° 28' E).

Microbial strains and culture conditions

The strain AH97 was grown at 30°C in tryptic soy broth

(TSB) (contents per liter: 17.0 g tryptose; 3.0 g soytone; 5.0 g NaCl; 15 g agar) fortified with 5% NaCl. The pH was adjusted to 7.3 prior to autoclaving.

For antibacterial and antifungal experiments, *Bacillus subtilis* ATCC 6633 and *Mucor ramannianus* NRRL1829 (sensitive strains) were used as the indicator microorganisms.

Taxonomic study

Cultural characteristics of strain AH97 were studied on yeast extract-malt extract agar (ISP2) (27), inorganic salts-starch agar (ISP4) (27), tryptic soy agar (TSA) (27) and complex medium agar (CMA) (26). All media were supplemented with 5% of NaCl. Colors of aerial and substrate mycelia were determined with the ISCC-NBS centroid color charts (National Bureau of Standards, USA 1964). The micromorphology of strain AH97 was examined under light microscopy for the mycelial organization and sporulation.

For the physiological study, fifty nine tests were considered, including the utilization of 20 carbohydrate compounds, the degradation of adenine, guanine, xanthine, hypoxanthine, milk casein, tyrosine, testosterone (6), tween 80, gelatine, starch, esculin and arbutin (20), the decarboxylation of nine organic acids (7) and the production of nitrate reductase (20). The strain AH97 was checked for salt requirement or tolerance by inoculation on TSA containing 0, 5, 7, 10, 15 and 20% of NaCl, as described by Kushner (11). The strain was also examined for its ability to grow on TSA (with 5% of NaCl), supplemented with 6 antibiotics or with lysozyme (8) and to grow at pH 5 and 9, and at 42°C.

For the chemotaxonomic analysis, biomass was obtained from culture grown on shake TSB medium supplemented with 5% of NaCl, and incubated at 30°C for 7 days. Diaminopimelic acid (DAP) isomer, whole-cell sugars pattern and phospholipids were analysed according to the methods of Becker *et al.* (1), Lechevalier and Lechevalier (14) and Minnikin *et al.* (21), respectively.

DNA preparation, PCR amplification and sequence analysis

DNA was prepared according to the method of Liu *et al.* (18). The strain AH97 was grown at 30°C for 7 days in a shake flask containing 100 mL of TSB medium supplemented with 5% of NaCl. PCR amplification of the 16S rDNA of the halophilic strain was performed using two primers: 27f (5'-AGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3'). The 16S rDNA was amplified by PCR using Invitrogen kit. The final volume of reaction mixture of 50 µL contained 1X PCR buffer (10 mmol of Tris-HCl, 50 mmol of KCl, pH 9.0 at 25°C), 1.5 mmol of MgCl₂, 200 mmol of each dNTP, 1 mmol 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: 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 ultraviolet fluorescence after ethidium bromide staining.

The sequencing reaction was performed by MilleGen Company (Toulouse, France). The same primers as before and an automated sequencer were used for this purpose. The obtained sequences were compared for similarity level with the reference species of bacteria contained in genomic database banks, using the 'NCBI Blast' available at <http://www.ncbi.nlm.nih.gov/>

Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using softwares included in MEGA version 3.0 package (10). The 16S rDNA sequence of the strain AH97 was aligned using the CLUSTAL W program (30) against corresponding nucleotide sequences of *Actinoalloteichus* reference species retrieved from GenBank. Evolutionary

distance matrices were generated as described by Jukes and Cantor (9) and a phylogenetic tree was inferred by the neighbor-joining method (24). Tree topologies were evaluated by bootstrap analysis (5) based on 1000 resamplings of the neighbor-joining dataset.

Antimicrobial activity of the strain AH97

The antimicrobial spectrum was determined by conventional agar method (agar cylinders method). The microorganisms used are listed in Table 2.

Strain AH97 was grown on TSA agar plates supplemented with 5% of NaCl for 7 days at 30°C, then calibrated agar cylinders (5 mm in diameter) were cut out and placed onto the agar surface (nutrient agar or Sabouraud agar, covered respectively by 10 mL of culture containing 10⁵ CFU mL⁻¹ for bacteria or 10⁴ CFU mL⁻¹ for fungi). A sterile cylinder of TSA supplemented with 5% of NaCl was used as control. The plates were incubated at 30°C for 24-48 h after a diffusion process for 4 h at 4°C. The diameters of any inhibition zones formed around the cylinder of actinomycete isolate were then measured.

Kinetic of growth and production of antimicrobial compounds

For the pre-culture, the strain AH97 was grown on TSA supplemented with 5% of NaCl, for 7 days at 30°C. The mycelium was scraped and inoculated into a 250 mL Erlenmeyer flask containing 50 mL of TSB supplemented with 5% of NaCl. Aliquots (5%, v/v) of this pre-culture were transferred into 500-mL Erlenmeyer flasks containing 100 mL of the same medium. The cultures were incubated on a rotary shaker (250 rpm) at 30°C for 7 days. The antibacterial and antifungal activities were assayed daily against *Bacillus subtilis* and *Mucor ramannianus* by the agar diffusion method (well technique). Each well of 10 mm in diameter was filled with 0.2 mL of the supernatant. The growth (dry weight of mycelium) was determined (22) and the pH was measured.

Production and extraction of antimicrobial compounds

For the production of active compounds, fermentations were carried out in 500 mL Erlenmeyer flasks containing 100 mL of TSB supplemented with 5% of NaCl (250 rpm, 30°C and 3 days).

The culture broth (10 L) was centrifuged at $5,000 \times g$ for 10 min to remove the biomass. The cell-free supernatant was extracted with an equal volume of *n*-butanol. The organic extract was concentrated to dryness, recuperated in methanol and bioassayed against *B. subtilis* and *M. ramannianus* by paper disk diffusion method.

Biological and chemical detection of antimicrobial compounds

Crude extract samples were subsequently subjected to thin-layer chromatography (TLC). Samples were spotted onto $20 \times 20\text{-cm}^2$ silica gel plates (Merck Art 5735, kieselgel 60F254), and then developed with *n*-butanol-acetic acid-water (B.A.W.) (3: 1: 1, v/v) as the solvent mixture. The active spot was detected by bioautography (3) on silica gel plates seeded with *M. ramannianus* or *B. subtilis*. Clear halos, due to growth inhibition of the microorganisms, indicated the location of antimicrobial compounds on the TLC plates, and the retention factor (Rf) values were recorded. The antimicrobial compounds were also detected under ultraviolet irradiations at 254 and 365 nm and by spraying with some chemical reagents such as naphthoresorcinol- H_2SO_4 and ninhydrin.

Purification of antimicrobial compounds

Concentrated extracts were purified on preparative TLC silica gel plates (Merck 60 F-254; 0.5 mm thick). The plates were developed with B.A.W. solvent system (3: 1: 1, v/v), and then air dried. The active band was visualized in ultraviolet light at 254 and 365 nm, scraped from the plates, eluted with methanol and concentrated using the Rotavapor. The antibiotic activity of the band against *B. subtilis* and *M. ramannianus* was measured by the paper disk method. Final purification of the antimicrobial compounds was carried out by a high-

performance liquid chromatography (HPLC) (Waters, Milford, MA, USA) system equipped in first step (in order to separate the hydrophilic fraction of the hydrophobic fraction) with a C18 column (Uptisphere UP5ODB, 250×7.8 mm; Interchim) and in second step with a C18 pyramid column (Nucleodur VP 250/10, 250×10 mm; Machery Nagel). The analysis conditions were as follows: a mobile phase with a continuous gradient (convex curve) solvent system from 0 to 100% methanol in water (30 min) to separate the active fractions with Uptisphere UP5ODB C18 column, then 80 to 100% methanol in water (30 min) to purify the hydrophobic active fraction with the same column, and 100% water (isocratic) (30 min) to purify the hydrophilic active fraction with C18 pyramid column; flow rate of 2 mL min^{-1} and UV detection at 220 nm. The final purification was achieved after the third re-injection in HPLC system.

Spectroscopic studies of antimicrobial compounds

The UV absorption spectra of the hydrophilic fraction F1 in distilled water and hydrophobic fraction F2 in methanol were determined with a Shimadzu UV 1605 spectrophotometer. Mass spectrum of fraction F1 was recorded on an ion-trap mass spectrometer (Finnigan MAT, San Jose, CA), equipped with a nanospray ion electro-spray ionisation (ESI) source (positif ion mode) and fraction F2 with electron impact by using Nermag R-10-10C spectrometer. Infrared (IR) spectra of the fractions (F1) and (F2) were obtained with a Perkin Elmer FT-IR 1760 spectrometer. ^1H and ^{13}C nuclear magnetic resonance spectra of the hydrophobic compound F2 (in MeOD) and hydrophilic compound F1 (in D_2O) were recorded on a Bruker AMX 400 spectrometer operating at 500 MHz.

RESULTS AND DISCUSSION

Taxonomy

The strain AH97 grew well on TSA and CMA (7 days at 30°C) supplemented with 5% of NaCl. The isolate had a

greenish blue to grayish blue (TSA) or pale yellowish (CMA) aerial mycelium. The substrate mycelium was light yellow on the two media used. Brown to black diffusible pigment was observed on TSA. No growth was observed on ISP2 and ISP4 media with 5% of NaCl. The aerial mycelium was branched and fragment into straight spore chains after 10 days of incubation at 30°C. Endospores, sclerotia, sporangia, synnemata and whirls were not observed.

Cell-wall hydrolysate of strain AH97 contained the DL-diaminopimelic acid isomer but glycine was not found. Galactose, mannose and glucose were the characteristic sugars of the whole-cell extract in addition to ribose. Thus, strain AH97 had cell-wall of type III and sugar pattern type C (14). The phospholipid profile was of type PII, characterized by the presence of phosphatidylethanolamine as major phospholipid (15). Based on the morphological and chemical characteristics, AH97 is classified in the genus *Actinoalloteichus* (19). The genus currently comprises only three species, *A. cyanogriseus* IFO14455^T (29), *A. spitiensis* DSM44848^T (28) and *A. hymeniacidonis* HPA177^T (29), and no one is halophilic.

Physiological characteristics

The physiological properties of strain AH97 are shown in Table 1. Optimal growth of strain was observed at 30°C at pH 7 and in presence of NaCl in range 5 to 7%. Strain was unable to grow at concentration 0 and 10% of NaCl, at pH 5 and at 42°C, and able to grow at pH 9.

The strain was able to hydrolyse a great number of compounds such as adenine, arbutin, casein, esculin, gelatine, starch, testosterone, tween 80, arabinose, cellobiose, fructose, galactose, glucose, mannitol, rhamnose, ribose, sucrose, trehalose, sodium propionate, sodium pyruvate and sodium succinate but did not utilize guanine, hypoxanthine, tyrosine, xanthine, adonitol, inositol, maltose, mannose, melibiose, raffinose, salicine, sorbitol, xylose, sodium acetate, sodium benzoate, sodium butyrate, sodium citrate, sodium oxalate,

sodium tartrate, alanine, proline and serine. It reduced nitrate and was resistant to novobiocin (5 µg mL⁻¹), streptomycin (10 UI) and vancomycin (30 µg mL⁻¹) and sensitive to chloramphenicol (30 µg mL⁻¹), erythromycin (15 UI) and lysozyme (0.005%).

Table 1. Physiological characters of strain AH97.

Tests	Strain AH 97	Tests	Strain AH97
Degradation of :			
Gelatine	+	Decarboxylation of sodium salts:	
Guanine	-	Acetate	-
Hypoxanthine	-	Benzoate	-
Starch	+	Butyrate	-
Testosterone	+	Citrate	-
Tween 80	+	Oxalate	-
Tyrosine	-	Propionate	+
Xanthine	-	Pyruvate	+
		Succinate	+
Carbon source utilization:		Tartrate	-
Adonitol	-		
Arabinose	+	Nitrate reductase	+
Cellobiose	+		
Fructose	+	Growth in the presence of NaCl (%):	
Galactose	+	0	-
Glucose	+	5	+
Inositol	-	7	+
Maltose	-	10	-
Mannitol	+		
Mannose	-	Sensitivity to:	
Melibiose	-	Lysozyme (0.005% w/v)	S
Raffinose	-	Chloramphenicol (30 µg mL ⁻¹)	S
Rhamnose	+	Erythromycin (15 UI)	S
Ribose	+	Novobiocin (5 µg mL ⁻¹)	R
Salicine	-	Streptomycin (10 UI)	R
Sorbitol	-	Vancomycine (30 µg mL ⁻¹)	R
Sucrose	+		
Trehalose	+	Growth at:	
Xylose	-	pH 5	-
		pH 7 and 9	+
Nitrogen source utilization:		42°C	-
Alanine	-		
Proline	-	Optimal growth temperature	30°C
Serine	-		

Note: +, positive test; -, negative test; S, sensibility; R, resistance.

Phylogenetic analysis

The 16S rDNA sequences of the strain AH97 (1248 nucleotides) was determined and deposited in GenBank under the accession number FJ379336. This strain was first analysed by a BLAST search and was aligned with those of *Actinoalloteichus* reference species available in the GenBank database, which confirmed its identification at the genus level. The position of strain AH97 in the 16S rDNA *Actinoalloteichus* tree is shown in Fig. 1

The neighbor-joining tree indicated that the novel strain fall into one distinct clade with the type strains of *Actinoalloteichus spitiensis* DSM44848^T (96.2%), *A.*

cyanogriseus IFO14455^T (95.8%), which were different from strain AH97 (similarity level < 97%), and *A. hymeniacidonis* HPA177^T (98.4%), the most closely related species. The results suggest that our strain represent a distinct phylogenetic line.

Strain AH97 differed from *A. hymeniacidonis* (31) by many characters. By contrast to our strain, *A. hymeniacidonis* was not halophilic, grew well on ISP2, had a brownish-purplish-grey aerial mycelium and a brownish-black substrate mycelium, was able to degrade maltose, mannose, sorbitol, xylose and sodium citrate, unable to degrade arabinose and sodium succinate and reduction of nitrate is negative.

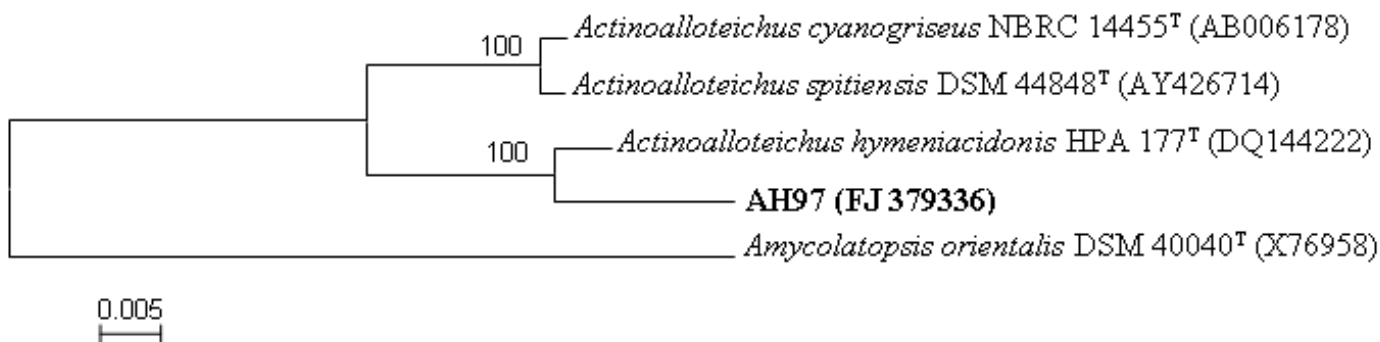


Figure 1. Neighbor-joining tree based on 16S rDNA sequences showing the relations between the moderately halophilic actinomycete isolate AH97 and type species of the genus *Actinoalloteichus*. The accession numbers of strain sequences are given in parentheses.

The numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1000 resampled datasets; only values over 50% are given. Bar: 0.005 nucleotide substitution per nucleotide position. *Amycolatopsis orientalis* is given as out group.

Antimicrobial activity on solid media

Strain AH97 showed a broad spectrum of antimicrobial activity (Table 2). It was strongly active against Gram-positive bacteria such as *Bacillus coagulans*, *B. subtilis* and *Staphylococcus aureus* and against fungi such as *Aspergillus alliaceus*, *A. carbonarius*, *A. flavus*, *A. ochraceus*, *Mucor ramannianus* and *Penicillium glabrum*. It was moderately

active against *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Salmonella enterica*, *Serratia marcescens*, *Kluyveromyces lactis*, *Saccharomyces cerevisiae* and *Aspergillus niger*. However, no activity was observed against *Pseudomonas aeruginosa*, *P. syringae*, *Agrobacterium tumefaciens*, *Candida albicans* and *Botrytis cinerea*.

Table 2. Antimicrobial activity of strain AH97 against various microorganisms.

Bioassay organisms	Diameter of inhibition zone (mm)
Gram-positive bacteria	
<i>Bacillus coagulans</i>	30
<i>Bacillus subtilis</i> ATCC 6633	30
<i>Enterococcus faecalis</i>	17
<i>Staphylococcus aureus</i> CIP 7625	25
Gram-negative bacteria	
<i>Klebsiella pneumoniae</i>	14
<i>Salmonella enterica</i>	18
<i>Serratia marcescens</i>	13
Yeasts	
<i>Kluyveromyces lactis</i>	13
<i>Saccharomyces cerevisiae</i>	18
Filamentous fungi	
<i>Aspergillus alliaceus</i>	24
<i>Aspergillus carbonarius</i> M333	23
<i>Aspergillus flavus</i>	20
<i>Aspergillus niger</i>	15
<i>Aspergillus ochraceus</i>	25
<i>Mucor ramannianus</i> NRRL 1829	30
<i>Penicillium glabrum</i>	20

* Activity was performed using agar cylinder method. The diameter of the cylinder agar does not include in diameters of inhibition zones. The strain AH97 is non-active against *Agrobacterium tumefaciens* n° 2410, *Pseudomonas aeruginosa*, *P. syringae* n° 2410, *Candida albicans* and *Botrytis cinerea*.

Growth kinetics and production of antimicrobial compounds

In TSB medium with 5% of NaCl, the biomass increased and reached the maximum at 3 days, followed by a stationary phase until 4 days, than decreased. The antifungal activity was detected at 1 day after inoculation and reached a maximum at 3 days. The antibacterial activity began and reached a maximum at 2 days of fermentation than decrease after this. The pH varied between 7.2 and 8.6 during the incubation (Fig. 2).

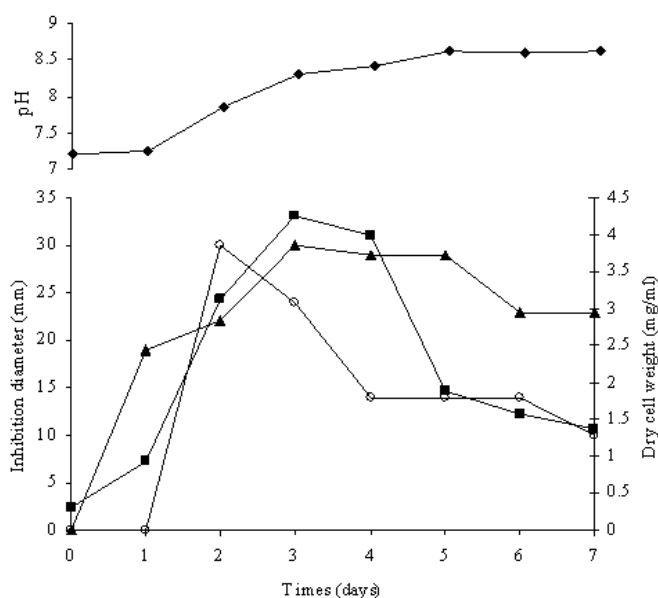


Figure 2. Time course of growth and antibiotic production in TSB medium with 5% of NaCl: (■) dry cell weight, (○) antibacterial activity against *Bacillus subtilis*, (▲) antifungal activity against *Mucor ramannianus*, (♦) pH.

Inhibition diameter values were measured without diameter of well (10 mm).

Extraction, purification and partial characterization of antimicrobial compounds

Various separation steps were applied to 10 L culture broth of strain AH97 grown in TSB medium supplemented with 5% of NaCl.

Antimicrobial compounds were extracted from broth culture with *n*-butanol. The active extract was concentrated to dryness using the Rotavapor, recuperated in methanol and analysed by TLC developed in B.A.W. solvent system. One active band, named fraction TM2, was detected by bioautography (at a R_f value of 0.44), which was active against *B. subtilis* and *M. ramannianus*.

The active band was scraped from the plates, eluted with methanol, concentrated and purified by HPLC in first with a C18 column (Uptisphere UP50DB). The chromatogram of the first round of HPLC injection showed two main peaks with

antibacterial and antifungal activity, which were designated F1 (retention time: RT, 5.00 min, hydrophilic and colorless fraction), and F2 (RT, 17.00 min, hydrophobic and colorless oil). Each fraction was collected separately and re-injected in HPLC system with a C18 pyramid column (Nucleodur) to purified F1 with 100% H₂O eluted phase and C18 column (Uptisphere UP5ODB) to purified F2 with 80 to 100% methanol in water eluted phase.

Fraction 1 (F1)

Chromogenic reactions with naphthoresorcinol-H₂SO₄ and ninhydrin were positive (presence of sugar and amine). The UV-visible spectrum in water (data not shown) of the pure product exhibited a maximum at 200 nm in water. The IR absorption spectrum (data not shown) exhibited the following bands: 3390 cm⁻¹ (O-H), 2933 and 673 cm⁻¹ (C-H), 1675 cm⁻¹

(C-OH) and 1543 cm⁻¹ (NH₂). The PM of the fraction F1 (data not shown) is 850 with fragments at m/z 685 and 522. The fragmentation of the majority peak in positive mode showed differences between the ions at m/z 162-164 that could correspond to C6 sugars.

The ¹H NMR (Figure 3) and ¹³C NMR spectra (data not shown) seem to indicate the presence of sugars, the vast majority of signals protons resonate between 3.3-4.2 ppm and the majority of carbon signals resonate between 50-80 ppm. These signals are characteristic of CH (OR), CH (OH) and CH₂ (OR) groups. There are also the signals around 100 ppm carbon (carbon bonded to two oxygens) and signals to 170-180 ppm identified to a group O=CH-O-CH₃. We also note a set of proton signals (6.6-6.7 ppm) and carbon (130-140 ppm) characteristic of C=C (alkene). These results suggest the presence of an aminoglycosidic compound.

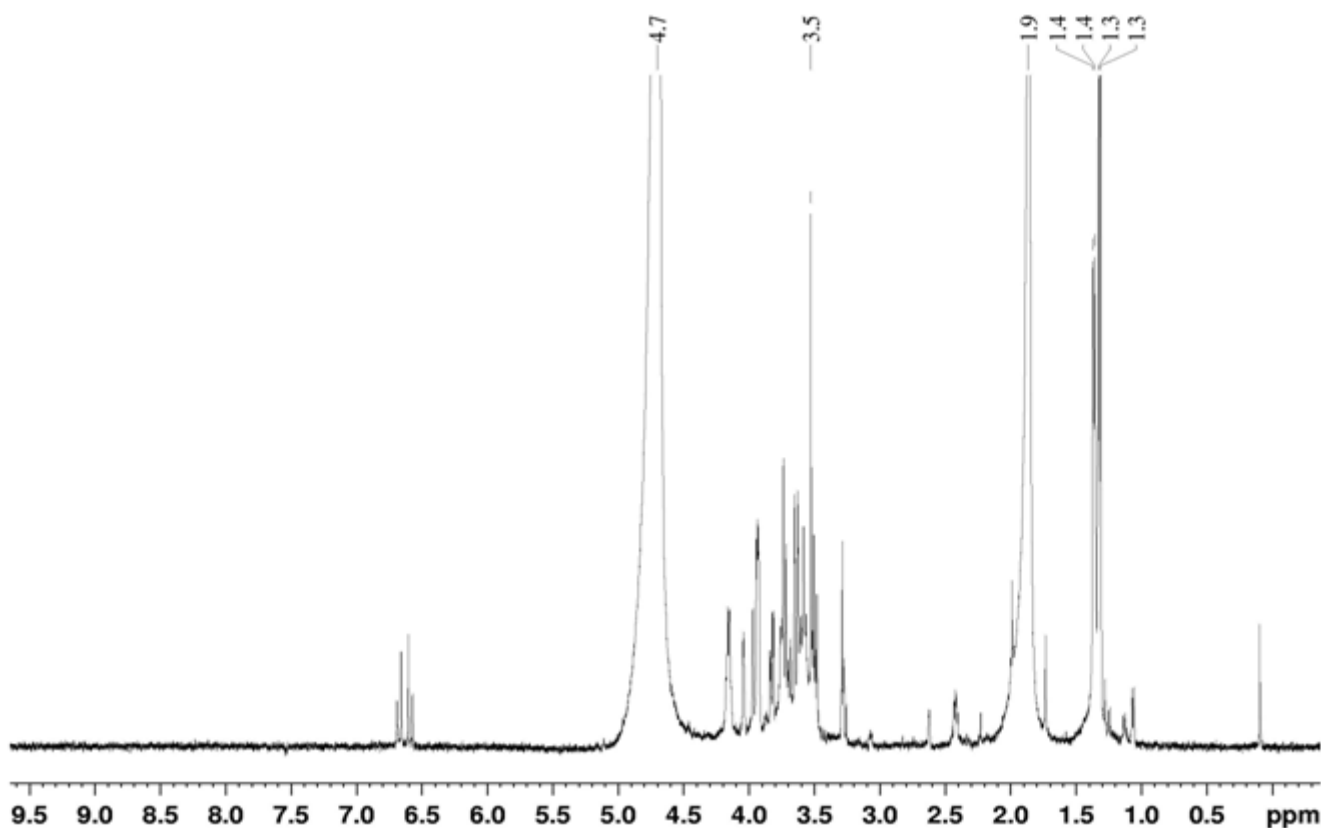


Figure 3. ¹H NMR of F1 compound produced by the strain AH97.

Fraction 2 (F2)

Chromogenic reactions with naphthoresorcinol-H₂SO₄ and ninhydrin were negative for F2 compound. UV-visible spectrum (data not shown) of the pure product F2 exhibited the maxima at 225 and 275 nm in methanol suggesting the presence of aromatic ring and the absence of polyenic structure.

Compound F2 was isolated as colorless oil, which had an IR absorption spectrum (data not shown): 3000-2800 cm⁻¹ (C-H), 1800-1600 cm⁻¹ (C-O), 1600-1580 cm⁻¹ (aromatic ring) and 1300-1200 cm⁻¹ (C-O). Molecular weight is 390.

¹H NMR spectrum of pure fraction F2 (Fig. 4) showed some common main signals regions indicating the presence of methyl group at 0.94-0.98 ppm, ethyl group at 1.37-1.47 ppm,

C-H at 1.70-1.71 ppm, O-CH₂ at 4.24-4.25 ppm and aromatic ring at 7.64-7.75 ppm. Based on the spectral data and search in literature, fraction F2 was identified as dioctyl phthalate (Fig. 5), which has the same UV-visible and infrared spectrum, the same molecular weight and the same ¹H NMR spectrum.

Phthalate compounds are petrochemicals used as plasticisers or solvents in a variety of industrial products. Nevertheless, many phthalate derivatives have been isolated from terrestrial and marine organisms including plants (16, 17), marine algae (25), and fungal and bacterial culture broth, particularly those belonging to the genus *Streptomyces*. Our experiments were repeated 3 times at differentes periods to confirm that the dioctyl phthalate produced by strain AH97 was a natural product and not an impurity.

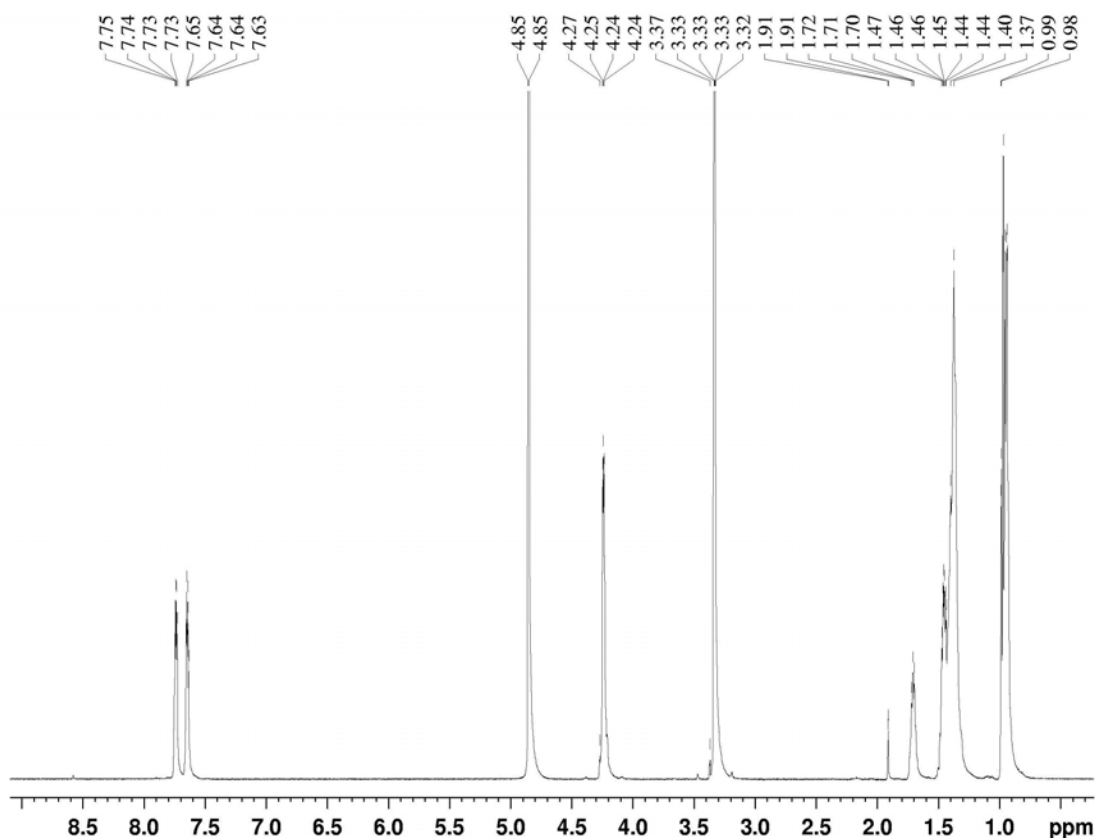


Figure 4. ¹H NMR of F2 compound produced by the strain AH97.

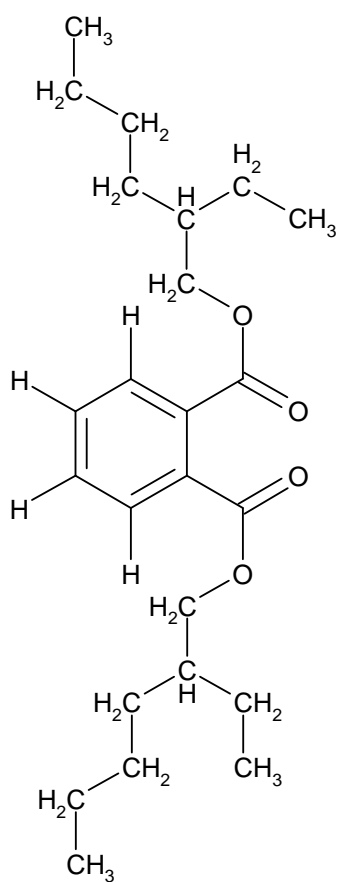


Figure 5. Chemical structure of the diethyl phthalate.

CONCLUSION

A moderately halophilic actinomycete strain (AH97), isolated from saline Saharan soil, was identified by a polyphasic study to the genus *Actinoalloteichus*. Analysis of the 16S rDNA sequence of this strain showed a similarity level at 98.4% with *A. hymeniacidonis* the most closely related which, in the other side, showed significant physiological characteristic differences. Strain AH97 showed an antibacterial and antifungal activity against broad spectrum of microorganisms.

Our results indicate that *Actinoalloteichus* sp. AH97 produced two bioactive compounds, F1 (hydrophilic) and F2 (hydrophobic). The comparison of their characteristics with those cited in the literature suggested that the product (F1) is an

aminoglycosidic compound and the product (F2) is the dioctyl phthalate. Further studies will be necessary to determine the structure of the F1 compound.

REFERENCES

1. 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. *J. Appl. Microbiol.*, 12: 421-423.
2. Berdy, J. (2005). Bioactive microbial metabolites. *J. Antibiot.*, 58: 1-26.
3. Betina, V. (1973). Bioautography in paper and thin layer chromatography and its scope in the antibiotic field. *J. Chromatogr.*, 78: 41-51.
4. Cai, Y. (2009). Classification and salt-tolerance of actinomycetes in the Qinghai lake water and lakeside saline soil. *J. Sustain. Develop.*, 2: 107-110.
5. Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution.*, 39: 783-791.
6. Goodfellow, M. (1971). Numerical taxonomy of some nocardioform bacteria. *J. Gen. Microbiol.*, 69: 33-90.
7. Gordon, R.E.; Barnett, D.A.; Handarhan, J.E.; Hor-Nay-Pang, C. (1974). *Nocardia coeliaca*, *Nocardia autotrophica* and the nocardin strains. *Int. J. Syst. Bacteriol.*, 24: 54-63.
8. 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.
9. 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.
10. Kumar, S.; Tamura, K.; Nei, M. (2004). MEGA3: Integrated software for molecular evolutionary genetic analysis and sequence alignment. *Brief. Bioinfo.*, 5: 150-163.
11. Kushner, D. J. (1993). Growth and nutrition of halophilic bacteria. In: Vreeland, L.I. (ed). *The biology of halophilic bacteria*, pp. 87-103.
12. Lamari, L.; Zitouni, A.; Boudjella, H.; Badji, B.; Sabaou, N.; Lebrhi, A.; Lefebvre, G.; Seguin, E. (2002). New dithiolopyrrolone antibiotics from *Saccharothrix* sp. Sa 233. I. Taxonomy, fermentation, isolation and biological activities. *J. Antibiot.*, 55: 696-701.
13. Lazzarini, A.; Cavaletti, L.; Toppo, G.; Marinelli, F. (2001). Rare genera of actinomycetes as potential producers of new antibiotics. *Ant. v. Leeuw.*, 78: 399-405.
14. Lechevalier, M.P.; Lechevalier, H.A. (1970). Composition of whole-cell hydrolysates as a criterion in the classification of aerobic actinomycetes. *Int. J. Syst. Bacteriol.*, 20: 435-443.

15. Lechevalier, M.P.; De Bièvre, C.; Lechevalier, H.A. (1977). Chemotaxonomy of aerobic actinomycetes: phospholipids composition. *Biochem. Syst. Ecol.*, 5: 249-260.
16. Lee, D.S., (2000). Dibutyl phthalate, a glucosidase inhibitor from *Streptomyces melanosporofaciens*. *J. Biosci. Bioeng.*, 89: 271-273.
17. Lee, K.H.; Kim, J.H.; Lim, D.S.; Kim C.S.; Kim C.H. (2000). Anti-leukaemic and antimutagenic effects of di(2-ethylhexyl) phthalate isolated from aloe linne. *J. pharm. Pharmacol.*, 52: 593-598.
18. Liu, D.; Coloe, S.; Baird, R.; Pedersen, J. (2000). Rapid mini-preparation of fungal DNA for PCR. *J. Clinical. Microbiol.*, 38: 471.
19. Liu, Z.; Zhang, Y.; Yan, X. (1984). A new genus of the order *Actinomycetales*. *Acta Microbiol. Sin.*, 24: 295-298.
20. Marchal, N.; Bourdon, J.L.; Richard, C.L. (1987). Milieux de culture pour l'isolement et l'identification biochimique des bactéries. Doin Press, Paris.
21. 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.
22. Pfefferle, C.; Theobald, U.; Gürtler, H.; Fiedler, H-P. (2000). Improved secondary metabolite production in the genus *Streptosporangium* by optimization of the fermentation conditions. *J. Biotechnol.*, 80: 135-142.
23. Sabaou, N.; Boudjella, H.; Bennadji, A.; Mostefaoui, A.; Zitouni, A.; Lamari, L.; Bennadji, H., Lefebvre G.; Germain, P. (1998). Les sols des oasis du Sahara algérien, source d'actinomycètes rares producteurs d'antibiotiques. *Sécheresse*, 9: 147-153.
24. Saitou, N.; Nei, M. (1987). The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, 4: 406-425.
25. Sastry, V.M.V.S.; Rao, G.R.K. (1995). Dioctyl phthalate and antibacterial compound from marine brown alga-*Sargassum wightii*. *J. Appl. Phycology*, 7: 185-186.
26. Sehgal, S.N.; Gibbons, N.E. (1960). Effect of some metal ions on the growth of *Halobacterium cutirubrum*. *Can. J. Microbiol.*, 6: 165-169.
27. Shirling, E.B.; Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *Int. Syst. Bacteriol.*, 16: 3313-3340.
28. Singla, A. K.; Mayilraj, S.; Kudo, T.; Krishnamurthi, S.; Prasad, G. S.; Vohra, R. M.(2005). *Actinoalloteichus spitiensis* sp. nov., a novel *Actinobacterium* isolated from a cold desert of the Indian Himalayas. *Int. J. Syst. Evol. Microbiol.*, 55: 2561-2564.
29. Tamura, T.; Zhiheng, L.; Yamei, Z.; Hanato, K. (2000). *Actinoalloteichus cyanogriseus* gen. nov., sp. nov. *Int. J. Syst. Evol. Microbiol.*, 50: 1035-1040.
30. 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.
31. Zhang, H.; Zheng, W.; Huang, J.; Luo, H.; Jin, Y.; Zhang, W.; Liu, Z.; Huang, Y. (2006). *Actinoalloteichus hymeniacidonis* sp. nov., an actinomycete isolated from the marine sponge *Hymeniacidon perleve*. *Int. J. Syst. Evol. Microbiol.*, 56: 2309.
32. Zitouni, A.; Boudjella, H.; Mathieu, F.; Sabaou, N.; Lebrihi, A. (2004a). Mutactimycin PR, a new anthracycline antibiotic from *Saccharothrix* sp. Sa 103. Taxonomy, fermentation, isolation and biological activities. *J. Antibiot.*, 57: 367-372.
33. Zitouni, A.; Lamari, L.; Boudjella, H.; Badji, B.; Sabaou, N.; Gaouar, A.; Mathieu, F.; Lebrihi, A. (2004b). *Saccharothrix algeriensis* sp. nov., isolated from Saharan soil. *Int. J. Syst. Evol. Microbiol.*, 54: 1377-1381.