# *Saccharothrix* sp. PAL54, a new chloramphenicol-producing strain isolated from a Saharan soil

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**Abstract** An actinomycete strain designated PAL54, producing an antibacterial substance, was isolated from a Saharan soil in Ghardaïa, Algeria. Morphological and chemical studies indicated that this strain belonged to the genus *Saccharothrix*. Analysis of the 16S rDNA sequence showed a similarity level ranging between 96.9 and 99.2% within *Saccharothrix* species, with *S. longispora* DSM 43749$^T$, the most closely related. DNA–DNA hybridization confirmed that strain PAL54 belonged to *Saccharothrix longispora*. It showed very strong activity against pathogenic Gram-positive and Gram-negative bacteria responsible for nosocomial infections and resistant to multiple antibiotics. Strain PAL54 secreted the antibiotic optimally during mid-stationary and decline phases of growth. One antibacterial compound was isolated from the culture broth and purified by HPLC. The active compound was elucidated by uv-visible and NMR spectroscopy and by mass spectrometry. The results showed that this compound was a D(−)-threo chloramphenicol. This is the first report of chloramphenicol production by a *Saccharothrix* species.

**Keywords** Actinomycete · Taxonomy · *Saccharothrix* · Antibacterial activities · Chloramphenicol

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## Introduction

Because of the increasing resistance of pathogenic microorganisms to antibiotics, research has intensified to discover new bioactive molecules. Several published studies have reported the emergence of new bacterial strains resistant to many antibiotics including some clinically used cephalosporins of 3rd and 4th generation (Katsumi et al. 2005; Sekhsokh et al. 2008). Studies on the emergence of multidrug-resistant bacteria responsible for nosocomial infections have also been reported in Algeria (Touati et al. 2006; Aggoune-Khinache et al. 2008; Messai et al. 2008). Recently, a new gene called New Delhi metallo-beta-lactamase (NDM-1) was discovered in several enterobacteria. It allows them to synthesize an enzyme inactivating most beta-lactam antibiotics used in therapy and thus constitutes a real health hazard (Kumarasamy et al. 2010).

Actinomycetes are particularly interesting for their high capacity to produce secondary metabolites with diverse chemical structures (Valan Arasu et al. 2008). It has been estimated that approximately two-thirds of natural antibiotics have been isolated from actinomycetes, and about 75% are produced by members of the genus *Streptomyces* (Solanki et al. 2008). However, in recent years, the rate of discovery of new antibiotics in the genus *Streptomyces* has been declining and isolation of other actinomycete genera

appears to be necessary to find novel strains producing commercially valuable antibiotics. Many interesting antibiotics are also produced by other genera of actinomycetes such as *Micromonospora, Nocardia*, *Nocardiopsis, Saccharothrix, Actinomadura, Actinoplanes, Amycolatopsis, Saccharopolyspora,* and *Streptosporangium* (Genilloud et al. 2011).

Intensive programs looking for antibiotics are running worldwide. The approaches considered in these research programs include the isolation of new antibiotics from actinomycetes other than the genus *Streptomyces* and the exploration of new and uncommon ecosystems. Algerian Saharan soils are exposed to an arid climate and represent particular ecosystems. In our laboratory, during a screening program of the search for new antibiotics from non-*Streptomyces* genera, selective isolation methods were used to isolate a number of active actinomycete strains from Algerian Saharan soils (Sabaou et al. 1998), and novel antibiotic molecules were obtained (Lamari et al. 2002; Zitouni et al. 2004b; Boudjella et al. 2010; Merrouche et al. 2010).

As part of this program, an actinomycete strain PAL54 was isolated and identified as belonging to the genus *Saccharothrix.* It showed interesting activity against pathogenic Gram-positive and Gram-negative bacteria responsible for nosocomial infections and resistant to many antibiotics.

This paper reports the taxonomy of the organism, and the production, purification and structure elucidation of the active molecule.

## Materials and methods

### Strain isolation

The actinomycete strain designated PAL54 was isolated from a Saharan soil collected in Ghardaïa (latitude, 32°24′N; longitude, 03°48′E; altitude, 468 m). The dry soil sample was suspended in sterile distilled water and diluted. Aliquots (0.2 mL) of each dilution were spread onto chitin-vitamins agar (Hayakawa and Nonomura 1987). The medium was supplemented with polymyxin (25 mg $L^{-1}$) and cycloheximide (80 mg $L^{-1}$) to inhibit the growth of bacteria and fungi respectively. The plates were incubated at 30°C for 2 weeks.

### Morphological and chemical characterization

The genus of the collected isolate was identified by morphological characteristics and chemical analysis of cellular components. The morphological and cultural features were observed by naked-eye examination of 14 day-old cultures grown on various International *Streptomyces* Project (ISP) media: yeast extract–malt extract agar (ISP-2), oatmeal agar (ISP-3), inorganic salts–starch agar (ISP-4), glycerol–asparagine agar (ISP-5) (Shirling and Gottlieb 1966) and on Bennett medium. The micromorphology and sporulation were observed by light microscopy. Colors of aerial and substrate mycelia were determined with the ISCC-NBS color charts (Kelly and Judd 1976).

For chemotaxonomic analyses, biomass was obtained from a culture grown in shake ISP-2 medium (Shirling and Gottlieb 1966) and incubated at 30°C for 4 days. Diaminopimelic acid isomers, whole-cell sugar pattern and phospholipids were analyzed according to the methods of Becker et al. (1964), Lechevalier and Lechevalier (1970) and Minnikin et al. (1977) respectively.

### Physiological characterization

Production of melanoid pigments was tested on peptone-yeast extract-iron agar (ISP-6) and tyrosine agar (ISP-7) media (Shirling and Gottlieb 1966). The assimilation of carbohydrate as sole carbon source was determined on ISP-9 medium (Shirling and Gottlieb 1966). The decomposition of hypoxanthine and xanthine and assimilation of amino acid as sole nitrogen source were evaluated as described by Locci (1989). Degradation of starch and production of nitrate reductase were determined as previously described (Marchal et al. 1987). Sensitivities to sodium chloride (4 and 7% w/v) and growth at 45°C were evaluated on Bennett medium.

### DNA extraction, 16S rDNA sequencing, phylogenetic analysis and DNA–DNA hybridization

DNA was extracted according to the method of Liu et al. (2000). The strain PAL54 was grown at 30°C for 4 days with agitation (250 rpm) in a 500 mL flask containing 100 mL of ISP-2 medium. The 16S rDNA was amplified by PCR using an Invitrogen kit and two primers: 27f (5′-AGAGTTT GATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTT ACGACTT-3′). The final 50-µL volume of reaction mixture contained 1× PCR buffer (Tris–HCl 10 mM; KCl 50 mM; pH 9.0 at 25°C), 1.5 mM of $MgC1_2$, 200 mM of each dNTP, 1 mM of each primer, 1.25 U of Taq DNA polymerase and 1 µL (500 ng) of the purified DNA. PCR amplification of the 16 rDNA was carried out on a Stratagene RoboCycler Gradient 96. The conditions for thermal cycling were as follows: denaturation of the target DNA at 98°C for 3 min followed by 30 cycles of 94°C for 1 min, primer annealing at 52°C for 1 min and primer extension at 72°C for 2 min. At the end of the cycling, the reaction mixture was held at 72°C for 10 min and then cooled to 4°C. The PCR product was detected by agarose gel electrophoresis and was visualized by ultraviolet (uv) fluorescence after ethidium bromide staining.

The sequencing reaction was performed by the Mille-Gen Company (Toulouse, France). The same primers as before and an automated sequencer were used for this purpose. The sequence obtained was compared for similarity level with available sequences of the reference species of bacteria contained in the GenBank database, using the ''NCBI Blast'' available at the ncbi-nlm-nih.gov website. Phylogenetic and molecular evolutionary analyses were carried out using software included in the MEGA version 3.0 (Kumar et al. 2004) package. The 16S rDNA sequence of strain PAL54 was aligned using the CLUSTAL W program (Thompson et al. 1994) against corresponding nucleotide sequences of representatives of the genus *Saccharothrix* recovered from GenBank. Evolutionary distance matrices were generated as described by Jukes and Cantor (1969) and a phylogenetic tree was constructed using the neighbor-joining method of Saitou and Nei (1987). The topology of the tree was evaluated by bootstrap analysis (Felsenstein 1985) using 1,000 resamplings.

DNA was extracted from cells using Marmur's method (1961) and purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). DNA–DNA relatedness between strains was determined as described previously (Zitouni et al. 2004a) in 5× SSC (1× SSC is 0.15 M sodium chloride and 0.015 M sodium citrate) and 20% dimethyl sulfoxide at 66°C (melting point −23°C) by the method of De Ley et al. (1970), using a Perkin Elmer Lambda 35UV/VIS spectrophotometer fitted with the Peltier temperature controller, PTP-1. The experiments were performed in duplicate.

## Antimicrobial activity

Antimicrobial activity was evaluated on ISP-2 and Bennett media by the streak method against various microorganisms. The experiment was done firstly by streaking a straight line of the PAL54 inoculum across the surface of medium on 90-mm-diameter plates and incubating at 30°C for 10 days. After the growth of the isolate PAL54, target microorganisms were seeded in streaks crossing the actinomycete culture. The antimicrobial activity was appreciated by measuring the length of inhibition between target microorganisms and actinomycete colony margins.

The target microorganisms, isolated from sick patients in hospitals of Algeria, were mostly multiresistant to antibiotics (Table 1). They included Gram-positive (*Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* S1) and Gram-negative (*Acinetobacter baumanii* E16, *Enterobacter cloacae* E10 and E13, *Escherichia coli* E52 and E195, *Klebsiella pneumoniae* E40 and K44, *Salmonella enterica* E32 and *Pseudomonas aeruginosa* IPA1) bacteria, yeasts (*Saccharomyces cerevisiae* ATCC 4226 and *Candida albicans* IPA200) and filamentous fungi (*Aspergillus carbonarius* M333 and *Fusarium culmorum* FC1). The measurements of distance of inhibition represent the average of two experiments.

## Time course of growth and antibiotic production

Fermentations were conducted in Bennett broth. A seed culture was prepared with the same medium and used to inoculate a 500 mL Erlenmeyer flask containing 100 mL

**Table 1** Resistance patterns of target bacteria

| Microorganisms | Resistance to | Sensitivity to |
|---|---|---|
| *Bacillus subtilis* ATCC 6633 | NEO | C, CAR, CHL, ERY, GEN, K, RIF, SPI, SSS, VAN |
| *Staphylococcus aureus* S1 | CAR, GEN, K, NEO, OLE, SPI, VAN | C, CHL |
| *Acinetobacter baumanii* E16 | AMC, ATM, CFP, CTX, FEP, GEN, PIP, TOB | AMC, C, CXC, FOX, TCC, TIC, TZP |
| *Enterobacter cloacae* E10 | AMC, ATM, CAZ, CFP, CTX, FEP, GEN, PIP, TCC, TIC, TOB | C, CXC, FOX, TZP |
| *Enterobacter cloacae* E13 | AMC, ATM, CFP, CTX, FEP, FOX, GEN, PIP, TCC, TIC, TOB, TZP | C, CXC |
| *Escherichia coli* E52 | AMX, ATM, CAZ, CFP, CTX, FEP, GEN, PIP, TIC, TOB | AMC, C, CXC, FOX, TCC, TZP |
| *Escherichia coli* E195 | AMC, AMX, CAZ, CF, CTX, CXM, FOX, TCC, TIC | C, CXC |
| *Klebsiella pneumoniae* E40 | AMX, CAZ, CFP, CTX, FEP, GEN, PIP, TIC, TOB | AMC, ATM, C, CXC, TCC |
| *Klebsiella pneumoniae* K44 | AMX, ATM, CF, CTX, CXM, GEN, K, MZ, SSS, TIC, TOB | AMC, C, CXC, TCC |
| *Salmonella enterica* E32 | ATM, CAZ, CFP, CTX, FEP, GEN, PIP, TIC, TOB | C, CXC, FOX, TCC, TZP |
| *Pseudomonas aeruginosa* IPA1 | CAR, ERY, GEN, NEO, SPI, SSS | C, CHL, K, RIF |

*AMC* amoxicillin + clavulanic acid, *AMX* amoxicillin, *ATM* aztreonam, *C* chloramphenicol, *CAR* carbenicillin, *CAZ* ceftazidim, *CF* cefalotin, *CFP* cefpirom, *CHL* chlortetracycline, *CTX* cefotaxime, *CXC* cefotaxime + clavulanic acid, *CXM* cefuroxime, *ERY* erythromycin, *FEP* cefepime, *FOX* cefoxitin, *GEN* gentamicin, *K* kanamycin, *MZ* mezlocillin, *NEO* neomycin, *OLE* oleandomycin, *PIP* piperacillin, *RIF* rifampicin, *SPI* spiramycin, *SSS* sulfonamide, *TCC* ticarcillin + clavulanic acid, *TIC* ticarcillin, *TOB* tobramycin, *TZP* piperacillin + tazobactam, *VAN* vancomycin

of Bennett. The cultures were incubated on a rotary shaker (250 rpm) at 30°C for 14 days. The antibacterial activities were assayed daily against *Bacillus subtilis* ATCC 6633 and *Klebsiella pneumoniae* E40 by the agar diffusion method (well technique). The growth (dry weight of mycelium) and the pH were also measured.

The amounts of the antibiotic produced were estimated from the 4th to 10th day by correlation between the diameters of inhibition obtained with the active compound present in the culture filtrate and those obtained with the purified active compound (as described in the following paragraph). In both cases the agar diffusion method (well technique) was used with *Bacillus subtilis* ATCC 6633 (which is the most sensitive) as target microorganism.

### Isolation and purification of antibiotic

The extraction of active compound took place on the day of optimal production rate. The Bennett culture broth (250 mL) was centrifuged to remove the biomass. The cell-free supernatant was extracted with an equal volume of dichloromethane. The organic extract was concentrated to dryness.

The resulting dry extract was recuperated in 0.5 mL of methanol and bioassayed against *Bacillus subtilis* ATCC 6633 and *Klebsiella pneumoniae* E40 by the paper disk diffusion method. Preparative chromatography with silica gel plates (Merck Art. 5735, Kiesselgel 60HF 254–366; 20 × 20 cm) was employed for the partial purification of antimicrobial products. 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 compounds were visualized under uv at 254 nm (absorbance) and at 365 nm (fluorescence), and the active spot was detected by bioautography (Betina 1973). The TLC plates were deposited in a plastic bioassay dish (23 cm × 23 cm × 2.2 cm, Fisher Scientific Labosi) and overlayed with 50 mL (per plate) of ISP-2 medium (containing 7 g $L^{-1}$ agar) seeded with *Bacillus subtilis* ATCC 6633 or *Klebsiella pneumoniae* E40 as target microorganisms, and incubated at 30°C for 24 h. A clear area due to the inhibition of the growth of target micro-organisms indicated the location of the antibiotic compound. The retention factor (Rf) of the active spot was measured.

The final purification of the antibiotic was performed by Waters reverse phase HPLC using an XBridge C18 (5 µm) column (200 × 10 mm, Waters) with a continuous linear gradient solvent system from 20 to 100% methanol in water, a flow rate of 2 mL $min^{-1}$ and uv detection at 220 and 254 nm. The final purification was achieved after the second re-injection in the HPLC system.

### Spectroscopic analysis of antibiotic

The uv absorption spectrum of the active molecule in methanol was determined with a Shimadzu uv 1,605 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 antibiotic compound in 600 µL of $CD_3OD$. 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

### Taxonomy

The strain PAL54 formed a well-developed aerial mycelium which fragmented anarchically into long, straight, flexuous, open loops and hooks. The spores were rod-shaped and 1.5–2.5 × 0.6–0.8 µm in size. The sporulation was better on ISP-2, ISP-5 and Bennett media. The substrate mycelium was branched and partially fragmented into rod-like elements. Endospores, sclerotic granules, synnemata and flagellated spores were not observed. The strain showed good growth on ISP-2, ISP-3, ISP-4 and Bennett media and moderate growth on ISP-5 medium. The aerial and substrate mycelia were white and yellowish brown respectively. No soluble pigment was produced on any of the media used.

The chemotaxonomic study of strain PAL54 showed the presence of DL-diaminopimelic acid isomer and the absence of glycin in the cell wall. The whole-cell hydrolysates contained rhamnose and galactose (in addition to glucose and ribose) as characteristic sugars, typical of cell wall type IIIE (Kroppenstedt and Evtushenko 2006). The diagnostic phospholipid detected was phosphatidylethanolamine, corresponding to phospholipid type PII (Lechevalier et al. 1977). Based on its morphological and chemical characteristics, strain PAL54 was classified in the

genus *Saccharothrix* (Labeda et al. 1984). This genus is reported to contain 11 species and two sub-species (Kim et al. 2011).

The 16S rDNA sequence (1,305 nucleotides) of strain PAL54 has been deposited in the GenBank data library and has been assigned the accession number JN225874.

This sequence was aligned with those of *Saccharothrix* reference species available in the GenBank database, which confirmed the identification of the strain at the genus level (similarity level between 96.9 and 99.2%). Its position in the 16S rDNA *Saccharothrix* tree is shown in Fig. 1. The similarity level was 99.2% with *Saccharothrix longispora* NRRL B-116116$^T$ (Grund and Kroppenstedt 1989), the most closely related species. The physiological properties of strain PAL54 and those of the type strain of *S. longispora* are summarized in Table 2. Two physiological differences are notable and involve melibiose and hypoxanthine degradation. Moreover, strain PAL54 has a white aerial mycelium, while the *S. longispora* aerial mycelium is blue. The level of DNA–DNA relatedness between strain PAL54 and *Saccharothrix longispora* DSM 43749$^T$ (=RRL B-116116$^T$) was 76.2% (based on the mean of duplicate determinations, 74.9 and 77.5%), which is above the 70% relatedness guideline proposed by Wayne et al. (1987) for delineation of separate species.

Based on the genotypic results, it was concluded that strain PAL54 belonged to the species *Saccharothrix longispora.* However, this strain could be distinguished from *S. longispora* DSM 43749$^T$ by some phenotypic properties such as the color of aerial mycelium and the degradation of melibiose and hypoxanthine.

## Antimicrobial activity

The antimicrobial activity of the strain PAL54 against target microorganisms is shown in Table 3. The strain showed a strong activity against Gram-negative and Gram-positive bacteria such *Bacillus subtilis* ATCC 6633, *Acinetobater baumanii* E16, *Staphylococcus aureus* S1, *Klebsiella pneumoniae* E40 and K44, *Escherichia coli* E52 and E195 and *Salmonella enterica* E32, but no activity against yeasts and filamentous fungi. Bennett medium was generally better than ISP-2 medium.

The activity of *Saccharothrix* species against Gram-positive bacteria has been widely published (Horvath et al. 1979; Takeuchi et al. 1992; Sabaou et al. 1998) but the activity against Gram-negative bacteria and fungi has been reported only rarely (Zitouni et al. 2005). Antitumoral activity has also been noted in several cases in strains of *Saccharothrix* (Vertesy et al. 2001; Murakami et al. 2009). No antibiotic activity is reported in the literature for *Saccharothrix longispora*, the species most closely related to strain PAL54.

## Time course of growth and antibiotic production

During the time course of fermentation in Bennett broth, antibiotic production, dry cell weight and pH parameters were monitored as shown in Fig. 2. The antibacterial activity of strain PAL54 started at the mid-stationary phase of growth (4 days for *Bacillus subtilis* ATCC 6663 and 5 days for *Klebsiella pneumonia*e E40) and reached a maximum after 9 days, during the decline phase. This confirmed that the activities were due to secondary

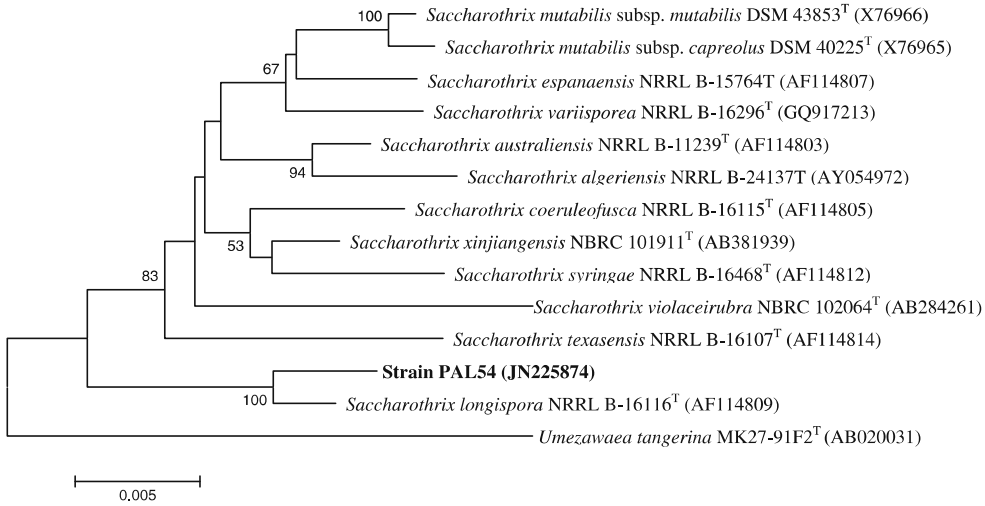


**Fig. 1** Neighbor-joining tree based on 16S rDNA sequences showing the relations between strain PAL54 and type species of the genus *Saccharothrix*. The *numbers* at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1,000 resampled data sets. *Bar*, 0.005 nt substitution per nt position. *Umezawaea tangerina* MK27-91F2$^T$ has been used as outgroup

**Table 2** Physiological properties of the strain PAL54 in comparison with those of *Saccharothrix longispora*

| Tests | Strains | |
|---|---|---|
| | PAL54 | *S. longispora* NRRL B-116116$^T$* |
| Carbon source utilization: | | |
| Arabinose | + | + |
| Fructose | + | + |
| Galactose | + | + |
| Inositol | – | – |
| Mannitol | – | – |
| Melibiose | + | – |
| Raffinose | – | – |
| Rhamnose | + | + |
| Salicine | + | + |
| Sucrose | + | + |
| Xylose | + | + |
| Nitrogen source utilization: | | |
| Alanine | ± | ± |
| Proline | + | + |
| Serine | + | + |
| Hydrolysis of: | | |
| Starch | + | + |
| Hypoxanthine | + | – |
| Xanthine | – | – |
| Nitrate reduction | + | + |
| Growth in the presence of NaCl: | | |
| 4% w/v | + | + |
| 7% w/v | – | – |
| Growth at 45 °C | – | – |

Tests: + positive;– negative; ± doubtful

* Data from Grund and Kroppenstedt (1989) and Labeda (2002)

metabolites. The pH kinetics showed slight variation (between 7.0 and 8.1) during the incubation.

The amounts of the active compound obtained during the 4th, 5th, 6th, 7th, 8th, 9th and 10th days were estimated to be 3.4, 4.2, 15, 26, 30, 31 and 31 mg $L^{-1}$, respectively.

## Isolation and purification of antibiotic

On silica gel thin-layer chromatogram, the dichloromethane extract migrated and gave one bioautographic compound, which was active against *Bacillus subtilis* ATCC 6633 and *Klebsiella pneumoniae* E40. The compound, named 54A (Rf = 0.74 in ethyl-acetate–methanol, 100–15 v/v), showed a strong antibacterial activity and a strong absorbance under uv at 254 nm. It was selected and purified by HPLC. The active fraction was eluted with 80% of methanol in water at a retention time of 22.80 min. This compound was recovered and re-injected until complete

**Table 3** Antimicrobial activity of strain PAL54

| Target microorganisms | Activity of strain PAL54 (in mm) | |
|---|---|---|
| | ISP-2 medium | Bennett medium |
| *Bacillus subtilis* ATCC 6633 | 40 | 39 |
| *Staphylococcus aureus* S1 | 37 | 47 |
| *Acinetobacter baumanii* E16 | 50 | 38 |
| *Enterobacter cloacae* E10 | 28 | 37 |
| *Enterobacter cloacae* E13 | 12 | 17 |
| *Escherichia coli* E52 | 33 | 45 |
| *Escherichia coli* E195 | 46 | 50 |
| *Klebsiella pneumoniae* E40 | 27 | 35 |
| *Klebsiella pneumoniae* K44 | 28 | 34 |
| *Salmonella enterica* E32 | 21 | 40 |
| *Pseudomonas aeruginosa* IPA1 | 22 | 10 |
| *Saccharomyces cerevisiae* ATCC 4226 | 0 | 0 |
| *Candida albicans* IPA200 | 0 | 0 |
| *Aspergillus carbonarius* M333 | 0 | 0 |
| *Fusarium culmorum* FC1 | 0 | 0 |

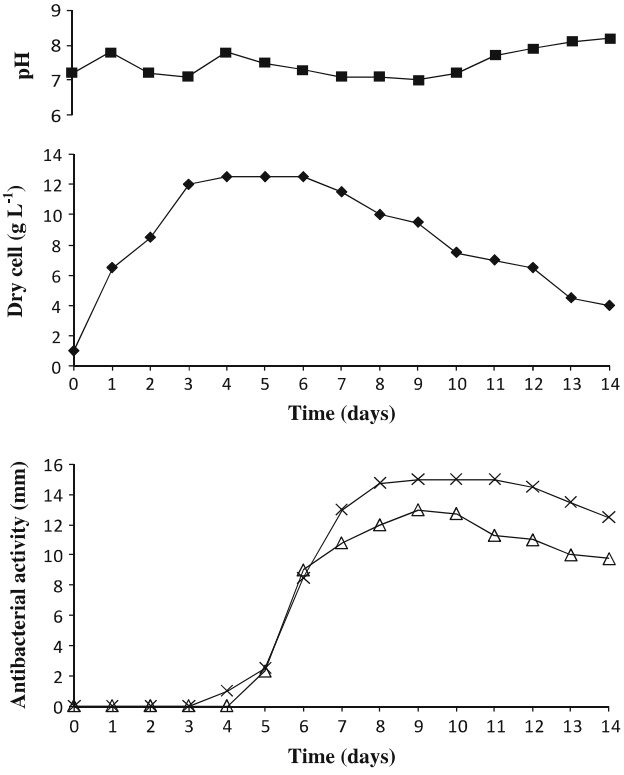


**Fig. 2** Time course of pH, growth and antibacterial activity in Bennett medium against *Bacillus subtilis* ATCC 6633 (*cross*) and *Klebsiella pneumoniae* E40 (*open triangle*). Measurements of activity against bacteria represent the diameters of inhibition without including the diameter of wells (10 mm). All experiments were repeated twice

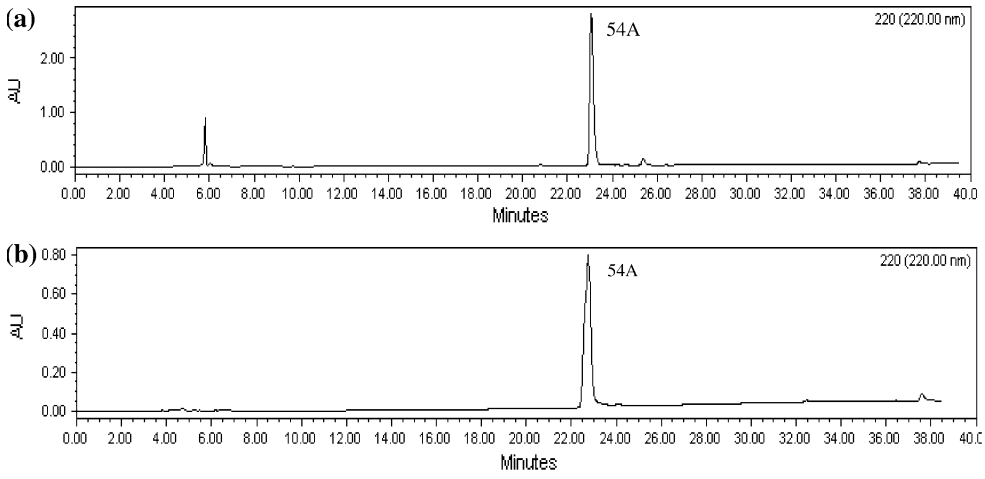


**Fig. 3** HPLC purification process for the antibiotic secreted by the strain PAL54. **a** First HPLC injection of the compound 54A (Rf = 0.74 in ethyl-acetate–methanol, 100–15 v/v) resulting from the TLC silica gel. **b** Final purification of the compound 54A after re-injection. Column, XBridge C18 (Waters); continuous linear gradient system, 20–100% methanol in water; flow rate, 2 mL $min^{-1}$; detection, 220 nm

purification (Fig. 3). A quantity of 5.5 mg of purified antibiotic was obtained from 250 mL of culture filtrate. The chromogenic reactions 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.

## Spectroscopic analysis of antibiotics

The uv-visible spectrum of antibiotic 54A showed maxima at 213 and 273 nm. The mass spectrum of the compound was obtained in positive and negative mode. The negative mode yielded a pseudo-molecular ion [M − H] = 321. Thus the molecular weight of antibiotic 54A is M = 322.

$^{1}H$ and $^{13}C$ NMR spectroscopy were used for the characterization of compound 54A. All the $^{1}H$ and $^{13}C$ signals were assigned on the basis of chemical shifts, spin–spin coupling constants, splitting patterns and signal intensities, and by using $^{1}H$-$^{1}H$ COSY45, $^{1}H$-$^{13}C$ HSQC and $^{1}H$-$^{13}C$ HMBC experiments. The $^{1}H$ and $^{13}C$ chemical shifts of compound 54A are given in Table 4 and Fig. 4.

The $^{13}C$ and HSQC spectra show 11 carbon signals. From the $^{13}C$ data, it was possible to discern one amide group ($\delta_c$ 165.2), five $sp^2$-hybridized carbons ($\delta_c$ from l50.2 to 122.8) and four $sp^3$-hybridized carbons bearing electronegative heteroatoms ($\delta_c$ 69.9–57.1). The $^{1}H$ NMR spectrum revealed AA'XX' system characteristic of dimeta substituted aromatic ring ($\delta_c$ 8.19 and 7.66, 4H, m) and a hydroxymethyl group with diastereostopic hydrogens ($\delta_c$ 3.63 and 3.83, 2H, dd, J = 6.0 Hz; 12.0 Hz). The hydrogens of the hydroxyl and amide groups were not observed due to rapid exchange with MeOD. The 2D $^{1}H$-$^{1}H$ and $^{1}H$-$^{13}C$ experiments, and especially the long range $^{1}H$-$^{13}C$ couplings observed in the HMBC spectrum (Fig. 5), permitted the connectivity between all the groups of the molecule to be established.

**Table 4** $^{1}H$ and $^{13}C$ NMR data assignments of 54A compound in $CD_3OD$ at 298 K

| $^{1}H$ and $^{13}C$ number | $^{1}H$ chemical shift ppm | $^{13}C$ chemical shift ppm |
|---|---|---|
| 1 | – | 147.2 |
| 2, 6 | 8.2 | 127.8 |
| 3, 5 | 7.7 | 127.0 |
| 4 | – | 150.2 |
| 7 | 5.2 | 69.9 |
| 8 | 4.2 | 57.1 |
| 9 | 3.6–3.8 | 60.8 |
| 10 | – | 165.2 |
| 11 | 6.3 | 66.0 |

See Fig. 4 for numbering of hydrogen and carbon atoms

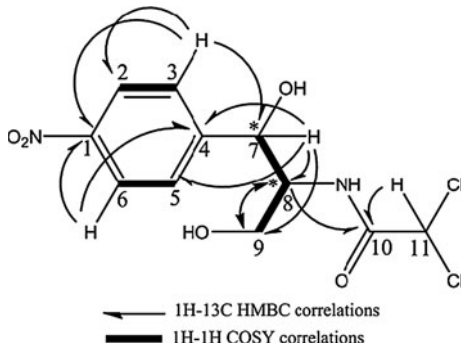


**Fig. 4** Hydrogen, carbon numbering, COSY and HMBC correlations of 54A compound. * asymmetric carbon atoms

The structure of compound 54A was determined by NMR and mass spectrometry to be 2,2-dichloro-N-(1,3-dihydroxy-1-(4-nitrophenyl)propan-2-yl)acetamide. This structure corresponds to that of chloramphenicol.

It can be seen that there are two asymmetric carbon atoms on chloramphenicol, leading to four possible stereoisomers. In this study, we used the Karplus relationship

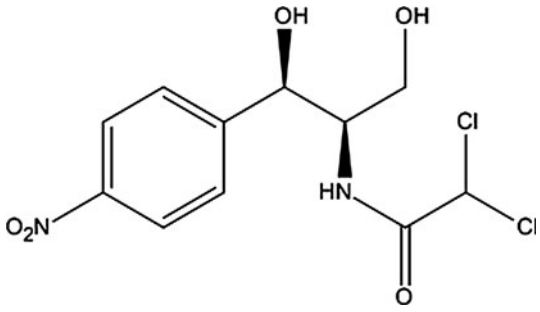


**Fig. 5** Structure of D(−)-threo chloramphenicol stereoisomer

in order to identify the stereoisomer 54A purified. The $^{3}$J-coupling constants for (H7-H8), (H7-C9) and (H8-C4) were around 3–4 Hz, which corresponds to a dihedral angle of 45–50°. Based on these results, there are 2 possible conformations: (R,R) or D(−)-threo form, and (S,S) or L(+)-threo form. Only the D(−)-threo form (the naturally occurring isomer) of chloramphenicol has biological activity (Yunis 1988). This suggests that the antibiotic 54A is D(−)-threo chloramphenicol (Fig. 5).

Chloramphenicol is an antibiotic belonging to the family of aromatic benzene. It is known to be produced by *Streptomyces venezuelae* and also *S. omiyaensis* and *S. phaeochromogenes*, but is also manufactured synthetically because of its relatively simple structure (Umezawa et al. 1949; Asselineau and Zalta 1973; Doull et al. 1983). It has never been reported in another microorganism. This antibiotic is active against Gram-positive and Gram-negative bacteria and against rickettsia, mycoplasma and chlamydia. It is bacteriostatic and inhibits peptide bond synthesis at the 50S ribosomal subunit by interfering with peptidyl transferase (Yunis 1988). The resistance to chloramphenicol of target bacteria used in this study might have allowed the selection of a chloramphenicol-producing strain.

*Saccharothrix* species have been reported to produce antibiotics belonging to aminoglycoside and benzoquinone (Takahashi et al. 1986), glycopeptide (Takeuchi et al. 1992), carboxylic nucleoside (Bush et al. 1993), dithiolopyrrolone (Lamari et al. 2002), heptadecaglycoside (Singh et al. 2000), anthracyclin (Zitouni et al. 2004b), macrolide (Murakami et al. 2009) and angucycline (Kalinovskaya et al. 2010) families. The fact that *Saccharothrix* produces chloramphenicol is itself quite original.

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