Streptomyces thinghirensis sp. nov., isolated from rhizosphere soil of *Vitis vinifera*

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A novel actinomycete, strain S10^T, was isolated from rhizosphere soil of wild *Vitis vinifera* in Thinghir, Ouarzazate Province, Southern Morocco. The taxonomic status of this strain was established using a polyphasic approach. Strain S10^T had white–grey aerial mycelium with long, spiral spore chains bearing smooth surfaced spores and produced a yellow diffusible pigment. Chemotaxonomic analyses showed that the cell wall of strain S10^T contained L-diaminopimelic acid and glycine. Phylogenetic analysis based on the almost complete 16S rRNA gene sequence indicated that strain S10^T belonged to the Group I streptomycetes, branching off next to *Streptomyces marokkonensis* LMG 23016^T from the *Streptomyces violaceoruber* group. DNA–DNA relatedness and phenotypic data distinguished strain S10^T from the phylogenetically closest related type strains. It is therefore proposed that strain S10^T (=CCMM B35^T=DSM 41919^T) represents the type strain of a novel species of the genus *Streptomyces*, for which the name *Streptomyces thinghirensis* sp. nov. is proposed.

Over the past decades, interest in the discovery of new sources of secondary metabolites with applications in medicine (Newman *et al.*, 2003) and agriculture (Copping & Menn, 2000) has significantly increased. Micro-organisms are an almost unlimited source of novel compounds. Among them, actinomycetes hold a prominent position due to their ability to produce various secondary metabolites, including antibiotics (Lazzarini *et al.*, 2000; Watve *et al.*, 2001; Donadio *et al.*, 2002), antitumour agents (Maskey *et al.*, 2003) and enzymes (Breccia *et al.*, 1995; Ko *et al.*, 2005). Actinomycetes are Gram-positive, aerobic bacteria. They form branching substrate and aerial mycelia that bear spores and possess DNA with a high G + C content. Many species of the genus *Streptomyces* are known to produce antibiotics (Chun *et al.*, 1997; Labeda *et al.*, 1997). Actinomycetes represent a high proportion of the soil microbial biomass and appear to be of importance

among the microbial flora of the rhizosphere (Sardi *et al.*, 1992). Associations between actinomycetes and plant organs can be deleterious or beneficial for the host. While some actinomycetes secrete herbicidal compounds (Tanaka & Omura, 1993) or cause plant diseases (Locci, 1994), others can fix atmospheric nitrogen symbiotically (Oakley *et al.*, 2004) or protect plants against fungal infections (Cao *et al.*, 2005). Several descriptive reports have shown that actinomycetes are a promising group of fungus-antagonistic and root-colonizing microbes. They protect several different plants from soil-borne fungal pathogens to various degrees (El-Tarabily & Sivasithamparam, 2006).

In the course of our screening programme for actinomycetes from Moroccan habitats that are active against many phytopathogens (Loqman *et al.*, 2009), one actinomycete strain, strain S10^T, was isolated from the rhizosphere soil of wild, healthy *Vitis vinifera* plants, collected from Thinghir, Ouarzazate Province, Southern Morocco. The strain was identified using a polyphasic approach.

Strain S10^T was isolated on soil extract agar as described in Ouhdouch *et al.* (2001). The strain was maintained on

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain S10^T is FM202482.

Micrographs showing spore chains and the spore shape of *Streptomyces thinghirensis* sp. nov. and a table detailing the fatty acid pattern of the novel strain and closely related species of the genus *Streptomyces* are available with the online version of this paper.

International *Streptomyces* Project (ISP) no. 2 (ISP 2) agar slants (glucose-yeast extract-malt extract agar) at 4 °C and as 20% (v/v) glycerol stocks at -20 °C. Biomass for chemical and molecular studies was obtained by growing strain S10^T in shake flasks in ISP 2 broth (28 °C, 1 week, 150 r.p.m.).

Physiological characteristics were determined after 2 weeks growth at 28 °C according to the methods prescribed by the ISP (Shirling & Gottlieb, 1966). Morphological properties were examined by light microscopy and scanning electronic microscopy. The colour of the aerial mycelium was determined from mature sporulating aerial mycelia according to the scale adopted by Prauser (1964) and the colour series was determined according to the system proposed by Nonomura (1974). Production of melanoid pigments was determined on ISP 6 and ISP 7 media. Analysis of cell-wall diaminopimelic acid isomers and whole cell sugars was performed according to the protocol described by Lechevalier & Lechevalier (1980). Fatty acid methyl esters and mycolic acid trimethylsilylestere were prepared and analysed as previously described (Klatte *et al.*, 1994) using the standard Microbial Identification System (MIDI Inc.) for automated GC analyses (Sasser, 1990).

Carbon source utilization was determined on ISP 9 medium supplemented with sterile carbon sources. Standard techniques were used for the determination of catalase, oxidase and nitrate reduction activities. Sensitivity to NaCl was established according to the method of Tresner *et al.* (1968). The temperature range for growth was determined on ISP 2 and Bennett medium (Jones, 1949). Antibiotic resistance was examined by the disc diffusion method on Olson's medium (Olson, 1968) with plates incubated at 28 °C for 21 h. The antimicrobial activity of strain S10^T was determined by the plate diffusion method (Bauer *et al.*, 1966). Bacterial test strains were incubated on nutrient agar at 37 °C for 24 h and fungal strains were incubated on Sabouraud agar medium at 28 °C for 24 h for yeasts and 48 h for moulds.

For 16S rRNA gene sequence analysis, strain S10^T was cultivated for 2 days at 28 °C with agitation in 500 ml flasks containing 100 ml of Hickey-Tresner medium, 1 g yeast extract l⁻¹, 1 g beef extract l⁻¹, 2 g NZamine A l⁻¹, 10 g dextrin l⁻¹, 20 mg CoCl₂ · 6H₂O l⁻¹ (Hopwood *et al.*, 1985). Biomass was harvested by centrifugation (8000 g for 10 min) and washed twice with double-distilled water. Mycelia (200 mg) were used for DNA extraction as described by Liu *et al.* (2000). The 16S rRNA gene was amplified by PCR using the universal primers PA and PH. Amplification was carried out in 50 µl reaction volumes containing 1.5 U of AmpliTaq Gold Taq polymerase (Applied Biosystems), dNTPs (0.25 mM each), 1 µM each primer and 100 ng genomic DNA. Reaction conditions were: 97 °C for 4 min, followed by 35 cycles of 97 °C for 45 s, 52 °C for 45 s and 72 °C for 45 s and a final incubation at 72 °C for 10 min. The amplified products

were visualized on a 0.8% (w/v) agarose gel stained with ethidium bromide. Sequencing reactions were performed by Macrogen. The primers used for sequencing are listed in Coenye *et al.* (1999). The sequences obtained were compared with sequences present in the public sequence databases as well as with EzTaxon, a web-based tool for the identification of prokaryotes based on 16S rRNA gene sequences from type strains (Chun *et al.*, 2007). BLAST analysis was performed at www.ncbi.nlm.nih.gov.

DNA-DNA hybridization analysis was performed between strain S10^T and its closest relatives based on the degree of 16S rRNA gene similarity and the inferred phylogeny. DNA-DNA hybridization with the high scoring (in EzTaxon) but ambiguous sequences for *Streptomyces almqvistii* NRRL B-1685^T (GenBank accession no. AY999782), *Streptomyces althoticus* NRRL B-3981^T (AY999791) and *Streptomyces matensis* NBRC 12889^T (AB184221) was not deemed necessary because of their distant location in the phylogenetic tree (Fig. 1). 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 performed 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-thermostatted 6 × 6 multicell changer and a temperature controller with *in situ* temperature probe (Varian), as described by Wayne *et al.* (1987). All DNA-DNA hybridizations were conducted in duplicate and the reported results give the mean of the two experiments.

For phylogenetic analysis, 16S rRNA gene sequences for the type strains of closely related *Streptomyces* species were retrieved from GenBank, aligned with CLUSTAL W (Larkin *et al.*, 2007), and analysed using the neighbour-joining, maximum-parsimony and maximum-likelihood tools from the PHYLIP package, version 3.6 (Felsenstein, 2005). Phylogenetic trees were visualized using Dendroscope (Huson *et al.*, 2007).

Strain S10^T had morphological characteristics that were consistent with members of the genus *Streptomyces*. From light and electron microscopic observations, it was found that strain S10^T had *Spirales*-type spore chains (see Supplementary Fig. S1a in IJSEM Online) with smooth spore surface ornamentation (Supplementary Fig. S1b). The chemotaxonomic characteristics of strain S10^T supported its classification as a member of the genus *Streptomyces*. LL-Diaminopimelic acid and glycine were detected in the cell-wall peptidoglycan. As usually found for streptomycetes, the fatty acid profile was comprised mainly of fatty acids with a length of 14–18 carbon atoms (Lechevalier, 1977), in particular saturated iso- and anteiso-branched chain fatty acids: ai-C_{15:0} (23.7%), i-C_{16:0} (19.5%), ai-C_{17:0} (13.7%), i-C_{15:0} (11.0%), i-C_{17:0} (6.5%) and i-C_{14:0} (2.5%), with only a few unbranched fatty acids, C_{16:0} (5.6%), C_{15:0} (2.3%), C_{17:0} (0.7%) and C_{14:0} (0.2%). A compar-

0.001

Fig. 1. Unrooted maximum-likelihood phylogenetic tree based on 1436 aligned positions of the 16S rRNA gene showing the phylogenetic relationships between strain S10^T and the most closely related type strains of the genus *Streptomyces*. Bootstrap values (%) above the branches were derived from 1000 replications of maximum-likelihood inferences, those below the branches give the maximum support by 1000 replications of maximum-parsimony, neighbour-joining and least squares (FITCH) inferences. Bar, 0.001 substitutions per nucleotide position.

ison of the fatty acid profiles of strain S10^T and closely related species is available in Supplementary Table S1 (available in IJSEM Online).

A BLAST search with the 1462 bp 16S rRNA gene sequence of strain S10^T showed that it displayed greater than 99 % sequence similarity to the 16S rRNA gene sequences of many members of the genus *Streptomyces*. The highest degree of similarity was found with *Streptomyces marokkonensis* LMG 23016^T (99.65 %), *S. almquistii* NRRL B-1685^T (99.58 %), *S. althioticus* NRRL B-3981^T (99.51 %), *S. matensis* NBRC 12889^T (99.51 %), *Streptomyces aurantiogriseus* NRRL B-5416^T (99.32 %), *Streptomyces lienomycini* NBRC 15425^T (99.24 %), *Streptomyces coelestis* DSM 40421^T (99.20 %) and *Streptomyces violaceolatus* DSM 40438^T (99.18 %). Five of these strains were selected for DNA–DNA hybridization experiments. Low levels of DNA–DNA relatedness to strain S10^T were found for all five strains: *S. coelestis* DSM 40421^T 4.6 ± 0.6 %, *S. aurantiogriseus* DSM 40138^T 6.7 ± 1.0 %, *S. lienomycini* DSM 41475^T 9.3 ± 0.3 %, *S. violaceolatus* DSM 40438^T 10.5 ± 4.5 % and *S. marokkonensis* DSM 41918^T 33.4 ± 0.4 %. When applying the recommended threshold of 70 % DNA–DNA relatedness as proposed by Wayne *et al.* (1987), strain S10^T could be differentiated from its five closest neighbours.

Phylogenetic analysis showed that strain S10^T was most closely related to *Streptomyces marokkonensis* LMG 23016^T and that both strains branched off separately from the *S. violaceoruber* species group (Fig. 1).

A comparison of the phenotypic characteristics of strain S10^T and the strains with the top BLAST results is shown in Table 1. It is clear from these comparisons that strain S10^T is phenotypically different from the most closely related *Streptomyces* species. Additional phenotypic properties of the new isolate are given in the species description.

From the phenotypic and genotypic data obtained, it is proposed that strain S10^T represents a novel species within the genus *Streptomyces*. The name *Streptomyces thinghirensis* sp. nov. is proposed with strain S10^T as the type strain.

Description of *Streptomyces thinghirensis* sp. nov.

Streptomyces thinghirensis (thin.ghi.ren'sis. N.L. masc. adj. *thinghirensis* of Thinghir, named after the town in Southern Morocco where the strain was isolated).

Hyphae are abundant and well-developed. A yellow diffusible pigment is produced on all test media and yellow substrate mycelium and white–grey aerial mycelium are visible. No melanin production is observed on peptone–yeast extract–iron agar (ISP 6) or tyrosine agar (ISP 7). Good growth is observed on ISP 2 agar. Gelatin is not liquefied. Milk is coagulated and peptonized. H₂S is not produced. Nitrate is reduced. D-Fructose, D-galactose, D-glucose, D-mannitol, D-mannose, *myo*-inositol, L-rhamnose and D-sorbitol are utilized as sole carbon sources. D-Sucrose, maltose, D-lactose and cellobiose are weakly

Table 1. Physiological characteristics of strain S10^T and its phylogenetic neighbours

Taxa: 1, S10^T; 2, *S. marokkonensis* LMG 23016^T; 3, *S. coelestis* DSM 40421^T; 4, *S. violaceolatus* DSM 40438^T; 5, *S. lienomycini* DSM 41475^T; 6, *S. aurantiogriseus* DSM 40138^T. +, Well utilized/present; w, weakly utilized; –, not utilized/absent; B, blue; Br, brownish; Cs, colourless; G, grey; GBr, grey–brown; RG, red–grey; WG, white–grey; Y, yellow.

Characteristic	1	2	3	4	5	6
Aerial mass colour	WG	G	G	Br	G	RG
Substrate mycelium	Y	G	GBr	Gr	Cs	B
Reverse side pigment	Y	–	+	+	–	–
Diffusible pigment	+	–	+	+	–	+
Melanin pigments	–	–	–	–	+	–
Utilization of						
D-Arabinose	–	w	+	+	+	+
myo-Inositol	+	–	+	–	+	+
Lactose	w	+	–	–	+	+
Raffinose	–	–	w	–	+	+
Rhamnose	+	+	+	–	+	+
Sucrose	w	+	+	–	+	+
Xylose	–	+	+	+	+	+
Degradation of aesculin	+	–	+	+	+	+
Growth in 7% (w/v) NaCl	+	+	–	–	+	–

utilized, while D-arabinose, D-xylose and raffinose are not utilized as sole carbon sources. Growth occurs from 28 to 42 °C, from pH 5 to pH 10, and in the presence of 7% (w/v) NaCl. Resistant to (µg ml⁻¹): ampicillin (10), amoxicillin (10), nalidixic acid (30), penicillin G (10), sulfamide (25) and rifampicin (5), but sensitive to novobiocin (30), gentamicin (10) and streptomycin (10). Active against the moulds *Aspergillus niger*, *Fusarium oxysporum* f. sp. *albedinis*, *Pythium ultimum*, *Sclerotium rolfsii* and *Verticillium dahliae*, the yeasts *Candida albicans*, *Candida tropicalis* and *Saccharomyces cerevisiae*, and the bacteria *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli* and *Streptomyces scabiei*.

The type strain, S10^T (=CCMM B35^T=DSM 41919^T), was isolated from the rhizosphere soil of wild *Vitis vinifera* plants.

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