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ORIGINAL ARTICLE

A new *Streptomyces* strain isolated from Saharan soil produces di-(2-ethylhexyl) phthalate, a metabolite active against methicillin-resistant *Staphylococcus aureus*

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Abstract An actinomycete strain designated G60 was isolated from a Saharan soil sample in Ghardaïa, Algeria, by a dilution agar plating method using chitin-vitamin agar medium supplemented with penicillin. Morphological and chemical studies indicated that this strain belonged to the genus *Streptomyces*. Analysis of the 16S rDNA sequence showed an identity level within *Streptomyces* species, with *S. coerulescens* ISP 5146^T and *S. bellus* ISP 5185^T the most closely related (100 % for each). However, the comparison of the morphological and physiological characteristics of the strain with those of the two nearest species showed significant differences. Strain G60 had a very strong activity against pathogenic staphylococci, including methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, other clinical isolates of MRSA and vancomycin resistant *S. aureus* (VRSA) S1. One antimicrobial compound was extracted by *n*-hexane from the ISP2 culture medium at 5 days of fermentation culture and purified by HPLC. The chemical structure of the compound was determined after spectroscopic (^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY and ^{1}H-^{13}C HMBC spectra), and spectrometric (mass spectrum) analyses. The bioactive compound was identified as di-(2-ethylhexyl) phthalate.

Keywords *Streptomyces* · Taxonomy · Antistaphylococcal activities · Di-(2-ethylhexyl) phthalate

Introduction

During the past two decades, the emergence of antibiotic-resistant microorganisms has become a problem in public health (Angebault and Andremont 2013). Among these microorganisms, *Staphylococcus aureus* is resistant to antibiotics such as aminoglycosides, macrolides, fluoroquinolones, tetracyclines and vancomycin (Green et al. 2012). The Gram-positive bacterium methicillin-resistant *S. aureus* (MRSA) is responsible for various infection diseases including sepsis pneumonia, osteomyelitis, endocarditis, pimples and bacteremia (Loomba et al. 2010; Tong et al. 2012). The treatment of infections caused by this virulent pathogen has become a serious problem due to the development of resistance to β-lactamin antibiotics (Fuller et al. 2005). The resistance mechanisms of MRSA includes enzymatic inactivation of the antibiotic (penicillinase and aminoglycoside-modification enzymes), alteration of the target with decreased affinity for the antibiotic (notable examples being penicillin-binding protein 2a of methicillin-resistant *S. aureus* and D-Ala-D-Lac of peptidoglycan precursors of vancomycin-resistant strains), trapping of the antibiotic (for vancomycin and possibly daptomycin) and efflux pumps (fluoroquinolones and tetracycline) (Pantosti et al. 2007). Therefore, new antistaphylococcal molecules are necessary to combat these resistant pathogenic staphylococci.

Three strategies have been employed to find new antibacterial antibiotics: first, the search for molecules derived from known

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antibiotics, such as clarithromycin and telithromycin (Clardy et al. 2006); second, the search for novel targets within the bacteria (Coates and Hu 2007); third, an intensified search for new molecules from natural sources (Von Nussbaum et al. 2006).

Until recently, the majority of antimicrobial compounds in current use for the treatment of various infectious diseases were isolated from microorganisms (Tawiah et al. 2012). The actinomycetes are an important group of microorganisms, especially the genus *Streptomyces*, which are best known for their ability to produce antibiotics (Watve et al. 2001). It has been estimated that approximately 45 % of natural antibiotics were isolated from actinomycetes, and about 75 % of bioactive molecules coming from actinomycetes are produced by members of the genus *Streptomyces* (Solecka et al. 2012).

The exploration of new soils and habitats from the extreme environments is one of several research programs established to obtain new strains and probably new bioactive metabolites (Thakur et al. 2007). The Algerian Saharan soils are particular ecosystems, which are rich and diversified in actinomycetes (Sabaou et al. 1998; Boubetra et al. 2013a; Meklat et al. 2013). New strains or species producing novel bioactive molecules were isolated from those soils in our laboratory (Boudjella et al. 2010; Aouiche et al. 2012; Boubetra et al. 2013b).

In the present study, we describe the taxonomy of a new *Streptomyces* strain isolated from an Algerian Saharan soil having antistaphylococcal activity. Fermentation on different culture media, extraction, purification and final structure of the active compound are reported as well.

Materials and methods

Isolation of the actinomycete strain

The strain G60 was isolated from Saharan soil collected in Metlili (Ghardaïa, center of Algeria, 32°16′N, 3°37′E). The dry soil sample was subjected to serial dilutions in sterile distilled water and 0.2 mL of each dilution was plated on chitin-vitamin agar medium (Hayakawa and Nonomura 1987). The culture medium was supplemented with penicillin (25 mg/L) and actidione (50 mg/L) as selective antibiotics to prevent growth of bacteria and fungi, respectively. The plates were incubated at 30 °C for 2 weeks and then examined by light microscopy. After isolation, the strain G60 was stored on agar slant of ISP2 medium (Shirling and Gottlieb 1966) at 4 °C.

Identification of the actinomycete strain

Morphological characteristics and chemotaxonomic studies

The genus of the collected isolate was determined by the morphological features and chemical analysis of cellular constituents. Its cultural characteristics were determined by naked eye examination of 14-day-old cultures grown on various media: yeast extract-malt extract agar (ISP2), oatmeal agar (ISP3) and inorganic salts-starch agar (ISP4) (Shirling and Gottlieb 1966), and on nutrient agar and Bennett agar (Waksman 1961). The micromorphology and sporulation were observed by light microscopy on ISP2 medium. The colors of aerial and substrate mycelia were determined with the ISCC-NBS centroid color charts (Kelly and Judd 1976). The diaminopimelic acid isomers, the whole-cell sugar pattern and the phospholipids were analyzed according to the methods of Becker et al. (1964), Lechevalier and Lechevalier (1970) and Minnikin et al. (1977), respectively.

Physiological characterization

The physiological characterization of the strain G60 was realized as described by Locci (1989). The physiological tests (n=60) concerned the hydrolysis of adenine, guanine, hypoxanthine, xanthine, gelatin, tyrosine, starch and esculin, the utilization of 20 carbohydrate compounds, the decarboxylation of seven organic acids, the assimilation of six amino acids, the reduction of nitrate, the sensitivity to phenol (0.001 % w/v), sodium azide (0.001 % w/v), sodium chloride (4 and 7 % w/v) and crystal violet (0.05 % w/v), and the growth at pH 5 and pH 9 and at 40 and 45 °C, and in the presence of seven antibiotics. 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 and 16S rDNA sequencing

The strain G60 was grown at 30 °C for 5 days in a shake flasks containing 100 mL ISP2 medium. DNA preparation was realized according to the method of Liu et al. (2000). PCR amplification of the 16S rDNA of the strain was performed using two primers: 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′- GGTTACCTTGTTACGACTT-3′). The 16S rDNA gene sequence was amplified by PCR using an Invitrogen kit (Invitrogen, Carlsbad, CA). The final 50 μL volume of reaction mixture contained 1×PCR buffer (10 mM Tris–HCl, 50 mM KCl, pH 9.0 at 25 °C), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1 μM of each primer, 1.25 U *Taq* DNA polymerase and 1 μL (500 ng) purified DNA. The amplification was performed on a thermal cycler (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 (UV) fluorescence after ethidium bromide staining. The PCR products obtained were sent to a commercial

company (MilleGen, Toulouse, France) for sequence determination. The same primers as above and an automated sequencer were used for this purpose.

Phylogenetic analyses

The sequence determined was compared for similarity level with the reference species of bacteria contained in genomic database banks, using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al. 2012). The phylogenetic analyses were conducted using Molecular Evolution Genetics Analysis (MEGA) software version 5.0 (Tamura et al. 2011). The 16S rDNA sequence of the strain G60 was aligned using the CLUSTAL W program (Thompson et al. 1994) against corresponding nucleotide sequences of representatives of the genus *Streptomyces* retrieved from the GenBank database. The evolutionary distance matrices were generated as described by Kimura (1980) and a phylogenetic tree was constructed via the neighbor-joining (NJ) algorithm (Saitou and Nei 1987). The tree topologies were evaluated by bootstrap analysis (Felsenstein 1985) based on 1,000 resamplings of the NJ data set.

Antistaphylococcal activity

Antibacterial activity was evaluated on ISP2 medium by the streak method against various strains of *S. aureus*. The experiment was done firstly by streaking a straight line of the G60 inoculum across the surface of medium on 90-mm diameter plates and incubating at 30 °C for 8 days. After growth of strain G60, the target staphylococci were seeded in streaks crossing the actinomycete culture. The antimicrobial activity was appreciated by measuring the length of inhibition between the target microorganisms and the actinomycete colony margins. Seven target staphylococci were tested, and chosen for their varying degrees of sensitivity and resistance to a range of antibiotics (ESM Table 1). They include five strains isolated from patients in hospitals of Algeria: *S. aureus* 38, *S. aureus* 39, *S. aureus* 636 and *S. aureus* S1 (multi-resistant to antibiotics), and *S. aureus* R2 (resistant to penicillin), and the two other staphylococci, *S. aureus* ATCC 25923 (methicillin sensitive) and *S. aureus* MRSA ATCC 43300 (methicillin resistant).

Effect of different culture media on antimicrobial activity

In order to choose the best medium for antibiotic production, 11 culture media were tested. Basal culture medium (BM), which contained the following components (in g/L distilled water): 2 $(NH4)_2SO_4$, 2 NaCl, 0.5 KH_2PO_4, 1 K_2HPO_4, 0.2 $MgSO_4$, was used to prepare the six first culture media. The composition of the culture media used is as follows (in g/L distilled water): GBM: 10 glucose and BM; SBM: 10 starch and BM; ABM: 10 L-asparagine and BM; MEBM: 2 meat extract and BM; PBM: 2 peptone from soya and BM; YEBM: 4 yeast extract and BM; GME: 10 glucose and 4 malt extract; GP: 10 glucose and 2 peptone; GYE: 10 glucose and 4 yeast extract; Bennett medium: 10 glucose, 2 peptone, 1 yeast extract, 1 meat extract (Shirling and Gottlieb 1966); ISP2: 4 glucose, 10 malt extract, 4 yeast extract (Shirling and Gottlieb 1966). The final pH of the media was adjusted at 7.3. Each 250-mL Erlenmeyer flask containing 60 mL medium was inoculated with 3 % (v/v) of the actinomycete culture grown in medium for 72 h at 30 °C. The cultures were incubated on a rotary shaker (250 rpm) at 30 °C for 7 days. The antistaphylococcal activity of each medium was measured by well diffusion method against two target microorganisms including *S. aureus* ATCC 25923 and *S. aureus* MRSA ATCC 43300. Each well of 10 mm in diameter was filled with 0.2 mL supernatant.

Time course of growth and bioactive compound production on liquid media

Based on the above results, fermentations were carried out in the four best broth media. The pH was adjusted to 7.3 before autoclaving. Each 500-mL Erlenmeyer flask containing 100 mL medium was inoculated with 5 % (v/v) of the actinomycete culture grown on medium for 72 h at 30 °C. The Erlenmeyer flasks were incubated at 30 °C for 10 days with shaking at 250 rpm. The antistaphylococcal activity was daily assayed against *S. aureus* MRSA ATCC 43300 by the well-diffusion method. The growth (dry weight of mycelium) and the pH were also measured.

Isolation and purification of the bioactive compound

The extraction of the bioactive compound was realized on the day of optimal production rate. The ISP2 culture broth (200 mL) was centrifuged to remove the biomass. Each 50 mL of the cell free supernatant was extracted with an equal volume of the following organic solvents: *n*-hexane, dichloromethane, *n*-butanol and ethyl acetate. The organic layer was dehydrated with Na_2SO_4 and concentrated using the rotavapor. The resulting dry extract was recuperated in 0.5 mL methanol and bioassayed against *S. aureus* MRSA ATCC 43300 by the paper disk diffusion method.

Preparative chromatography with silicagel plates (Merck Art. 5735, Kiesselgel 60HF 254–366; 20×20 cm) was employed for the partial purification of antimicrobial product. A dry crude extract, dissolved in methanol, was spotted and developed in the solvent system ethyl acetate-methanol (100:15 v/v). The developed TLC plates were air-dried overnight to remove all traces of solvents. The separated

compound was visualized under UV at 254 nm and 365 nm, and the active spot was detected by bioautography (Betina 1973). The TLC plates were deposited in a plastic bioassay dish (23×23×2.2 cm, Fisher Scientific Labosi, Elancourt, France) and overlayed with 50 mL (per plate) ISP2 medium (containing 7 g/L agar) seeded with *S. aureus* MRSA ATCC 43300 as target microorganism, and incubated at 30 °C for 24 h. A clear area due to the inhibition of the growth of the target microorganisms indicated the location of the bioactive compound. The retention factor (Rf) of the active spot was measured. The purification of this compound was performed by JASCO reverse phase HPLC using an interchrom C18 column (15.0 μm, 250×7.8 mm) with a continuous linear gradient solvent system from 0 to 100 % methanol in water, a flow rate of 1 mL/min and ultra violet detection at 220 nm. The final purification was achieved after the second re-injection in the HPLC system.

Antibacterial activity of the purified compound

The tests were carried out by the conventional paper-disk method. Bacteria were grown on nutrient agar medium. The purified compound was dissolved in methanol and a paper disk (6 mm in diameter) containing 50 μg of the sample was placed on the agar plates seeded with the test-microorganism. Growth inhibition was examined after 24 h incubation at 30 °C. The antimicrobial activity was estimated by measuring the diameter of the inhibitory zone.

Spectroscopic analyses of the antistaphylococcal compound

The UV-visible of the active compound was determined in methanol solution with a JASCO V-660 k spectrophotometer. The mass spectrum was recorded on an ion-trap mass spectrometer (Finnigan MAT, San Jose, CA), equipped with a nanospray ion electro-spray ionization (ESI) source (negative ion mode). An NMR sample was prepared by dissolving 2 mg of the bioactive compound in 600 μL CD_3OD. All spectra were recorded on a Bruker Avance 500 spectrometer equipped with a 5 mm triple resonance inverse Z-gradient probe (TBI 1H, 31P, BB). All chemical shifts for ^{1}H and ^{13}C were relative to TMS using ^{1}H (residual) or ^{13}C chemical shifts of the solvent as a secondary standard. The temperature was set at 298 K. Gradient-enhanced ^{1}H COSY45 was performed including 36 scans per increment. ^{1}H-^{13}C correlation spectra using a gradient-enhanced HSQC sequence (delay optimized for 1JCH of 145 Hz) were obtained with 120 scans per increment. A gradient enhanced HMBC experiment was performed allowing 62.5 ms for long-range coupling evolution (240 scans were accumulated). Typically, 2,048 t2 data points were collected for 256 t1 increments.

Results and discussion

Morphological characteristics, chemotaxonomy, and identification of the genus

The strain G60 showed good growth and abundant mycelia on all media used after 8 days at 30 °C except in nutrient agar medium, in which moderate growth was observed (Table 1). The aerial mycelium was greenish grey on ISP2, ISP3 and ISP4, greyish white on Bennett, and light grey on nutrient agar. It produced long spiraled chains of spores (10–50 spores per chain) carried by sporophores. The substrate mycelium was light yellow on ISP2, Bennett and nutrient agar media, but was colorless on ISP3 and ISP4 media. Diffusible pigments were not produced on all media used. Melanoid pigments were also not produced on ISP6 and ISP7 media. Endospores, sclerotia, sporangia, synnemata and whirls were not observed.

The chemotaxonomic study of the strain G60 showed the presence of LL-diaminopimelic acid isomer and the presence of glycine in the cell wall. Characteristic sugars (arabinose, xylose, madurose and rhamnose) were not detected in the whole-cell hydrolysates. Strain G60 corresponds to chemotype IC according to Williams et al. (1989). The diagnostic phospholipid detected was phosphatidylethanolamine, corresponding to phospholipids type PII (Lechevalier et al. 1977).

Based on the morphological and chemical characteristics, the strain G60 was classified in the genus *Streptomyces* (Holt et al. 1994). This genus was reported to contain 615 species including 38 subspecies (Labeda et al. 2012).

Physiological characteristics

The physiological properties of the strain G60 are shown in Table 2. Its optimal growth was observed at 30 °C and at pH 7. It was able to grow at 4 % of NaCl but not at 7 %. It was unable to grow in the presence of crystal violet (0.05 %), phenol (0.001 %) and sodium azide (0.001 %), at pH 5 and pH 9, and at 45 °C. The strain was able to hydrolyze all organic compounds tested except erythritol, L-alanine, guanine and sodium tartrate. It was resistant to erythromycin (10 μg/mL), chloramphenicol (25 μg/mL) and vancomycin (5 μg/mL), but sensitive to kanamycin (25 μg/mL), penicillin (25 μg/mL), rifampicin (5 μg/mL) and streptomycin (10 μg/mL).

Phylogenetic analysis

The 16S rDNA sequence (1,445 nucleotides) of the strain G60 was determined and has been deposited with the GenBank data library under the accession number KC414016. This sequence was aligned with those of *Streptomyces* reference

Table 1 Cultural characteristics of *Streptomyces* sp. G60 on different media after 14 days of incubation at 30 °C

Medium	Growth[a]	Aerial mycelium	Substrate mycelium
Yeast extract-malt extract agar (ISP2)	+++	Greenish grey	Light yellow
Oatmeal agar (ISP3)	+++	Greenish grey	Colorless
Inorganic salt-starch agar (ISP4)	+++	Greenish grey	Colorless
Bennett	+++	Greenish white	Light yellow
Nutrient agar	++	Light grey	Light yellow

[a] +++ Abundant, ++ moderate

species available in the GenBank database, which confirmed that the isolate G60 belonged to the genus *Streptomyces*. Its position in the 16S rDNA *Streptomyces* tree is shown in Fig. 1. The identity level was 100 % with *S. coerulescens* ISP 5146^T and *S. bellus* ISP 5185^T (Kämpfer 2012), the most closely related species. However, strains showing a 16S rDNA sequence identity of 100 % do not necessarily belong to the same species. Indeed, sequence identity of 100 % was found between several representatives of validly described *Streptomyces* species, such as the type strains of *S. rochei*

Table 2 Physiological properties of G60 strain[a]

Characteristics	Strain G60	Characteristics	Strain G60
Carbon source utilization (1 % w/v)		Hypoxantine	+
L-Arabinose	±	Tyrosine	+
Adonitol	+	Xanthine	+
Cellobiose	+	Gelatine	+
Erythritol	−	Decarboxylation of sodium salts	
D-Fructose	+	Acetate	+
Galactose	+	Benzoate	+
Glucose	+	Citrate	+
myo-Inositol	+	Oxalate	+
Lactose	+	Pyruvate	+
Maltose	+	Succinate	+
D-Mannitol	+	Tartate	−
D-Mannose	+	Resistance to antibiotics (μg/L)	
Melibiose	+	Chloramphenicol (25)	+
D-Raffinose	+	Erythromycin (10)	+
L-Rhamnose	+	Kanamycin (25)	−
Ribose	+	Penicillin (25)	−
Sucrose	+	Rifampicin (5)	−
Sorbitol	+	Streptomycin (10)	−
Trehalose	+	Vancomycin (5)	+
D-Xylose	+	Growth with (% w/v)	
Utilization of aminoacids (0.1 % w/v)		Crystal violet (0.05)	−
L-Proline	+	Phenol (0.001)	−
L-Serine	+	Sodium azide (0.001)	−
L-Alanine	−	NaCl (4)	+
L-Phenylalanine	+	NaCl (7)	−
L-Arginine	+	Growth at	
L-Histidine	+	pH 5	−
Utilization of organic compounds		pH 9	−
Adenine	+	40 °C	+
Starch	+	45 °C	−
Esculin	+	Nitrate reduction	+
Guanine	−	Production of melanoid pigment	−

[a] Tests: + positive; − negative; ± doubtful

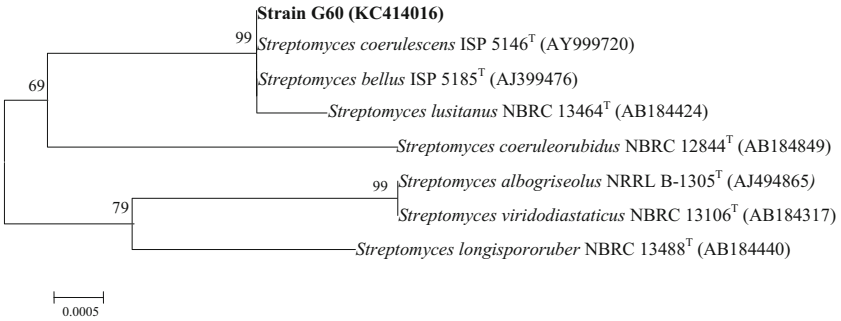

Fig. 1 Phylogenetic tree derived from nearly complete 16S rDNA gene sequences showing relationships between the strain G60 and the related type species of the genus *Streptomyces*. The tree was constructed using the neighbor-joining (NJ) method (Saitou and Nei 1987). Bootstrap values are indicated at nodes. *Bar* 0.0005 substitutions per nucleotide position

and *S. plicatus*, *S. gibsonii* and *S. rangoonensis*, *S. coerulescens* and *S. bellus*, *S. werraensis* and *S. biverticillatus*, *S. galilaeus* and *S. bobili*, *S. californicus* and *S. floridae*, *S. asterosporus* and *S. calvus*, etc. (Kämpfer 2012). However, our strain could be distinguished from *S. coerulescens* and *S. bellus* by some phenotypic properties such as the color of aerial mycelium (blue for these two species), the absence of the production of melanoid pigment on ISP6 and ISP7 media, the utilization of raffinose and rhamnose (differs from *S. coerulescens*), and the decarboxylation of sodium citrate and sodium succinate (differs from *S. bellus*) (Kämpfer 2012).

Antistaphylococcal activity

The antistaphylococcal activity of the strain G60 on ISP2 medium is shown in Table 3. The strain was active against all target staphylococci, with diameter of inhibitory zone varied from 21 to 30 mm. It showed stronger activity against *S. aureus* ATCC 25923, *S. aureus* ATCC 43300, *S. aureus* 636 and *S. aureus* S1, and strong to moderate activity against *S. aureus* 38, *S. aureus* 39 and *S. aureus* R2. Several strains of *Streptomyces* belonging to different species were already reported as active against strains of *S. aureus* (Smaoui et al. 2012; Mercy Rajan et al. 2013).

Table 3 Antistaphylococcal activities of the strain G60 on ISP2 medium

Target staphylococci	Activity of G60 (in mm) [a]
Staphylococcus aureus ATCC 25923	27.2±0.6
S. aureus ATCC 43300	26.5±1.0
S. aureus 38	20.0±1.0
S. aureus 39	21.0±1.0
S. aureus 636	30.7±1.2
S. aureus S1	27.5±1.0
S aureus R2	21.5±1.0

[a] Average ± standard deviation from three replicates per treatment

Effect of different culture media on bioactive compound

The effect of different culture media on antimicrobial production of the strain G60 was carried out in batch culture. The strain was able to grow in all the tested media. The antistaphylococcal activity was detected in all broth culture media (ESM Fig. 1). The strain was slightly more active against sensitive *S. aureus* ATTC 25923 than methicillin-resistant *S. aureus* MRSA ATTC 43300 on all the culture media used. It showed a good activity on ISP2, Bennett and GBM, moderate activity on SBM, PBM, YEBM, GYE and GME, and weak activity in ABM, MEBM and GP culture media. The activity on ISP2 medium was generally better than on Bennett and GBM media.

Time course of growth and bioactive compound production

Fermentations were carried out in the broth culture media showing good antimicrobial activity, including ISP2, Bennett and GBM. During the time course of fermentation in those broth media, antistaphylococcal activity, dry cell weight and pH parameters were monitored as shown in Fig. 2. The activity of the strain G60 against *S. aureus* ATCC 43300 started at the mid-exponential phase of growth (2 days) in ISP2, Bennett and GBM media, and reached a maximum after 4 days (in ISP2) and 5 days (on Bennett and GBM media). These activities were persistent until the end of the incubation. The pH kinetics showed variation between 5.6 and 8.4 during the incubation. The maximum dry mycelia weight (8.56 g/L) was reached at 4 days of fermentation on ISP2. The best activity was obtained in ISP2 medium, which was chosen as the production medium for the antibacterial compound.

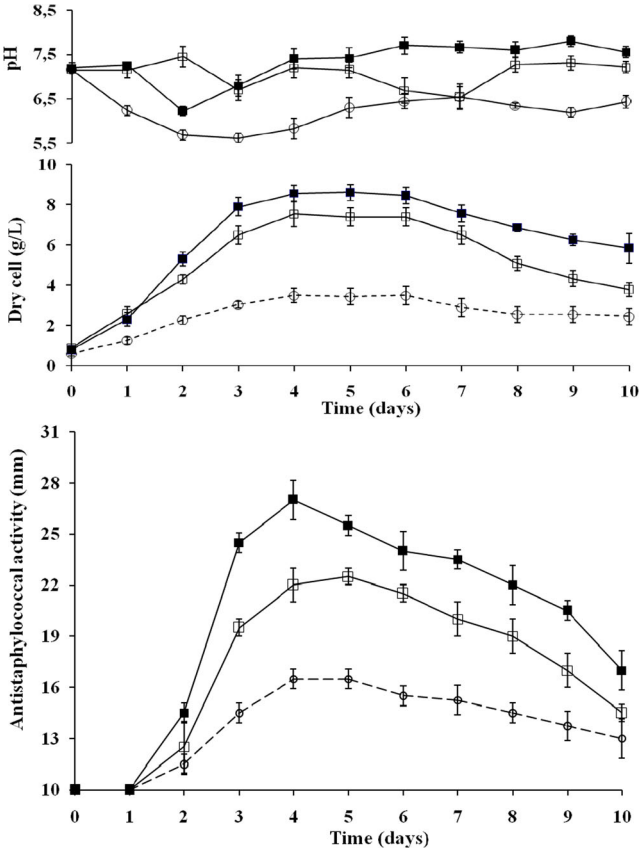

Fig. 2 Time course of pH, growth and antistaphylococcal activity against *Staphylococcus aureus* ATCC 43300 on ISP2 (*black squares*), Bennett (*white squares*) and glucose-basal medium (GBM; *white circles*) broth media. Measurements of activity against bacteria are given as diameter of inhibition, including the diameter of wells (10 mm). Each measure represents average ± standard deviation from three replicates per treatment

Isolation and purification of the bioactive compound

Antistaphylococcal activity was detected on all organic extracts tested, but was highly active on the *n*-hexane extract against *S. aureus* ATCC 43300, producing a maximum inhibitory zone of 18 mm. On a silica gel thin-layer chromatogram, the resolved *n*-hexane extract gave one bioautographic compound, which was active against *S. aureus*. The compound (Rf=0.8 in ethyl-acetate-methanol, 100–15 v/v), showed a strong antistaphylococcal activity and a strong absorbance under UV at 254 nm. This active compound was selected and purified by HPLC using a reverse-phase column. The HPLC profiles obtained showed a peak of antistaphylococcal activity, which was designated G60H (retention time: 39.4 min, with 80 % methanol in water). This peak was collected and re-injected into the HPLC system until total purification was achieved (ESM Fig. 2a, b). The pure bioactive compound had a light yellowish color.

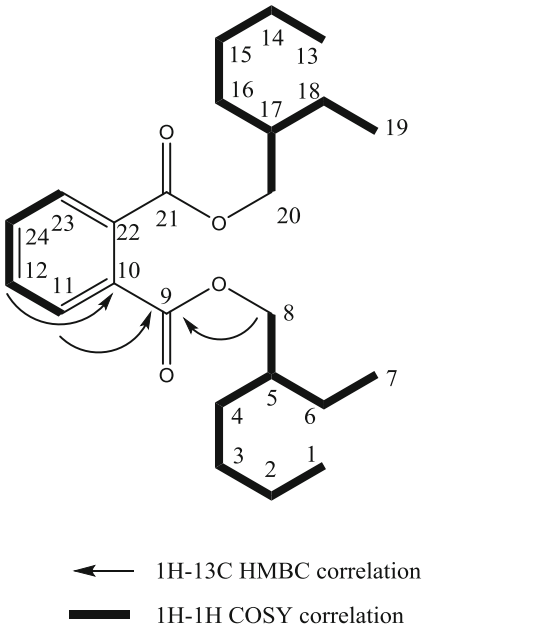

Fig. 3 Structure of the compound G60H and COSY and HMBC correlation

Table 4 ^{1}H and ^{13}C NMR data assignments of compound G60H in CD_3OD at 298 K (see Fig. 3 for numbering of hydrogen and carbon atoms)

^{1}H and ^{13}C number	^{1}H chemical shift, ppm	^{13}C chemical shift, ppm
1–13	0.94 (6H, t, 7.7Hz)	13.0
2–14	1.36 (4H, m)	22.6
3–15	1.37 (4H, m)	28.8
4–16	1.40 (4H, m)	30.2
5–17	1.71 (2H, sep, 6.0Hz)	38.7
6–18	1.45 (4H, m)	23.5
7–19	0.96 (3H, t, 7.4Hz)	10.0
8–20	4.22–4.25 (4H, dd, 11.0, 6.0Hz)	67.6
9–21	–	167.9
10–22	–	132.1
11–23	7.74 (2H, dd, 5.6, 3.4Hz)	128.4
12–24	7.64 (2H, dd, 5.6, 3.4Hz)	130.8

Antistaphylococcal activity of the purified compound

The purified compound G60H exhibited activity against all strains of *S. aureus*. The inhibition diameters (including the diameter of the disk) were 24, 23, 19, 20, 26, 24 and 20 mm against *S. aureus* ATCC 25923, *S. aureus* ATCC 43300, *S. aureus* 38, *S. aureus* 39, *S. aureus* 636, *S. aureus* S1 and *S. aureus* R2, respectively.

Spectroscopic studies

The UV-visible spectra of the pure product G60H exhibited maxima at 209.1, 247.3 and 291.5 nm, suggesting the presence of an aromatic ring. Its mass spectrum was obtained in positive and negative mode. The positive mode yielded with sodium [M+Na]=413.5 (ESM Fig. S3). Thus, the molecular weight of the antibiotic is M=390.5. The ^{1}H and ^{13}C chemical shifts of compound G60H are given in Table 4 and its structure can be seen in Fig. 3. The ^{1}H NMR spectrum revealed an AA'XX' system characteristic of a disubstituted ortho aromatic ring (δH 7.64 and 7.74. 4H, m). The HSQC and HMBC spectra show 24 carbon signals for the G60H molecule. It was possible to discern two ester groups (δ_c, 167.90), 16 sp^3-hybridized carbons (δ_c 13.0 to 67.6) and 2 sp^2-hybridized carbons (δ_c 132.20). The 2D ^{1}H-^{1}H and ^{1}H-^{13}C values established the connectivity between the groups of the G60H molecule, which has a di-(2-ethylhexyl) phthalate (DEHP) structure.

The DEHP is a bioactive compound belonging to the family of phthalates containing aromatic benzene. This family of petrochemical products is known as plasticizers or solvents in a variety of industrial products (Namikoshi et al. 2006).

DEHP is produced by some species and strains of *Streptomyces*: *S. melanosporofaciens* (Si-Kwan et al. 1991), *S. bangladeshensis* (Alim Al-Bari et al. 2005), a marine *Streptomyces* sp. 195–02 (Li et al. 2008), *Streptomyces* sp. SB9 (Lyutskanova et al. 2009), a psychrotolerant, *Streptomyces* sp. 6803 (Chen et al. 2010), *Streptomyces* sp. TN17 (Smaoui et al. 2011) and *S. mirabilis* strain NSQu-25 (El-Sayed 2012), and also by *Nocardia levis* MK-VL_113 (Kavitha et al. 2009), *Actinoalloteichus* sp. AH97 (Boudjelal et al. 2011) and a brown algae *Sargassum wightii* (Sastry and Rao 1995). In addition to its antibacterial activity, it has been reported that di-(2-ethylhexyl) phthalate had other biological activities such as anti-leukemic and anti-mutagenic effects (Lee et al. 2000). However, dibutyl phthalate (DBP)—another derivative of the phthalate family—is produced by *Streptomyces albidoflavus* (Roya 2006), *Streptomyces* sp. TN256 (Smaoui et al. 2012) and *Streptomyces nasri* (El-Naggar 1997). DBP was reported as antimicrobial product, and was also used as a peroxisome proliferator. It is an effective compound against demodicidosis (Yuan et al. 2001) as well as an endocrine disruptor with estrogenic compound (Marchetti et al. 2002) and also as antitumor compound (Mabrouk et al. 2008). Furthermore, other phthalate derivatives were also isolated from terrestrial and marine organisms including medicinally plants (Rowshanul Habib and Rezaul Karim 2009) and fungal culture broths (Amade et al. 1994).

Our experiments were repeated three times at different periods to confirm that the DEHP produced by strain G60 was a natural product and not an impurity.

References

Alim Al-Bari AA, Bhuiyan SA, Flores ME, Petrosyan P, Garcia-Varela M, Islam AU (2005) *Streptomyces bangladeshensis* sp. nov., isolated from soil, which produces bis-(2-ethylhexyl) phthalate. Int J Syst Evol Microbiol 55:1973–1977

Amade P, Mallea M, Bouaicha N (1994) Isolation, structural identification and biological activity of two metabolites produced by *Penicillium olsonii* Bainier and Sartory. J Antibiot 47:201–207

Angebault C, Andremont A (2013) Antimicrobial agent exposure and the emergence and spread of resistant microorganisms: issues associated with study design. Eur J Clin Microbiol Infect Dis 32:581–595

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 Microbiol Biotechnol 28:943–951

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

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

Boubetra D, Sabaou N, Zitouni A, Bijani C, Lebrihi A, Mathieu F (2013a) Taxonomy and chemical characterization of new antibiotics produced by *Saccharothrix* SA198 isolated from a Saharan soil. Microbiol Res 168:223–230

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

Boudjelal F, Zitouni A, Mathieu F, Lebrihi A, Sabaou N (2011) Taxonomic study and partial characterization of antimicrobial compounds from a moderately halophilic strain of the genus *Actinoalloteichus*. Braz J Microbiol 42:835–845

Boudjella H, Zitouni A, Coppel C, Mathieu F, Monje MC, Sabaou N, Lebrihi A (2010) Antibiotic R2, a new angucyclinone compound from *Streptosporangium* sp. Sg3. J Antibiot 63:709–711

Chen M, Xie LJ, Zhou JR, Song YY, Wang RL, Chen S, Su YJ, Zeng RS (2010) Collection, purification and structure elucidation of allelochimcals in *Streptomyces* sp. 6803. Allelopathy J 25:93–105

Clardy J, Fischbach MA, Walsh CT (2006) New antibiotics from bacterial natural products. Nat Biotechnol 24:1541–1550

Coates ARM, Hu Y (2007) Novel approaches to developing new antibiotics for bacterial infections. Br J Pharmacol 152:1147–1154

El-Naggar MYM (1997) Dibutyl phthalate and the antitumor agent F5A1, two metabolites produced by *Streptomyces nasri* submutant H35. Biomed Lett 55:125–131

El-Sayed MH (2012) Di-(2-ethylhexyl) phthalate, a major bioactive metabolite with antimicrobial and cytotoxic activity isolated from

the culture filtrate of newly isolated soil *Streptomyces* (*Streptomyces mirabilis* strain NSQu-25). World Appl Sci J 20:1202–1212

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

Fuller E, Elmer C, Nattress F, Ellis R, Horne G, Cook P, Fawcett T (2005) β-Lactam resistance in *Staphylococcus aureus* cells that do not require a cell wall for integrity. Antimicrob Agents Ch 49:5075–5080

Green BN, Johnson CD, Egan JT, Rosenth M, Griffith EA, Evans MW (2012) Methicillin-resistant *Staphylococcus aureus*: an overview for manual therapist. J Chiropr Med 1:64–76

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, Kreig NR, Sneath PHA, Staley JT, Williams S (1994) Bergey's Manual of Determinative Bacteriology, 9th edn. Williams and Wilkins, Baltimore

Kämpfer P (2012) Genus I. *Streptomyces* Waksman and Henrici 1943. In: Goodfellow A et al (eds) Bergey's manual of systematic bacteriology, 5th edn. Springer, New York, pp 1455–1767

Kavitha A, Prabhakar P, Vijayalakshmi M, Venkateswarlu Y (2009) Production of bioactive metabolites by *Nocardia levis* MK-VL_113. Lett Appl Microbiol 49:484–490

Kelly KL, Judd DB (1976) Color. Universal Language and Dictionary of Names–National bureau of standards, special publication 440. US Department of Commerce, Washington

Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, Park SC, Jeon YS, Lee JH et al (2012) Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol 62:716–721

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Labeda DP, Goodfellow M, Brown R, Ward AC, Lanoot C, Vanncanneyt M, Swings J, Kim S-B, Liu Z et al (2012) Phylogenetic study of the species within the family *Streptomycetaceae*. Antonie Van Leeuwenhoek 101:73–104

Lechevalier HA, Lechevalier MP (1970) Composition of whole-cell hydrolysates as a criterion in the classifcation of aerobic actinomycetes. In: Prauser H (ed) The actinomycetales. Fischer, Jena, pp 393–404

Lechevalier MP, de Bievre C, Lechevalier HA (1977) Chemotaxonomy of aerobic actinomycetes: phospholipid composition. Biochem Syst Ecol 5:249–260

Lee KH, Kim JH, Lim DS, Kim CH (2000) Antileukaemic and antimutagenic effects of di (2.ethylhexyl) phthalate isolated from *Aloe vera* Linne. J Pharm Pharmacol 52:593–598

Li CY, Ding WJ, She ZG, Lin YC (2008) Secondary metabolites of a marine actinomycete *Streptomyces* sp. (No. 195–02) from South China Sea. J Chin Med Mater 31:645–647

Liu D, Coloe S, Baird R, Pedersen J (2000) Rapid mini-preparation of fungal DNA for PCR. J Clin Microbiol 38:471

Locci R (1989) Streptomycetes and related genera. In: Williams ST, Sharpe ME, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 4. Williams and Wilkins, Baltimore, pp 2451–2492

Loomba PS, Taneja J, Mishra B (2010) Methicillin and vancomycin resistant *S. aureus* in hospitalized patients. J Glob Infect Dis 2: 275–283

Lyutskanova D, Ivanova V, Stoilova-Disheva M, Kolarova M, Aleksieva K, Peltekova V (2009) Isolation and characterization of a psychrotolerant *Streptomyces* strain from permafrost soil in spitsbergen, producing phthalic acid ester. Biotechnol Biotec Eq 23: 1220–1224

Mabrouk AM, Kheiralla ZH, Hamed ER, Youssry AA, Abdel Aty AA (2008) Production of some biologically active secondary metabolites from marine-derived fungus*Varicosporina ramulosa*. Malays J Microbiol 4:14–24

Marchetti L, Sabbieti MG, Menghi M, Materazzi S, Hurley MM, Manghi G (2002) Effect of phthalate esters on actin cytoskeleton of Py1a rat osteoblasts. Histol Histopathol 17:1061–1066

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 Van Leeuwenhoek 103: 771–776

Mercy Rajan B, Kannabiran K (2013) Antimicrobial activity of *Streptomyces albofaciens* against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus* multi-drug resistant species. Res J Pharm Biol Chem 4:1248–1257

Minnikin DE, Patel PV, Alshamahony L, Goodfellow M (1977) Polar lipid composition in the classifcation of *Nocardia* and related bacteria. Int J Syst Bacteriol 27:104–117

Namikoshi M, Fujiwara T, Nishikawa T, Ukai K (2006) Natural abundance ^{14}C content of dibutyl phthalate (DBP) from three marine algae. Mar Drugs 4:290–297

Pantosti A, Sanchini A, Monaco M (2007) Mechanisms of antibiotic resistance in *Staphylococcus aureus*. Future Microbiol 2:323–34

Rowshanul Habib M, Rezaul Karim M (2009) Antimicrobial and cytotoxic activity of Di-(2-ethylhexyl) phthalate and anhydrosophoradiol-3-acetate isolated from *Calotropis gigantea* (Linn.) Flower. Mycobiology 37:31–36

Roya RN, Laskar S, Sena SK (2006) Dibutyl phthalate, the bioactive compound produced by *Streptomyces albidoflavus* 321.2. Microbiol Res 161:121–126

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

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

Sastry VMVS, Rao GRK (1995) Dioctyl phthalate, an antibacterial compound from the marine brown alga *Sargassum wightii*. J Appl Phycol 7:185–186

Shirling EB, Gottlieb D (1966) Methods for characterization of *Streptomyces* species. Int J Syst Bacteriol 16:313–340

Si-Kwan K, Kim SS, Kim KS, Chung YR, Kim CH (1991) New antibiotics produced by *Streptomyces melanosporofaciens*. II. Antimicrobial activities and isolation, purification, and structure determination of the active compound. Kor J Appl Microbiol Biotechnol 19:235–241

Smaoui S, Mellouli L, Lebrihi A, Coppel Y, Ben Fguiraa LF, Mathieu F (2011) Purification and structure elucidation of three naturally bioactive molecules from the new terrestrial *Streptomyces* sp. TN17 strain. Nat Prod Res 25:806–814

Smaoui S, Mathieu F, Elleuch L, Coppel Y, Merlina G, Karray-Rebai I, Mellouli L (2012) Taxonomy, purification and chemical characterization of four bioactive compounds from new *Streptomyces* sp. TN256 strain. World J Microb Biotechnol 28:793–804

Solecka J, Zajko J, Postek M, Rajnisz A (2012) Biologically active secondary metabolites from actinomycetes. Cent Eur J Biol 7:373–390

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Tawiah AA, Gbedema SY, Adu F, Boamah VE, Annan K (2012) Antibiotic producing microorganisms from River Wiwi, Lake Bosomtwe and the Gulf of Guinea at Doakor Sea Beach, Ghana. BMC Microbiol 12:234–242

Thakur D, Yadav A, Gogoi BK (2007) Isolation and screening of *Streptomyces* in soil of protected forest areas from the state of

Assam and Tripura, India for antimicrobial metabolites. J Mycol Med 17:242–249

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Tong SYC, Chen LF, Fowler VG (2012) Colonization, pathogenicity, host susceptibility, and therapeutics for *Staphylococcus aureus*: what is the clinical relevance. Semin Immunopathol 34:185–200

Von Nussbaum F, Brands M, Hinzen B, Weigand S, Habich D (2006) Antibacterial natural products in medicinal chemistry—exodus or revival? Angew Chem Int Edn Engl 45:5072–5129

Waksman SA (1961) The actinomycetes, classification, identification and descriptions of genera and species, vol 2. Williams and Wilkins, Baltimore

Watve MG, Tickoo R, Jog MM, Bhole BD (2001) How many antibiotics are produced by the genus *Streptomyces*? Arch Microbiol 176:386–390

Williams ST, Goodfellow M, Alderson G (1989) Genus *Streptomyces* Waksman and Henrici. In: Williams ST, Sharpe ME, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 4. Williams and Wilkins, Baltimore, pp 2452–2492

Yuan FS, Guo SL, Qin ZX, Deng SH, Huang GH (2001) Effect of dibutyl phthalate on demodicidosis. Chin J Parasitol Parasit Dis 19:160–162