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Bioactive potential of a new strain of *Streptomyces* sp. PP14 isolated from Canadian soil

Noureddine Bouras[1,2]*, Atika Meklat[1,3], Omrane Toumatia[1], Salim Mokrane[1], Michael D. Holtz[2,4], Stephen E. Strelkov[2] and Nasserdine Sabaou[1]

[1]Laboratoire de Biologie des Systèmes Microbiens (LBSM), Ecole Normale Supérieure de Kouba, B.P. 92, 16 050 Vieux-Kouba, Algiers, Algeria.
[2]Department of Agricultural, Food and Nutritional Science, 410 Agriculture/Forestry Centre, University of Alberta, Edmonton, Alberta, T6G 2P5, Canada.
[3]Département de Biologie, Faculté des sciences Agronomiques, Vétérinaires et Biologiques, Université Saâd Dahleb, Blida, Algeria.
[4]Field Crop Development Centre, Alberta Agriculture and Rural Development, 6000 C & E Trail, Lacombe, Alberta, T4L 1W1, Canada.

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A new actinomycete strain designated PP14 was isolated from a Canadian soil sample, by a dilution agar plating method using a chitin-vitamins B medium supplemented with different antibiotics as selective agents. This actinomycete produced antimicrobial substances and exhibited strong antifungal activity against mycotoxigenic fungi including *Aspergillus carbonarius* (M333) and *Penicillium expansum*, and also against phytopathogenic fungi such as *Fusarium oxysporum* f. sp. *lini* (Foln 3-5) and *F. culmorum*. In addition, the strain showed strong activity against the yeast *Saccharomyces cerevisiae* (ATCC 4226). On the other hand, the strain exhibited an interesting antibacterial activity against bacteria including multi-resistant *Klebsiella pneumoniae* (E40). The highest antimicrobial activities were obtained on ISP2 medium. The *n*-butanol extract contained three bioactive spots detected on thin layer chromatography (TLC) plates. Strain PP14 was identified by morphological, chemotaxonomic and phylogenetic analyses to the genus *Streptomyces*. The 16S rRNA gene sequence similarities showed that strain PP14 is closely associated with members of the *Streptomyces violaceoruber* species group (*S. violaceoruber*, *Streptomyces violaceolatus*, *Streptomyces tricolor*, *Streptomyces humiferus*, *Streptomyces coelescens* and *Streptomyces anthocyanicus*). Furthermore, the comparison of physiological characteristics of strain PP14 with the *S. violaceoruber* species group showed significant differences. Our results showed that strain PP14 represents a distinct phyletic line suggesting a new genomic species.

Key words: Actinomycetes, *Streptomyces*, antimicrobial compounds, extremophile microorganisms, multi-resistant bacteria, Canadian soil.

INTRODUCTION

Actinomycetes, filamentous bacteria that naturally inhabit soils, continue to serve as an important source of various kinds of antimicrobial molecules. It has been estimated that approximately two-thirds of natural antibiotics have been isolated from these mycelial bacteria, and about 75% are produced by members of the genus

*Corresponding author. E-mail: noureddine_bouras@yahoo.fr. Tel: + 213 29 75 11. Fax: + 213 28 20 67.

Streptomyces (Buckingham, 1997; Newman et al., 2003). The search for new antibiotics continues to be of greatest importance in screening programs around the world because of the increase of resistant pathogenic bacteria and toxicity of some used antibiotics (Berdy, 1989). As with every screening program, the probability of finding bioactive molecules with desired properties depends on the number and diversity of strains isolated and screened (Mazza et al. 2003). Moreover, one of the strategies for enhancing the likelihood of obtaining particular strains and secondary bioactive metabolites is to analyze uncommon ecosystems which exist under extreme conditions and to consider geographical regions that have been poorly studied in the past.

Canadian soils are exposed to an extreme climate and could represent particular ecosystems. Extreme environments have been found by many investigators to be inhabited by microorganisms which are especially adapted to these ecological niches (Sabaou et al., 1998). Many of these organisms may represent new taxa and thus can provide a valuable resource for use in future biotechnological processes (Aguilar, 1996; Bouras et al., 2006; Boubetra et al., 2013; Meklat et al., 2013). As in any isolation program, the strains have to be identified. Physiological characteristics together with morphological and chemical features are sufficient for accurate identification of many genera and species, but may fail to correctly identify to the species level, strains that have the similar phenotypic characteristics. The introduction of molecular tools has markedly improved the identification techniques. The 16S rRNA sequence-based phylogenetic analysis is widely used to determine taxonomic positions of many microorganisms. Assisted by the polymerase chain reaction (PCR) technique, the 16S rRNA sequence of investigated microorganisms can be easily obtained and compared with the sequences available in public databases.

As part of our program to study the extremophilic actinobacteria, we described the isolation and characterization of a new bacterial strain from Canadian soil, and its identification by conventional and molecular methods. In addition, we reported on production and extraction of three compounds which shows antimicrobial activities against mycotoxigenic and phytopathogenic fungi, Gram-positive and negative bacteria and yeasts.

MATERIALS AND METHODS

Soil sample and bacterial strain isolation

A canola rhizospheric soil sample (5 to 10 cm of depth) was collected aseptically from an Edmonton field, Alberta province, western Canada. The sample was placed in sterile polyethylene bag, closed tightly and stored at 4°C until analysis. A novel strain, PP14, was selectively isolated by serial dilution agar plating method. One gram of soil was dried at 110°C during 30 min (to increase a chance to isolate Gram-positive bacteria such as actinomycetes, by destroying Gram-negative bacteria which are not able to sporulate), followed by phenol treatment (1.5%) during 15 min (to select actinomycete strains which are resistant to chemicals). The sample was aseptically added to 9 ml sterile deionized water. The suspension was vortexed and diluted. Aliquots (0.2 ml) of each dilution were spread on the surface of chitin-B vitamins agar medium (Hayakawa and Nonomura, 1987) consisting of (per liter of deionized water): 2 g of chitin, 0.35 g K_2HPO_4, 0.15 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.3 g NaCl, 0.02 g $CaCO_3$, 10 mg $FeSO_4.7H_2O$, 1 mg $ZnSO_4.7H_2O$, 1 mg $MnCl_2.4H_2O$ 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, capantothenate, p-aminobenzoic acid (0.5 mg/l for each) and biotin (0.25 mg/l) were added to the autoclaved medium. The antifungal cycloheximide (50 mg/l) and nystatin (30 mg/l) were used to inhibit development of invasive fungi. The following antibacterials were also added to the isolation medium as selective agents: rifampicin (5 mg/l), erythromycin, streptomycin, novobiocin (10 mg/l), chloramphenicol, kanamycin and penicillin (25 mg/l). These antibiotics were chosen on the basis of good results obtained previously during our work on the selective isolation of extremophile actinomycetes (Sabaou et al., 1998; Zitouni et al., 2004a, 2005; Badji et al., 2011). The plates were incubated at 30°C for 21 days. After isolation, strain PP14 was stored on agar slant of ISP2 medium (1% malt extract, 0.4% yeast extract and 0.4% glucose) at 4°C.

Morphological and cultural characteristics

Morphological and cultural characteristics of strain PP14 were determined by naked-eye examination according to the method described by Shirling and Gottlieb (1966). Cultural characteristics were studied on various International *Streptomyces* Project (ISP) media: yeast extract-malt extract agar (ISP2), oatmeal agar (ISP3), inorganic salts-starch agar (ISP4), nutrient agar and Bennett media at 30°C for 21 days. Colors of aerial and substrate mycelia were determined with the ISCC-NBS centroid color charts (Kelly and Judd 1976). Furthermore, the micromorphology and sporulation were observed on the same media by optical microscopy.

Chemical analysis of cellular constituents

Strain PP14 was grown on ISP2 broth for five days at 30°C. Mycelia were harvested by centrifugation, at 7000 *g* for 10 min, and washed by deionized water. These were then used for chemical (chemotaxonomic) analyses of diaminopimelic acid (DAP) isomers and glycin according to the procedure of Becker et al. (1964), and analysis of whole-cell sugars according to the procedure of Lechevalier and Lechevalier (1970). Phospholipids were extracted and analyzed according to the procedure of Minnikin et al. (1977).

Physiological characteristics

Carbohydrate assimilation as sole carbon source was determined as described by Goodfellow et al. (1971). The production of melanoid pigments was tested on peptone-yeast extract-iron agar (ISP6) and tyrosine agar (ISP7) media (Shirling and Gottlieb, 1966).

DNA preparation, PCR amplification and 16S rRNA sequencing

DNA extraction was realized according to the method of Liu et al. (2000). The strain PP14 was grown at 30°C for 7 days on a rotary shaker (250 rpm) in a 500 ml flask containing 100 ml of ISP2 medium. PCR amplification of the 16S rRNA of the strain was performed using two primers: 27f (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1492r (5´GGTTACCTTGTTACGACTT-3´). The PCR amplification was conducted using a GeneAmp PCR System 9700 Thermocycler (Applied Biosystems, Foster City, CA) in a 25-µl volume containing 1 U of Platinum *Taq* DNA polymerase, 2 mM $MgCl_2$, 1 × PCR buffer

(20 mM Tris-HCl pH 8.4 at 25°C, 50 mM KCl) (Invitrogen Life Technologies, Carlsbad, CA), 0.4 µM each forward and backward primer, 0.2 mM each dNTP, and and 1 µl (500 ng) of the purified DNA.

The amplification cycle consisted of 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. PCR product (10 µl of reaction mix per loading well) was resolved on ethidium bromide- stained 1% agarose gel electrophoresis in 1× Tris acetate-EDTA buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.3), and was visualized under ultraviolet (UV) fluorescence using a Syngene BioImaging System (Synoptics Inc., Frederick, MD). The PCR products obtained were submitted to Department of Medical Genetics at the University of Alberta (Edmonton, Alberta, Canada). The same primers as above and an automated sequencer were used for this purpose. The obtained sequence was compared for similarity level with available sequences in the public databases such as GenBank (using NCBI Blast), as well as with EzTaxon, a web-based tool for the identification of prokaryotes based on 16S rRNA gene sequences from type strains (Chun et al., 2007).

Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using software included in MEGA version 4.0 (Tamura et al., 2007) package. The 16S rRNA sequence of strain PP14 was aligned using the CLUSTAL W program (Thompson et al., 1994) against corresponding nucleotide sequences of representatives of the genus *Streptomyces*. Evolutionary distance matrices were generated as described by Jukes and Cantor (1969) and a phylogenetic tree was inferred by the neighbour-joining method (Saitou and Nei, 1987). Tree topologies were evaluated by bootstrap analysis (Felsenstein 1985) based on 1000 re-samplings of the neighbour-joining dataset.

Antimicrobial assay

The first screening of antimicrobial activity of the isolated actinomycete was determined by the streak assay method against several microorganisms. The strain was first cultivated in a straight line on ISP2 (containing 12 g/l agar) plates, of 90 mm diameter, and incubated for 10 days at 30°C. After the growth of the strain, the target microorganisms were seeded in streaks perpendicular to those of actinomycetes cultivation. The antimicrobial activity was evaluated, by measuring the distance of inhibition between target microorganism and actinomycetes colony margins, after incubation at 30°C for 24 h for bacteria and yeasts, and 48 h for filamentous fungi.

The target microorganisms used were several mycotoxigenic and phytopathogenic fungi (*Aspergillus carbonarius* M333, *Aspergillus niger*, *Aspergillus flavus*, *Penicillium expansum*, *Penicillium glabrum*, *Fusarium moniliforme*, *Fusarium culmorum*, *Fusarium oxysporum* f. sp. *lini*, *Fusarium graminearum*, *Fusarium solani* and *Mucor ramannianus* NRLL 1829), two yeasts (*Candida albicans* IPA 200 and *Saccharomyces cerevisiae* ATCC 4226), three Gram-positive bacteria (*Bacillus subtilis* ATCC 6633, *Micrococcus luteus* ATCC 9314 and *Listeria monocytogenes* Institut Pasteur), and several Gram-negative bacteria (*Escherichia coli* ATCC 10536, *E. coli* ATCC 25925, *E. coli* K12, *Agrobacterium tumefasciens* # 2410 LB and *Klebsiella pneumoniae* CIP 82.91). In addition, a total of eleven bacteria responsible for nosocomial infections and resistant to multiple antibiotics were included in this study: four strains of *K. pneumoniae* (Kz8, Kz19, K44 and E40), two *Enterobacter cloacae* (E10 and E13), two strains of *E. coli* (E52 and E195), one *Salmonella enterica* (E32), one *Pseudomonas aeruginosa* (CIP A22) and one *Acinetobacter baumanii* (E16). All these 11 multi-resistant bacteria were isolated from sick patients in hospitals of Algeria (Touati et al., 2006; Messai et al., 2008; Aouiche et al., 2011). The target microorganisms without accession number are from our laboratory collection. The measurements of distance of inhibition represent the average of two experiments.

Kinetics of antimicrobial products of strain PP14

In the investigation of the culture medium that would enable optimal production of antimicrobial activities, the cultivation of the strain was carried out in two liquid culture media: ISP2 (composition mentioned above) and Bennett (0.1% meat extract, 0.1% yeast extract, 0.2% peptone and 1% glucose). The pH of each medium was adjusted to 7.2 prior to autoclaving. Each flask containing 100 ml of each medium (ISP2 and Bennett) was inoculated with 5% v/v of strain pre-culture grown in ISP2 medium for 72 h at 30°C under agitation of 250 rpm. The inoculated flasks were incubated at 30°C for 10 days with shaking at 250 rpm. The aliquots were collected each day by centrifuging (Microlitre Centrifuges, Heraeus Instruments, Biofuge) 4 ml of homogenized culture broth in Eppendorf tubes for 10 min at 16000 ×*g*. The antimicrobial activities were daily assayed for a duration of 10 days, against ten target microorganisms (which represent the most sensitive microorganisms): five filamentous fungi (*A. carbonarius* M333, *A. niger*, *P. expansum*, *P. glabrum* and *M. ramannianus* NRRL 1829), one yeast (*S. cerevisiae* ATCC 4226) and four bacteria (*B. subtilis* ATCC 6633, *M. luteus* ATCC 9314, *E. cloacae* E10 and E13) by the conventional agar diffusion method (wells technique). Wells (10 mm in diameter diameter) made in the ISP2 agar plates (containing 10 g/l of agar) were filled with 200 µl of the cell-free supernatant samples. Accordingly, the amounts of the active compounds present in the culture filtrate were estimated by measuring the diameters of inhibition.

Production, extraction and detection of antimicrobial compounds

For the production of antimicrobial compounds, fermentations were carried out in 500-ml Erlenmeyer flasks each containing 100 ml of the optimal medium (ISP2). The inoculated cultures were incubated at 30°C under constant agitation of 250 rpm. The extraction of active compounds took place on the day of optimal production (after 7 days of fermentation). The culture broth was centrifuged for 20 min at 8000 ×*g* to remove the mycelium. The cell-free supernatant was divided into 3 equal volumes of 80 ml, and then each was extracted with an equal volume of organic solvent. A range of extraction solvents was screened for effectiveness, including ethyl acetate, dichloromethane and *n*-butanol. Each organic extract was evaporated to dryness under vacuum on a Rotavapor (Laborota 4000). The resulting dry extracts were recuperated in 1 ml of methanol and subjected to biological assay (paper disk of 6 mm in diameter, Institut Pasteur) against *B. subtilis* ATCC 6633 (30 µl per disk) and *M. ramannianus* NRRL 1829 (40 µl per disk). Inhibition zones were expressed as diameter and measured after incubation at 37°C for 24 h for *B. subtilis* ATCC 6633, and at 28°C for 48 h for *M. ramannianus* NRRL 1829.

After that, the organic solvent which gave the highest inhibition diameter (*n*-butanol) was subsequently used for further experiments. The culture broth was centrifuged to remove the biomass. The cell-free supernatant (40 ml) was extracted with an equal volume of *n*-butanol and the butanolic layer was dehydrated with Na_2SO_4 and concentrated to dryness using a Rotavapor. The crude extract was recuperated in 1 ml of methanol and bio-assayed by paper disk diffusion method (as described above) against four filamentous fungi (*A. carbonarius* M333, *A. niger*, *P. expansum* and *P. glabrum*), one yeast (*Saccharomyces cerivisea* ATCC 4226) and three bacteria (*M. luteus* ATCC 9314 and *E. cloacae* E10 and E13).

Table 1. Growth and some phenotypic characteristics of strain PP14 on different media at different incubation time.

Agar medium	Growth*	Color of mycelium		Soluble pigment†
		Aerial	Substrate	
Characteristics after 7 days of incubation				
Yeast extract-malt extract (ISP 2)	++	Pale yellow green	Deep brown	Yellow ++
Oat meal (ISP 3)	++	Light olive gray	Light grayish olive	–
Inorganic salts-starch (ISP 4)	+++	Light olive gray	Dark yellow grayish	–
Nutrient agar	+	Light gray	Grayish yellow	Absent
Bennett	+++	Moderate gray	Grayish yellow	Green yellow +
Characteristics after 14 days of incubation				
Yeast extract-malt extract (ISP 2)	++	Yellowish gray	Deep brown	Brown +
Oat meal (ISP 3)	+++	Light olive gray	Light grayish olive	Brown +
Inorganic salts-starch (ISP 4)	+++	Light olive gray	Moderate olive brown	Brown +/–
Nutrient agar	++	Yellowish gray	Grayish yellow	Brown +/–
Bennett	+++	Moderate gray	Moderate olive brown	Brown +
Characteristics after 21 days of incubation				
Yeast extract-malt extract (ISP 2)	+++	Yellowish gray	Deep brown	Brown ++
Oat meal (ISP 3)	+++	Light olive gray	Light grayish olive	Brown +
Inorganic salts-starch (ISP 4)	+++	Light olive gray	Moderate olive brown	Brown +
Nutrient agar	++	Yellowish gray	Grayish yellow	Brown +
Bennett	+++	Moderate gray	Moderate olive brown	Brown +

*+++, Very good growth; ++, good growth; +, weak growth; †++, very strong color; +, moderate color; +/–, weak growth; –, no pigment.

The butanolic extract was analyzed by thin layer chromatography (TLC). For this, 30 µl (*B. subtilis*) or 40 µl (*F. oxysporum* f. sp. *lini*) of butanolic extract was spotted onto 20 × 20-cm silica gel plates (Merck Art. 5735, Kiesselgel 60HF 254-365, Darmstaadt, Germany), and then developed with solvent systems: ethyl acetate / methanol (100:15 v/v), and *n*-butanol/acetic acid/deionized water (60:20:20 v/v/v). The developed TLC plates were air-dried overnight at 40°C to remove all traces of solvents. The separated compounds were visualized as colored spots and also under ultraviolet (UV) at 254 (absorbance) and at 365 nm (fluorescence). The active spots were detected by bioautography (Betina 1973). The TLC plates were then placed in a plastic bioassay dish (23 × 23 × 2.2 cm^3, Fisher Scientific Labosi) and overlaid with 150 ml/plate of ISP2 medium (containing 7 g/l of agar) seeded with *B. subtilis* or *Fusarium oxysporum* f. sp. *lini* as target microorganisms. After the agar had set, the dish was incubated at 30°C. After 48 h, reddish brown-colored fungal growth or opaque bacterial growth was visible. Clear areas due to inhibition of microorganism growth indicated the location of antimicrobial compounds on the TLC plates. The retention factor (Rf) values of each active spot were recorded.

RESULTS

Identification of strain PP14

Strain PP14 was obtained with the addition of antibiotics in the medium as selective agents. The strain was characterized based on its cultural properties (color of aerial mycelium, substrate mycelium and diffusible pigments) and chemotaxonomic characters. The description of strain morphology and the growth on various media, at 30°C for 21 days, is summarized in Table 1. The non-fragmented substrate mycelium was brown or greenish-yellow. The well-developed aerial mycelium tended to be white to gray and differentiated into straight [*rectus-flexibilis* (RF)] chains of spores. Mature spore masses belonged to the grey color series. The strain showed good growth on ISP4 and Bennett, and moderate growth on ISP3 and ISP2 media, and poor growth on nutrient agar, after 7 days of incubation at 30°C. Abundant greenish-yellow to brown soluble pigment was produced, after 21 days of incubation, on all media used. Endospores, sclerotic granules, synnemata and flagellated spores were not observed. The physiological characteristic studies revealed that strain PP14 did not produce melanoid pigments on any of the media used (ISP6 and ISP7). The strain utilized glucose, mannose, raffinose, saccharose, fructose, arabinose and mannitol as sole carbon sources, but not ramnose, galactose, xylose or *myo*-inositol. The cell-wall peptidoglycan of strain PP14 contained only LL-diaminopimelic acid isomer and glycine, indicating that strain has a cell-wall chemotype I which is characteristic of the *Streptomyces* genus (Lechevalier and Lechevalier, 1970). The whole-cell hydrolysates contained galactose, ribose, rhamnose, with traces of mannose and xylose, but no characteristic sugars were detected indicating that the strain has a sugar type C. The phospholipid profile was of type II *sensu* (Lechevalier et al., 1977), characterized by the presence of phosphatidyl-ethanolamine, diphosphatidyl-glycerol and phosphatidyl-inositol as diag-

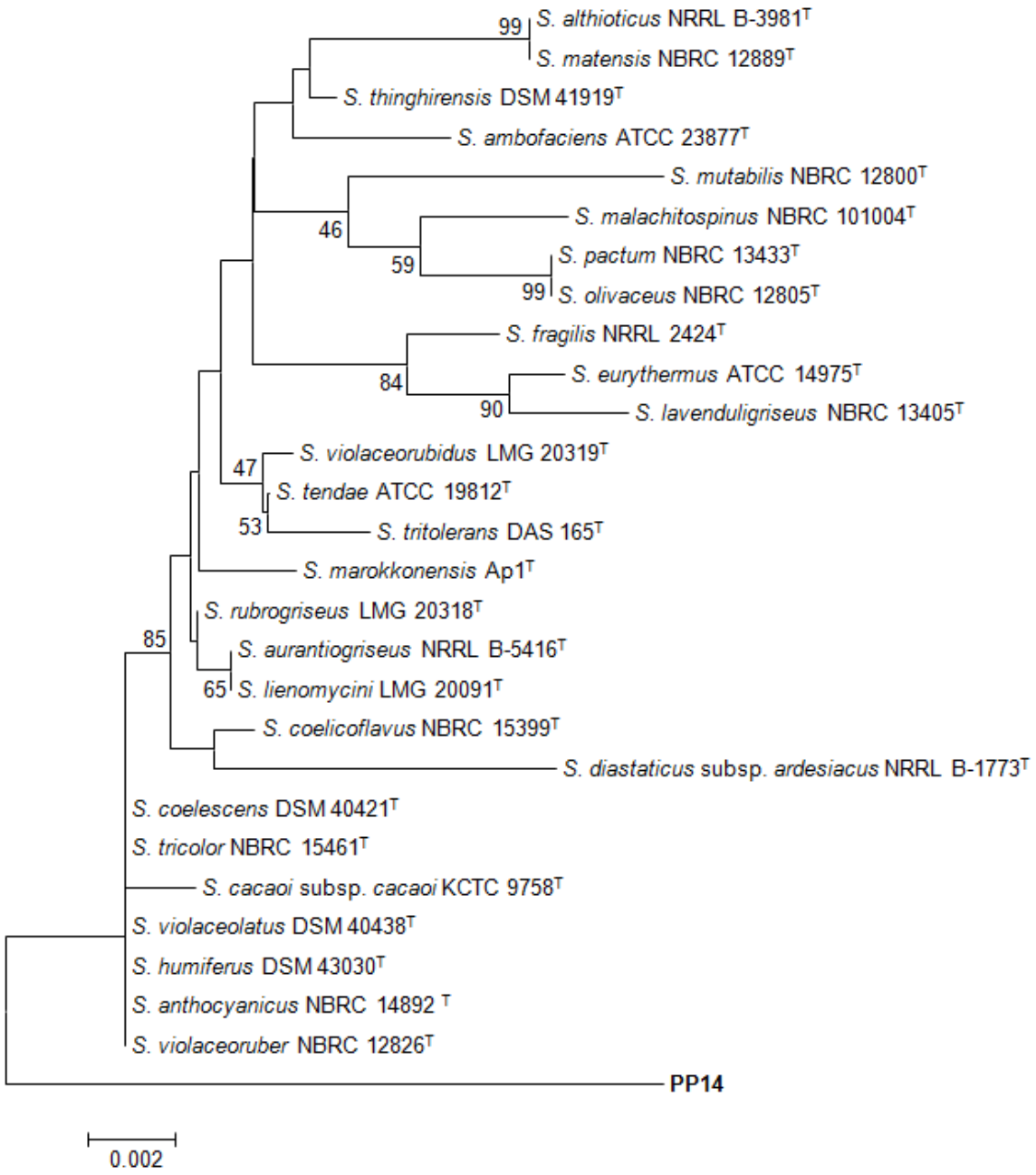

Figure 1. Neighbor-joining tree, based on 16S rRNA gene sequences showing the relationships between strain PP14 and the most close 27 type strain species of *Streptomyces*. The numbers at the nodes indicate the percentages of bootstrap support based on neighbor-joining analyses of 1000 resampled data sets; only values over 45% are given. Bar: 0.002 nucleotide substitution per nucleotide position.

nostic phospholipids. The obtained morphological and chemo-taxonomical characteristics strongly indicated that strain PP14 belonged to the genus *Streptomyces*.

The 16S rRNA sequence (1282 nucleotides) of strain PP14 has been determined and deposited in the Gen-Bank database under the accession number GQ374176.1. Phylogenetic relation of strain PP14 sequence with 16S rRNA of the highest 27 similar bacteria was carried out. The phylogenetic tree obtained by applying the neighbor joining method is illustrated in Figure 1. Comparison with sequences of the reference species of bacteria contained in genomic database banks exhibited a similarity level of only 98.3% with members of the *S. violaceoruber* species group (*S. violaceoruber*, *S. violaceolatus*, *S. tricolor*, *S. humiferus*, *S. coelescens* and S. *anthocyanicus*) which are the most closely related species. The physiological tests of strain PP14 and those of the *S. violaceoruber* clade are summarized in Table 2.

Table 2. Physiological characteristics of strain PP14 in comparison with those of closest phylogenetic neighbours (*S. violaceoruber* clade).

Characteristic*	PP14	*S. violaceoruber* clade†
Utilization of sole carbon source (1 %, w/v):		
Xylose	-	+
Rhamnose	-	+
Galactose	-	+
Glucose	+	+
Mannose	+	+
Meso-Inositol	-	+
Raffinose	+	+
Sucrose	+	v
Fructose	+	+
Arabinose	+	+
Mannitol	+	+

*+: Utilized, -: not utilized, v: variable. †Data for the reference species were taken from Williams et al. (1983, 1989) and Duangmal et al. (2005).

Antimicrobial activities

The antimicrobial activity of strain PP14 against various target microorganisms is shown in Table 3. Strain PP14 exhibited a very strong activity against tested Gram-positive bacteria (*M. luteus*), Gram-negative bacteria (*K. pneumoniae* E40 and *E. coli* E16, E52 and E195); however, almost no activity was observed for other tested Gram-negative bacteria. In addition, the strain exhibited a strong activity against *B. subtilis*, *E. cloacae* E10 and E13. An anti-yeast activity was also observed against *S. cerevisiae* but the growth of *C. albicans* was not affected. Moreover, an interesting antifungal activity was recorded against all mycotoxigenic and phytopathogenic fungi tested: a very strong activity against *F. oxysporum* f. sp. *lini*, *F. culmorum*, *F. graminearum*, *A. carbonarius* and *P. expansum*, and strong activity against *F. moniliforme*, *F. solani*, *M. ramannianus*, *A. flavus*, *A. niger* and *P. glabrum*.

Kinetics of antimicrobial products of strain PP14 in two different media

During the time course of fermentation in ISP2 broth and Bennett media, the antimicrobial activity was evaluated, by measuring the diameter of inhibition zone. The anti-fungal activity (against *A. carbonarius*, *A. niger*, *P. expansum*, *P. glabrum*, *M. ramannianus* and *S. cerevisiae*) and the antibacterial activity (against *B. subtilis*, *M. luteus*, *E. cloacae* E10 and E13) of strain PP14 was detected on day 3 of fermentation, but increased slowly between days 4 and 6, and reached a maximum always after 6, 7 or 8 days of fermentation; however, the anti-microbial activity was strongly decreased after day 8. The antimicrobial activity (evaluated by measuring the distance of inhibition) is given at the time of maximal values during 10 days of fermentation as reported in Table 4.

Production, extraction and detection of antimicrobial compounds

Table 5 shows the antimicrobial activity of different organic solvents (ethyl acetate, dichloromethane and *n*-butanol) obtained on the day of optimal production rate (after 7 days of fermentation), and the obtained results showed clearly that *n*-butanol is the best organic solvent for extraction. Moreover, the butanolic extract was also tested by measuring the distance of inhibition (mm) against eight other target-microorganisms. The obtained results are as follows: 24 mm for *M. luteus*, 17 mm for *E. cloacae* E13, 16 mm for *E. cloacae* E10, 52 mm for *S. cerivisea*, 33 mm for *P. expansum*, 23 mm for *P. glabrum*, 23 mm for *A. niger* and 39 mm for *A. carbonarius*.

On silica gel thin layer chromatography, the *n*-butanol extract, obtained on the day of optimal production rate (after 7 days), migrated and gave three bioautographic spots, which were active against *B. subtilis* and *F. oxysporum* f. sp. *lini*. One spot showed a yellow color: 14A (Rf = 0.81 in ethyl acetate/methanol, 100:15 v/v), with two non-colored spots: 14B (Rf = 0.72) and 14C (Rf = 0.60) exhibited a strong antibacterial and antifungal activities. Furthermore, Table 5 shows that the best organic solvent for extraction was *n*-butanol.

DISCUSSION

Various selective media and pretreatment procedures were applied to assess the optimal conditions for the isolation of rare actinomycetes and non-common strains of *Streptomyces* from different habitats (Naikpatil and Rathod, 2011). The pretreatment procedures could be realized, for example, by using the electromagnetic waves, by dry (or wet) heating of soil samples or by using chemicals such as antibiotics or phenol, etc. Several studies

Table 3. Antimicrobial activity of *Streptomyces* sp. PP14 against various microorganisms on solid ISP2 medium.

Bioassay microorganism[Ŧ]	Distance of inhibition (mm) [†]
Gram-positive bacteria	
Bacillus subtilis (ATCC 6633)	21
Micrococcus luteus (ATCC 9314)	29
Listeria monocytogenes (Institut Pasteur)	00
Gram-negative bacteria	
Agrobacterium tumefasciens (N° 2410 LB)	00
Escherichia coli (ATCC 10536)	01
E. coli (ATCC 25925)	01
E. coli (K12)	01
E. coli (E52)*	25
E. coli (E195)*	25
Enterobacter cloacae (E10)*	15
E. cloacae (E13)*	16
Acinetobacter baumanii (E16)*	30
Klebsiella pneumoniae (CIP 82.91)	02
K. pneumoniae (Kz8)*	00
K. pneumoniae (Kz19)*	00
K. pneumoniae (K44)*	01
K. pneumoniae (E40)*	33
Salmonella enterica (E32)*	05
Pseudomonas aeruginosa (CIP A22)*	01
Yeasts	
Candida albicans (IPA 200)	02
Saccharomyces cerevisiae (ATCC 4226)	45
Filamentous fungi	
Fusarium oxysporum f. sp. *lini* (Foln 3-5)	41
F. culmorum	45
F. graminearum	25
F. moniliforme	18
F. solani	16
Mucor ramannianus (NRRL 1829)	16
Aspergillus flavus	17
A. niger	25
A. carbonarius (M333)	39
Penicillium expansum	45
P. glabrum	16

*Resistant to multiple antibiotics (Aouiche et al., 2011); [†]Each value represents the average of two measurements; the inhibition distance of 1 to 8 mm was considered as weak, 9 to 14 mm as moderate, 15 to 24 mm as strong and 25 to 50 mm as very strong activity. The target microorganisms without accession number resulted from our laboratory collection.

studies reported that these techniques are extremely useful in the isolation of rare strains of actinomycetes (Kim et al., 1994; Naikpatil and Rathod, 2011).

The strain PP14 was first recognized on the basis of its morphological features. This strain was obtained with addition of 7 antibiotics as selective agents. The screening, isolation and characterization of new strains of actinomycetes producing new potential antibiotics has been a major area of research by our group for many years (Sabaou et al., 1998; Lamari et al., 2002; Zitouni et al., 2004b, 2005; Boudjella et al., 2006; Bouras et al., 2007, 2008; Aouiche et al., 2011; Badji et al., 2011; Meklat et

Table 4. Antimicrobial activity of *Streptomyces* sp. PP14 against various microorganisms on ISP2 and Bennett liquid media.

Bioassay microorganism	Distance of inhibition (mm)*†					
	ISP2			Bennett		
	6th day	7th day	8th day	6th day	7th day	8th day
B. subtilis ATCC 6633	11	12	11	12	12	11
M. luteus ATCC 9314	10	14	11	11	19	11
E. cloacae E10	16	16	17	13	13	14
E. cloacae E13	16	18	17	11	12	11
S. cerevisiae ATCC 4226	23	29	17	33	27	14
M. ramannianus NRRL 1829	26	18	17	15	15	15
A. niger	14	13	13	11	11	11
A. carbonarius M333	37	28	27	33	25	21
P. expansum	23	19	17	19	11	11
P. glabrum	15	13	13	13	11	11

*The antimicrobial activity (evaluated by measuring the distance of inhibition) is given at the time of maximal values during 10 days of fermentation. †Value include the diameter of wells (10 mm), and represents the average of two measurements.

Table 5. Effect of different organic solvents on antimicrobial activity of *Streptomyces* sp. PP14 against *B. subtilis* ATCC 6633 and *M. ramannianus* NRRL 1829.

Bioassay microorganism	Distance of inhibition (mm) by using different solvents*†		
	Ethyl-acetate	Dichloromethane	*n*-Butanol
B. subtilis ATCC 6633	18	15	19
M. ramannianus NRRL 1829	25	29	33

*The antimicrobial activity on ISP2 medium (evaluated by measuring the distance of inhibition) is given on the day of optimal production rate (after 7 days of fermentation). †Value include the diameter of wells (10 mm), and represents the average of two measurements.

al., 2012; Mokrane et al., 2013). The use of many antibiotics in combination with other selective agents (such as phenol) may lead in the future to the establishment of an adequate method for the selection of some species of actinomycete. The characterization of *Streptomyces* species is mainly based on the color of aerial and substrate mycelia, the soluble pigment and the shape and ornamentation of spore surface because of their stability (Shirling and Gottlieb, 1972; Holt et al., 1989).

The description of growth and some phenotypic characteristics of strain PP14 are summarized in Table 1.

On the basis of its morphological and chemical properties, strain PP14 was classified in the genus *Streptomyces*. Modern *Streptomyces* identification systems are based on 16S rRNA sequence data, which have provided precious information on systematic, and then have been used to identify several newly isolated *Streptomyces* (Lee et al., 2005; Kim et al., 2006). The 16S rRNA sequence of strain PP14 was compared with those of other *Streptomyces* species; it showed a sequence similarity of only 98.3% with members of the *Streptomyces violaceoruber* species clade (*S. violaceoruber*, *S. violaceolatus*, *S. tricolor*, *S. humiferus*, *S. coelescens* and S. *anthocyanicus*) which are the most closely related species. Much higher or similar 16S rRNA similarities have been found between representatives of validly described *Streptomyces* species, such as the type strains of *Streptomyces marokkonensis* DSM 41918^T, *S. humiferus* DSM 43030^T (99.1%), *S. thinghirensis* DSM 41919^T and *S. coelescens* DSM 40421^T (99.2%), *S. thinghirensis* DSM 41919^T and *violaceolatus* DSM 40438^T (99.2%), *S. marokkonensis* DSM 41918^T and *S. violaceoruber* DSM 40438^T (99.3%), *S. marokkonensis* DSM 41918^T and *S. coelescens* DSM 40421^T (99.3%), and *S. marokkonensis* DSM 41918^T and *S. violaceolatus* DSM 40438^T (99.3%) as reported by Bouizgarne et al. (2009) and Loqman et al. (2009). Moreover, the obtained results showed that strain PP14 could also be distinguished from the members of *S. violaceoruber* clade by several characters such as the color of aerial and substrate mycelia, the production of diffusible pigments and melanoid pigments; the use of xylose, rhamnose, galactose and *meso*-inositol as sole carbon source (Table 2). These results strongly suggest that strain PP14 is a new genomic species.

The obtained results in Table 3 showed the broad antimicrobial spectrum of strain PP14 against various target microorganisms especially against some phytopathogenic and mycotoxigenic fungi and multi-resistant bacteria. A

broad-spectrum antibacterial activity was observed against multi-resistant bacteria such as *K. pneumoniae* (E40), *A. baumanii* (E16), *E. coli* (E52) and *E. coli* (E195) which are known to be resistant respectively to 11, 8, 10 and 9 antibiotics among 15 tested antibiotics (Aouiche et al. 2011). However, the strain was moderately active against *E. cloacae* (E10) and *E. cloacae* (E13), which are resistant respectively to 11 and 12 tested antibiotics. In addition, the strain exhibited strong activity against Gram-positive bacterium, *M. luteus* (ATCC 9314), and yeast *S. cerevisiae* (ATCC 4226), and weak activity against Gram-negative bacterium *S. enterica* (E32) resistant to 9 tested antibiotics. Furthermore, strain PP14 showed a strong activity against phytopathogenic fungi such as *F. oxysporum* f. sp. *lini* (Foln 3-5), *F. culmorum* and *F. gramenearum*; and against mycotoxigenic fungi such as *Aspergillus carbonarius* and *A. niger* (both producers of ochratoxin A) and *Penicillium expansum*. However, the activity of the strain was moderate to *F. moniliforme*, *F. solani*, *P. glabrum*, *M. ramannianus* and *A. flavus* (producer of dangerous aflatoxins B1 and B2).

In general, Gram-negative bacteria are more resistant to antibacterial compounds than Gram-positive bacteria. Several studies showed that the outer cell membrane in Gram negative bacteria (double membranes) contains many protective mechanisms against antibiotics (Gupta, 2011). The activity of *Streptomyces* species against Gram-positive bacteria has been widely published (Park et al., 2009) but it seems that the activity against Gram-negative bacteria, yeasts and fungi has been rarely reported (Sabaratnam and Traquair, 2002; Baginski et al., 2005; Nguyen et al., 2010). Antitumoral activity has also been noted in several strains and species of *Streptomyces* (Pamboukian and Candida, 2004).

Table 4 shows the time of maximal antimicrobial activity (obtained always after 7, 8 or 9 day of fermentation) of strain PP14 obtained from two different culture media. Complex media have often been used for production of antibiotics, and the obtained results showed that ISP2 medium was generally better than Bennett medium to generate bioactive compounds (Boudjella et al., 2006). This variation reflects the importance of medium optimization to attain higher productivity. The chromogenic reactions of these compounds were negative with $FeCl_3$, naphtoresorcinol-H_2SO_4, ninhydrine, formaldehyde-H_2SO_4 and Dragendorff reagents, suggesting the absence of phenol, osidic residues, free amine groups, polycyclic aromatics and alkaloids.

Conclusion

The obtained findings give support to the biotechnological use of the rare and extremophile actinomycetes as sources of interesting bioactive natural molecules against mycotoxigenic and phytopathogenic fungi, Gram-positive and Gram negative bacteria and yeasts. Furthermore, these antimicrobial molecules could be beneficial to undertake a more comprehensive search for rare forms of antibiotic-producing organisms in extremophile bio-systems; however, further studies will be necessary to determine the structures of the active compounds.

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REFERENCES

Aguilar A (1996). Extremophile research in the European Union: from fundamental aspects to industrial expectations. FEMS Microbiol. Rev. 18:89-92.

Aouiche A, Sabaou N, Meklat A, Zitouni A, Bijani C, Mathieu F, Lebrihi A (2012). *Saccharothrix* sp. PAL54, a new chloramphenicol-producing strain isolated from a Saharan soil. World J. Microb. Biot. 28:943-951.

Badji B, Mostefaoui A, Sabaou N, Mathieu F, Lebrihi A (2011). Identification of a new strain of Actinomadura isolated from Saharan soil and partial characterization of its antifungal compounds. Afr. J. Microbiol. Res. 10:13878-13886.

Baginski M, Sternal K, Czub J, Borowski E (2005). Molecular modelling of membrane activity of amphotericin B, a polyene macrolide antifungal antibiotic. Acta Biochim. Polon. 52:655-658.

Becker B, Lechevalier MP, Gordon RE, Lechevalier HA (1964). Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole cell hydrolysates. Appl. Microbiol. 12:421-423.

Berdy J (1989). The discovery of new bioactive microbial metabolites: screening and identification. In *Bioactive microbial metabolites*, Edited by M. E. Bushell & U. Graefe. Amsterdam: Elsevier. pp.3-25.

Betina V (1973). Bioautography in paper and thin layer chromatography and its scope in the antibiotic field. J. Chromatogr. 78:41-51.

Boubetra D, Zitouni A, Bouras N, Mathieu F, Lebrihi A, Schumann P, Spröer C, Klenk HP, Sabaou N (2013). *Saccharothrix hoggarensis* sp. nov., an actinomycete isolated from Saharan soil. Int. J. Syst. Evol. Evol. Microbiol. 63:549-553

Boudjella H, Bouti K, Zitouni A, Mathieu F, Lebrihi A, Sabaou N (2006). Taxonomy and chemical characterization of antibiotics of *Streptosporangium* Sg 10 isolated from a Saharan soil. Microbiol. Res. 161:288-298.

Bouizgarne B, Lanoot B, Loqman S, Spröer C, Klenk HP, Swings J, Ouhdouch Y (2009). *Streptomyces marokkonensis* sp. nov., isolated from rhizosphere soil of *Argania spinosa* L. *Int.* J. Syst. Evol. Evol. Microbiol. 59:2857-2863.

Bouras N, Mathieu F, Sabaou N, Lebrihi A (2006). Effect of amino acids containing sulfur on dithiolopyrrolone antibiotic productions by *Saccharothrix algeriensis* NRRL B-24137. J. Appl. Microbiol. 100:390-397.

Bouras N, Mathieu F, Sabaou N, Lebrihi A (2007). Influence on dithiolopyrrolone antibiotic production by organic acids in *Saccharothrix algeriensis* NRRLB-24137. Process Biochem. 42:925-933.

Bouras N, Merrouche R, Lamari L, Mathieu F, Sabaou N, Lebrihi A (2008). Precursor-directed biosynthesis of new dithiolopyrrolone analogs by *Saccharothrix algeriensis* NRRL B-24137. Process Biochem. 43: 1244-1252.

Buckingham J (1997). Dictionary of Natural Products. England: Chapman and Hall, CRC.

Chun J, Lee JH, Jung Y, Kim M, Kin S, Kim BK, Lim YW (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. Int. J. Syst. Evol. Microbiol. 57:2259-2261.

Duangmal K, Ward AC, Goodfellow M (2005). Selective isolation of members of the *Streptomyces violaceoruber* clade from soil. FEMS Micobiol. Lett. 245:321-327.
Felsenstein J (2005). PHYLIP (phylogeny inference package) version 3.6. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.
Goodfellow M (1971). Numerical taxonomy of some nocardioform bacteria. J. Gen. Microbiol. 69:33-90.
Gupta RS (2011). Origin of diderm (Gram-negative) bacteria: antibiotic selection pressure rather than endosymbiosis likely led to the evolution of bacterial cells with two membranes. Antonie Leeuwenhoek. 100:171-182.
Hayakawa M, Nonomura H (1987). Humic acid-vitamin agar, a new medium for the selective isolation of soil actinomycetes. J. Ferment. Technol. 65:501-509.
Holt JG, Sharpe ME, Williams ST (1989). Bergey's Manual of Systematic Bacteriology. Williams and Williams. Baltimore, London.
Jukes TH, Cantor CR (1969). Evolution of protein molecules. In: Munro HN (ed) Mammalian protein metabolism. Academic Press, London, pp.21-132.
Kelly KL, Judd DB (1976). Color, Universal language and dictionary of names. National Bureau of Standards Special Publication 440. U.S. Department of Commerce, Washington D.C.
Kim HJ, Sung CL, Byung KH (2006). *Streptomyces cheonanensis* sp. nov., a novel streptomycete with antifungal activity. Int. J. Syst. Evol. Microbiol. 56:471-475.
Kim CJ, Lee KH, Kwon OS, Shimazu A, Yoo ID (1994). Selective isolation of actinomycetes by physical pretreatment of soil sample. K. J. Appl. Microbiol. Biotechnol. 22:222-225.
Lamari L, Zitouni A, Dob T, Sabaou N, Lebrihi A, Germain P, Seguin E, Tillequin F (2002). New dithiolopyrrolone antibiotics from *Saccharothrix* sp. SA 233. II. Physicochemical properties and structure elucidation. J. Antibiot. 55:702-707.
Lechevalier HA, Lechevalier MP (1970). Chemical composition as a criterion in the classification of aerobic actinomycetes. Int. J. Syst. Bacterio. 20:435-443.
Lechevalier MP, De Bievre C, Lechevalier HA (1977). Chemotaxonomy of aerobic actinomycetes: phospholipid composition. Biochem. Syst. Ecol. 5:249-260.
Lee JY, Jung HW, Hwang BK (2005). *Streptomyces koyangensis* sp. nov., a novel actinomycete that produces 4-phenyl-3-butenoic. Int. J. Syst. Evol. Microbiol. 55:257-262.
Liu D, Coloe S, Baird R, Pedersen J (2000). Rapid mini-preparation of fungal DNA for PCR. J. Clin. Microbiol. 38:471.
Loqman S, Bouizgarne B, Ait Barka E, Clement C, von Jan M, Spröer C, Klenk HP, Ouhdouch Y (2009). *Streptomyces thinghirensis* sp. nov., isolated from rhizosphere soil of *Vitis vinifera*. Int. J. Syst. Evol. Evol. Microbiol. 59:3063-3073.
Mazza P, Monciardini P, Cavaletti L, Sosio M, Donadio S (2003). Diversity of *Actinoplanes* and related genera isolated from an Italian soil. Microb. Ecol. 45:362-372.
Meklat A, Sabaou N, Bouras N, Zitouni A, Spröer C, Klenk HP, Mathieu F, Lebrihi A (2012). A novel strain of *Actinopolyspora mortivallis* with antibacterial activity isolated from a Saharan soil. Ann. Microbio. 62:1049-1057.
Meklat A, Bouras N, Zitouni A, Mathieu F, Lebrihi A, Schumann P, Spröer C, Klenk HP, Sabaou N (2013). *Actinopolyspora saharensis* sp. nov., a novel halophilic actinomycete isolated from a Saharan soil of Algeria. Antonie Leeuwenhoek, 103:771-776.
Messai Y, Iabadene H, Benhassine T, Alouache S, Tazir M, Gautier V, Arlet G, Bakour R (2008). Prevalence and characterization of extended-spectrum b-lactamases in *Klebsiella pneumoniae* in Algiers hospitals (Algeria). Pathol. Biol. 56:319-325.
Minnikin DE, Patel PV, Alshamahony L, Goodfellow M (1977). Polar lipid composition in the classification of *Nocardia* and related bacteria. Int. J. Syst. Bacteriol. 27:104-117.
Mokrane S, Bouras N, Sabaou N, Mathieu F (2013). Actinomycetes from saline and non-saline soils of Saharan palm groves: Taxonomy, ecology and antagonistic properties. Afr. J. Microbiol. Res. 7:2167-2178.
Newman DJ, Cragg GM, Snader KM (2003). Natural products as sources of new drugs over the period 1981–2002. J. Nat. Prod. 66:1022-1037.
Nguyen HC, Karray F, Lautru S, Gagnat J, Lebrihi A, Huynh TD, Pernodet JL (2010). Glycosylation steps during spiramycin biosynthesis in *Streptomyces ambofaciens*: involvement of three glycosyltransferases and their interplay with two auxiliary proteins. *Antimicrob.* Agents Chemother. 54:2830-2839.
Naikpatil SV, Rathod JL (2011). Selective isolation and antimicrobial activity of rare actinomycetes from mangrove sediment of Karwar Antimicrob. J. Ecobiotech. 10:48-53.
Pamboukian CRD, Candida M (2004). Production of the antitumoral retamycin during continuous fermentations of *Streptomyces olindensis.* Process Biochem. 39:2249-2255.
Park JS, Yang HO, Kwon HC (2009). Aldgamycin I, an antibacterial 16-membered macrolide from the abandoned mine bacterium, *Streptomyces* sp. KMA-001. J. Antibiot. 62:171-175.
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.
Sabaratnam S, Traquair JA (2002). Formulation of a *Streptomyces* biocontrol agent for the suppression of *Rhizoctonia* damping-off in tomato transplants. Biol. Control. 23:245-253.
Saitou N, Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-425.
Shirling EB, Gottlieb D (1966). Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16:313-340.
Shirling EB, Gottlieb D (1972). Cooperative description of type strains of *Streptomyces*. V. Additional descriptions. Int. J. Syst. Bacteriol. 22:265-394.
Tamura K, Dudley J, Nei M, Kumar S (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24:1596-1599.
Thompson JD, Higgins DG, Gibson TJ (1994). CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighing, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673-4680.
Touati A, Benallaoua S, Forte D, Madoux J, Brasme L, de Champs C (2006). First report of CTX-M-15 and CTX-M-3 beta-lactamases among clinical isolates of *Enterobacteriaceae* in Bejaia, Algeria. Int. J. Antimicrob. Agents 27:397-402.
Williams ST, Goodfellow M, Alderson G, Wellington EMH, Sneath PHA, Sackin MJ (1983). Numerical classification of *Streptomyces* and related genera. J. Gen. Microbiol. 129:1743-1813.
Williams ST, Goodfellow M, Alderson G (1989). Genus *Streptomyces* Waksman and Henrici 1943, 339AL In: Bergey's Manual of Systematic Bacteriology (Williams ST, Sharpe ME and Holt JG, Eds.), Williams and Wilkins, Baltimore, USA. 4:2452-2492.
Zitouni A, Boudjella H, Lamari L, Badji B, Mathieu F, Lebrihi A, Sabaou N (2005). *Nocardiopsis* and *Saccharothrix* genera in Saharan soils in Algeria: isolation, biological activities and partial characterization of antibiotics. Res. Microbiol. 156:984-993.
Zitouni A, Lamari L, Boudjella H, Badji B, Sabaou N, Gaouar A, Mathieu F, Lebrihi A, Labeda DP (2004a). *Saccharothrix algeriensis* sp. nov., isolated from Saharan soil. Int. J. Syst. Evol. Microbiol. 54:1377-1381.
Zitouni A, Mathieu F, Coppel Y, Pont F, Sabaou N, Lebrihi A (2004b). Mutactimycin PR, a new anthracycline antibiotic from *Saccharothrix* sp. SA 103. II. Physico-chemical properties and structure elucidation. J. Antibiot. 57:373-378.