Streptomyces marokkonensis sp. nov., isolated from rhizosphere soil of Argania spinosa L.

B. Bouizgarne,1 B. Lanoot,2 S. Loqman,1 C. Spröer,3 H.-P. Klenk,3 J. Swings2 and Y. Ouhdouch1

1Laboratoire de Microbiologie, Faculté des Sciences Semlalia, Université Cadi Ayyad, P.B. S-15, Marrakech, Morocco
2BCCM/LMG Bacteria Collection, Gent University, Belgium
3DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany

The novel actinomycete strain Ap1T was isolated from rhizosphere soil of the argan tree (Argania spinosa L.) in the south of Morocco. Strain Ap1T has been reported as a novel producer of the pentaene polyene macrolide isochainin, which strongly inhibits the growth of pathogenic yeasts and phytopathogenic fungi. Strain Ap1T shows a greyish-white aerial mycelium with chains of smooth-surfaced spores of the Spiralis type and a cell wall containing L-9-diaminopimelic acid. Based on chemotaxonomy and morphological features, strain Ap1T was identified as a member of the genus Streptomyces. 16S rRNA gene sequence similarities based on almost-complete 16S rRNA gene sequences showed that strain Ap1T is closely associated with members of the Streptomyces violaceoruber species group (S. violaceoruber, S. coelestis, S. violaceorubidus, ‘S. caesius’, ‘S. lividans’, S. violaceolatus and S. humiferus) and others (Streptomyces aurantiogriseus, S. lienomycini, S. chattanoogensis, S. rubrogriseus and S. tendae). However, protein profiling, DNA–DNA hybridization and BOX-PCR fingerprinting proved a relationship above the species level. In addition, the phenotype also allowed for the differentiation of strain Ap1T from its closest neighbours. As a result of this polyphasic approach, we conclude that strain Ap1T represents a novel species of the genus Streptomyces, for which the name Streptomyces marokkonensis sp. nov. is proposed. The type strain is Ap1T (=R-22003T =LMG 23016T =DSM 41918T).

Actinomycetes are widespread Gram-positive bacteria with filamentous growth and high DNA G+C content (60–78 mol%). They are important micro-organisms that produce various useful enzymes (George et al., 2001; Nascimento et al., 2002) and secondary metabolites such as immunomodulators, antitumour compounds and antibiotics (Chater & Hopwood, 1993; Mann, 2001; Maskey et al., 2003; Cragg et al., 2005). Currently, over 23 000 microbial secondary metabolites are known, 42% of which are produced by actinobacteria and fungi each, whereas all other bacteria contribute 16% (Lazzarini et al., 2000). Among the actinobacteria, members of the genus Streptomyces produce 70–80% of known bioactive natural products (Berdy, 2005) and they are identified as the largest producers of antibiotics (Wathe et al., 2001). Members of the genus contribute about 55% of the 12 000 known secondary metabolites with antibiotic activity known to date (Woodruff, 1999).

Actinomycetes are powerful biocontrol tools against soil-borne fungal plant pathogens (Misato et al., 1977; Tanaka & Omura, 1993). The role of antibiotic production by actinomycetes in the control of plant pathogens has been known for a long time (Rothrock & Gottlieb, 1984). Some antifungal compounds are commercially available, e.g. cycloheximide from Streptomyces griseus, kasugamycin from Streptomyces kasugaensis, Blastcidin-S from Streptomyces griseochromogenes (Agrios, 1988), Rhizovit from Streptomyces rimosus (Marten et al., 2001) and Mycostop from Streptomyces griseoviridis (Tahvonen, 1982; Kortemaa et al., 1997).

In this paper, strain Ap1T, with antagonistic activity towards some phytopathogens (Bouizgarne et al., 2006), was shown to present a colonial morphology and cell-wall chemistry consistent with its assignment to the genus Streptomyces. In the present investigation, strain Ap1T was the subject of a polyphasic taxonomic study, to establish whether it represented a novel species of the genus...
**Streptomyces.** This investigation included morphological and physiological properties as well as 16S rRNA gene sequencing, DNA–DNA hybridization, protein profiling and BOX-PCR fingerprinting (Louws et al., 1994; Rademaker et al., 1998). The two latter techniques have proven to be powerful tools in **Streptomyces** taxonomy, giving a good correlation with DNA–DNA hybridization data for species delineation (Lanoot et al., 2002, 2004).

Strain Ap1T was isolated from a soil sample collected from the rhizosphere of an argan tree (*Argania spinosa* L.) as described by Bouizgarne et al. (2006). The isolate was grown on oatmeal agar [International *Streptomyces* Project (ISP) medium 3; Kuster, 1959] at 28 °C for 2 weeks and stored at room temperature. For long-term preservation, the strain was conserved in a 20 % glycerol suspension.

Morphological characteristics and cell chemistry of strain Ap1T were examined using procedures recommended by the ISP (Shirling & Gottlieb, 1966). Morphological characteristics were assessed by light microscopy and scanning electron microscopy using cultures grown at 28 °C for 3 weeks on Bennett's agar (Jones, 1949) and oatmeal agar. The colour of aerial mycelium was determined from mature sporulating aerial mycelium according to the scale adopted by Prauser (1964) and the colour was determined according to the system proposed by Nonomura (1974). Production of melanoid pigments was assayed on yeast extract-iron agar (ISP medium 6) and tyrosine agar (ISP medium 7) incubated at 28 °C for 21 h (Shirling & Gottlieb, 1966).

Carbohydrate utilization was determined on Pridham–Gottlieb carbon utilization agar medium (ISP medium 9) supplemented with sterile carbon sources (Shirling & Gottlieb, 1966). Gelatin hydrolysis and nitrate reduction were assessed according to Williams & Cross (1971). Sensitivity to NaCl was established by the method of Tresner et al. (1968). The response of growth to temperature was assayed on ISP medium 2 and Bennett's medium (Jones, 1949). Sensitivity to antibiotics by the disc-diffusion method was conducted on Olson's medium (Jones, 1949). Sensibility to antibiotics by the disc-diffusion method was conducted on Olson’s medium (Jones, 1949) with incubation at 28 °C for 21 h. The antimicrobial activity of strain Ap1T was determined by the plate diffusion method (Bauer et al., 1966). Bacterial cultures were incubated on nutrient agar at 37 °C for 24 h. Yeasts and other fungi were incubated on Sabouraud agar medium at 28 °C for 24 h for yeasts and 48 h for other fungi.

Detection of cell-wall dianipimelic acid isomers and the whole-cell sugar pattern was performed according to the protocol described by Lechevalier & Lechevalier (1980). Analysis of fatty acid methyl esters was carried out using the Microbial Identification System (Microbial ID) (Sasser, 1990). Determination of the G+C content was done using the protocol of Tamaoka & Komagata (1984). DNA was hydrolysed into nucleosides with nuclease P1 and bacterial alkaline phosphatase (Sigma) followed by separation using reversed-phase HPLC (Waters).

Genomic DNA was prepared according to the protocol of Lanoot et al. (2004). The 16S rRNA gene was amplified by using a combination of one of the conserved forward primers pA (5′-AGAGTTTGTGATCCCTGCGCAG-3′), MH1 (5′-AGTTTGTATCGTGGCTCAG-3′) or ARI C/T (5′-CTGGTGTAGGA(C/T)GAACGCTG-3′) and one of the reverse primers MH2 (5′-TACCTTGTTACGTTCACCTACCA-3′) or pH (5′-AAGGAGGTGATCCAGCCCAG-3′), respectively hybridizing at positions 8–27, 10–27, 19–38, 1507–1485 and 1541–1522, according to the *Escherichia coli* numbering system. PCR products were purified using NucleoFast 96 PCR Plates (Macherey-Nagel) according to the manufacturer’s instructions. Sequencing reactions were performed using the BigDye Terminator cycle sequencing kit (Applied Biosystems) and purified using the Montage SEQ service Sequencing Reaction Cleanup kit (Millipore), based on the protocols of the manufacturer. Sequencing was achieved using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Primers used for sequencing are listed in Coenye et al. (1999). Sequence assembly was performed using the program AutoAssembler (Applied Biosystems). The BioNumerics software package version 5.1 (Applied Maths) was used for multiple alignment with available almost-complete sequences of type strains of the family *Streptomycetaceae* and with available sequences of *Streptomyces* species downloaded from EMBL/GenBank. The neighbour-joining algorithm (Saitou & Nei, 1987) was used to infer the phylogenetic tree, taking into account the correction of Jukes & Cantor (1969). Tree topology was evaluated by bootstrap analyses (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings.

rep-PCR fingerprinting, using the BOX AIR primer, including normalization and numerical analysis, was performed as described by Lanoot et al., (2004).

For 16S–ITS restriction fragment length polymorphism (RFLP) analysis, both the 16S rRNA gene and adjacent 16S–23S intergenic transcribed spacer (ITS) region were amplified using conserved primers, followed by digestion with restriction enzymes *BstUI* and *HaeIII*, and then separated on an 8 % polyacrylamide gel. The fingerprints were compared with a corresponding database (Lanoot et al., 2005) containing more than 450 *Streptomyces* species with validly published names using the software package BioNumerics version 4.0.

Protein extracts were prepared according to the protocol of Manchester et al. (1990) with some modifications, as given in Lanoot et al. (2002). SDS-PAGE of whole-cell proteins, scanning and normalization of electrophoretic patterns were performed as described by Pot et al. (1994). The similarity between the patterns was calculated using Pearson’s product–moment correlation coefficient with the GelCompar software version 4.2. From the similarity matrix obtained, a dendrogram was constructed using the UPGMA algorithm.

DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on
hydroxyapatite as described by Cashion et al. (1977). DNA–DNA hybridization was carried out as described by De Ley et al. (1970), incorporating the modifications described by Huß et al. (1983), using a model Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostat 6 × 6 multicell changer and a temperature controller with in situ temperature probe (Varian).

Strain Ap1\textsuperscript{T} exhibited a range of chemotaxonomic and phenotypic properties typical of members of the genus \textit{Streptomyces}. Both aerial and vegetative mycelium are present in strain Ap1\textsuperscript{T} grown at 28 °C for 21 days on oatmeal agar. Electron microscopy observation of the greyish-white aerial mycelium showed hyphae bearing long spore chains of more than 20 spores (Fig. 1). Spore chains are of the \textit{Spiralis} type (Fig. 1). The spores are cylindrical with a smooth surface. No fragmentation of the vegetative mycelium or flagellated spores was observed. Cultural characteristics of strain Ap1\textsuperscript{T} are summarized in Table 1. Whole-cell hydrolysates of strain Ap1\textsuperscript{T} contained L-diaminopimelic acid, alanine, glycine and glutamic acid as the main components (cell wall type I; Lechevalier & Lechevalier, 1980). No characteristic sugars were found in cell hydrolysates. The fatty acid profile comprised mainly saturated straight-chain and iso- and anteiso-branched fatty acids (fatty acid type 2c \textit{sensu} Kroppenstedt, 1985), specifically \textit{i-C}_{16:0} (17.7 %), \textit{ai-C}_{17:0} (10.9 %), \textit{ai-C}_{15:0} (23.1 %), \textit{i-C}_{15:0} (6.4 %), \textit{C}_{16:0} (14.7 %), \textit{i-C}_{17:0} (1.9 %), \textit{i-C}_{14:1} (3.6 %), \textit{i-C}_{17:1\textit{o}9c} (1.7 %), \textit{ai-C}_{17:1\textit{o}9c} (3.7 %), \textit{i-C}_{16:1\textit{H}} (2.6 %) and \textit{C}_{16:1\textit{t}07c} (9.9 %).

An almost-complete 16S rRNA gene sequence (1479 nt) was determined for strain Ap1\textsuperscript{T}. Primary sequence analysis with those of representatives of the family \textit{Streptomycetaceae} confirmed that strain Ap1\textsuperscript{T} was closely related to members of the genus \textit{Streptomyces}. The highest 16S rRNA gene sequence similarity values were found with the type strains of the genus \textit{Streptomyces} (Table 1). The highest 16S rRNA gene sequence similarity was predicted by 16S rRNA gene sequencing. Significant differences were found between the RFLP patterns of strain Ap1\textsuperscript{T} and those of the type strains of \textit{S. violaceorubidus} and \textit{S. chattanoogensis} (Fig. 3), predicting a more distant relationship to strain Ap1\textsuperscript{T}.

Using 16S-ITS RFLP fingerprinting, strain Ap1\textsuperscript{T} was found to be most closely related to the type strains of \textit{S. humiferus}, \textit{S. coelescens}, \textit{S. violaceolatus} and \textit{S. rubrogriseus}, as predicted by 16S rRNA gene sequencing. Significant differences were found between the RFLP patterns of strain Ap1\textsuperscript{T} and those of the type strains of \textit{S. violaceorubidus} and \textit{S. chattanoogensis} (Fig. 3), predicting a more distant relationship to strain Ap1\textsuperscript{T}.

Protein profiling, BOX-PCR fingerprinting and selected phenotypic traits were used to determine the exact relationship between strain Ap1\textsuperscript{T} and its closest neighbours at the species level. The protein profiles of strain Ap1\textsuperscript{T} and its closest neighbours in 16S rRNA gene sequencing (the type strains of \textit{S. violaceolatus}, \textit{S. tendae}, \textit{S. coelescens}, \textit{S. violaceoruber}, \textit{S. rubrogriseus} and \textit{S. lienomycini}) showed significant differences (Fig. 4). The highest correlation was found with the species \textit{S. rubrogriseus} and \textit{S. lienomycini} (r=0.82), but this was still far below the 0.90 cut-off level recommended for speciation (Lanoot et al., 2002). This corresponds with DNA–DNA relatedness values (21 % with \textit{S. rubrogriseus} DSM 41477\textsuperscript{T}, 17 % with \textit{S. lienomycini} DSM 41475\textsuperscript{T} and 8 % with \textit{S. violaceoruber} DSM 40783\textsuperscript{T}) far below the 70 % cut-off point recommended by Wayne et al. (1987). Hence, strain Ap1\textsuperscript{T} should be considered as representing a separate species. In addition, the BOX-PCR pattern of strain Ap1\textsuperscript{T} was unique after comparison with an in-house database containing the BOX patterns of 473 \textit{Streptomyces} type strains (Lanoot et al., 2004), supporting its novelty. Furthermore, the strain was distinguished phenotypically in many features from its closest neighbours, especially \textit{S. rubrogriseus} and \textit{S. lienomycini} (Gause et al., 1983) (Supplementary Table S1, available in IJSEM Online). \textit{S. lienomycini} LMG 20091\textsuperscript{T} differs from strain Ap1\textsuperscript{T} in producing no aerial mycelium on glycerol-nitrate agar or glycerol-asparagine agar. It also differs from strain Ap1\textsuperscript{T} in producing a yellowish substrate mycelium on inorganic salts-starch agar, glycerol asparagine agar and mineral agar Gause 1 and a colourless substrate mycelium on oatmeal agar and glycerol-nitrate agar. In addition, it differs from Ap1\textsuperscript{T} in producing melanin pigment (Gause et al., 1983). \textit{S. rubrogriseus} LMG 20318\textsuperscript{T} differs from Ap1\textsuperscript{T} in producing red substrate mycelium on oatmeal agar and glycerol-nitrate agar and a yellowish, red or crimson substrate mycelium on inorganic salts-starch agar (Gause et al., 1983) (Table 1).
Based on the genotypic, chemotaxonomic and phenotypic evidence, strain Ap1T belongs to a novel species in the genus *Streptomyces*, for which the name *Streptomyces marokkonensis* sp. nov. is proposed.

### Description of *Streptomyces marokkonensis* sp. nov.

*Streptomyces marokkonensis* (ma.ro.ko.nen’sis. N.L. masc. adj. marokkonensis pertaining to Marokko, the Dutch name of Morocco, from where the type strain was isolated).

Aerobic, Gram-positive, non-motile actinomycete that forms a branched substrate mycelium and greyish white aerial mycelium with long spore chains of the *Spiralis* type with more than 20 cylindrical, non-flagellated spores with smooth surfaces. Aerial spore mass colour is grey on yeast extract-malt extract agar (ISP medium 2), oatmeal agar (ISP medium 3), inorganic salts-starch agar (ISP medium 4), Bennett’s agar (Jones, 1949) and nutrient agar and white–grey on glycerol-asparagine agar (ISP medium 5), glucose asparagine agar, sucrose nitrate agar, glucose Czapek’s agar and Olson’s agar. Diffusible pigments are not formed on ISP medium 4 or 5, and melanin pigments are not produced on tryptone-yeast extract agar (ISP medium 1), peptone-yeast extract-iron agar (ISP medium 6) or tyrosine agar (ISP medium 7). Temperature range for growth determined on yeast extract-malt extract agar is 15–40°C, with optimal growth at 28–30°C. The pH range for growth is pH 4.5–10. No growth is observed in the presence of sodium azide (0.01 %, w/v) or potassium tellurite (0.01 %, w/v) or in the presence of NaCl concentrations greater than 9 %. Nitrate is reduced. H₂S is not produced. Coagulation of skimmed milk and liquefaction of gelatin are positive. Casein, gelatin and starch are degraded but not aesculin, xanthine or urea. D-Glucose, cellobiose, D-fructose, D-galactose, lactose, D-mannitol, D-mannose, L-rhamnose, salicin, sucrose, D-xylose, glycerol and maltose are utilized as sole carbon sources. L-Arabinose and inositol are utilized poorly and raffinose and sodium citrate are not utilized. Resistant to the β-lactams penicillin G (10 IU), amoxicillin (10 IU), oxacillin (1 IU), ampicillin (10 IU) and cefalotin (30 IU) and the sulfamides trimetoprim sulfoxide (10 U) and sulfamide (25 IU); sensitive to the aminoside antibiotics kanamycin (30 IU), amikacin (30 IU), neomycin (50 µg ml⁻¹), gentamicin (100 µg ml⁻¹), streptomycin (20 µg ml⁻¹) and tobramycin (10 µg ml⁻¹) and also to erythromycin (15 IU), novobiocin (30 IU), bacitracin (10 IU) and polymyxin B (300 IU). Shows activity against the yeasts *Candida albicans*, *Candida tropicalis* and *Saccharomyces cerevisiae*, the moulds *Fusarium oxysporum* f. sp. *albedinis*, *Fusarium oxysporum* f. sp. *lycoperdii*, *Verticillium dahliae* and *Aspergillus niger*, the Gram-positive bacteria *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus* and *Micrococcus luteus* and the Gram-negative bacteria *Escherichia coli*, *Salmonella typhi* and *Klebsiella pneumoniae*. The G+C content of the DNA of the type strain is 70 mol%.

### Table 1. Cultural characteristics of strain Ap1T, *S. lienomycini* LMG 20099T and *S. rubrogriseus* LMG 20318T

Data were obtained in this study. No pigments were formed on the agars listed.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Ap1T</th>
<th><em>S. lienomycini</em> LMG 20099T</th>
<th><em>S. rubrogriseus</em> LMG 20318T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral agar CaSe 1</td>
<td>Grey</td>
<td>White, later grey</td>
<td>Grey</td>
</tr>
<tr>
<td>Glycerol-nitrate agar</td>
<td>Whith grey</td>
<td>Whith grey</td>
<td>Grey</td>
</tr>
<tr>
<td>Oatmeal agar (ISP medium 3)</td>
<td>Grey</td>
<td>Grey</td>
<td>Grey</td>
</tr>
<tr>
<td>Inorganic salts-starch agar (ISP medium 4)</td>
<td>Grey</td>
<td>Grey</td>
<td>Grey</td>
</tr>
<tr>
<td>Glycerol-asparagine agar (ISP medium 5)</td>
<td>Whith grey</td>
<td>Not formed</td>
<td>Pink</td>
</tr>
</tbody>
</table>
The type strain, Ap1\textsuperscript{T} (=R-22003\textsuperscript{T} = LMG 23016\textsuperscript{T} = DSM 41918\textsuperscript{T}), was isolated from a soil sample from rhizosphere soil of *Argania spinosa* L. in the south of Morocco.

**Acknowledgements**

The authors are grateful to Barte Hoste and Katrien Vandemeulebroecke of BCCM/LMG for sequencing of the 16S rRNA gene of strain Ap1\textsuperscript{T} and to Jean Euzéby for his help in naming the species. Professor Abdelkader Outzoughite (Faculty of Science, Marrakesh, Morocco) is acknowledged for his help in the SEM observation of Ap1\textsuperscript{T}. Dr Stefanie Van Trappen from the BCCM/LMG Bacteria Collection is acknowledged for reviewing the manuscript. Larissa Therekhova (Gauze Institute of New Antibiotics, Moscow, Russia), Victor Chepurnov (UGent) and Lyudmila Evtushenko (VKM) are acknowledged for translating the original descriptions of *S. lienomycini* and *S. rubrogriseus* from Russian into English. Members of the LBVRN Laboratory (Faculty of Science, Agadir, Morocco) are acknowledged for their help during revision of this manuscript.

**References**


