Streptomyces youssoufiensis sp. nov., isolated from a Moroccan phosphate mine

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A novel actinomycete, strain X4^T, was isolated from a phosphate mine in Youssoufia, 100 km north of Marrakesh, Morocco. The taxonomic status of this strain was evaluated by a polyphasic approach. Strain X4^T had white aerial mycelium with *Rectiflexibiles* spore chains bearing smooth-surfaced spores and did not produce diffusible pigments. Chemotaxonomic analysis showed that the cell wall of strain X4^T contained LL-diaminopimelic acid and glycine. Phylogenetic analysis based on the almost complete 16S rRNA gene sequence indicated that strain X4^T belongs to the Group I streptomycetes, branching off next to *Streptomyces ramulosus* NRRL B-2714^T and *Streptomyces kasugaensis* M338-M1^T. DNA–DNA relatedness and phenotypic data enabled strain X4^T to be distinguished from the phylogenetically most closely related type strains. It is therefore proposed that strain X4^T represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces youssoufiensis* sp. nov. is proposed; the type strain is X4^T (=CCMM B709^T =DSM 41920^T).

Since the first description of plant-growth-promoting rhizobacteria (Kloepper & Schroth, 1978), the benefits attributed to these bacteria have been defined more precisely. Micro-organisms of the rhizosphere can contribute to plant fitness by increasing the efficiency of biological nitrogen fixation (Meunchang *et al.*, 2006) or phosphate solubilization (Gyaneshwar *et al.*, 2002; Caravaca *et al.*, 2005; Duponnois *et al.*, 2005). Reports in the literature indicate that *Serratia marcescens* EB 67, *Pseudomonas* sp. CDB 35 (Hameeda *et al.*, 2006), *Enterobacter* sp. and *Bacillus subtilis* (Toro *et al.*, 1997) contribute to an improvement in plant growth as rock-phosphate-solubilizing bacteria. Soil-borne micro-organisms can also be beneficial for plants by increasing the availability of trace elements such as iron, zinc, etc., in the rhizosphere (Cakmakci *et al.*, 2006). Among soil micro-organisms, actinobacteria are of special interest because they possess many properties that can contribute to plant growth or fitness (Lehr *et al.*, 2008), such as the production of phytohormones that stimulate plant growth (e.g. indole acetic acid; Vivas *et al.*, 2006) and/or excretion of substances such as siderophores and/or

anti-fungal agents (Jain & Jain, 2007; Hamdali *et al.*, 2008a, b, c) that limit the development and/or infectivity of various plant pathogens. These mostly filamentous and sporulating bacteria strongly adhere to soil particles and may form endophytic relationships with plants (Cao *et al.*, 2005; Conn *et al.*, 2008).

In the course of our screening programme for rock-phosphate-solubilizing actinomycetes (Hamdali *et al.*, 2008a), strain X4^T was isolated from a Moroccan phosphate mine in Youssoufia, in the Marrakesh region of southern Morocco. The strain was identified using a polyphasic approach and the results showed that it should be classified as a representative of a novel species within the genus *Streptomyces*.

Strain X4^T was isolated on soil extract agar as described by Ouhdouch *et al.* (2001). The strain was maintained on International *Streptomyces* Project (ISP) 2 agar slants (Shirling & Gottlieb, 1966) at 4 °C and as 20% (v/v) glycerol stocks at –20 °C. Biomass for chemical and molecular studies was obtained by growing strain X4^T in shake flasks of ISP 2 broth (28 °C, 1 week, 150 r.p.m.).

Physiological characteristics were determined after growth for 2 weeks at 28 °C according to the methods described by the ISP (Shirling & Gottlieb, 1966). Morphological properties were examined by light microscopy (Olympus

Abbreviation: ISP, International *Streptomyces* Project.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DSM 41920^T is FN421338.

A supplementary table is available with the online version of this paper.

microscope) and scanning electronic microscopy (JEOL). The colour of the aerial mycelium was determined from mature sporulating aerial mycelium 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 were prepared and analysed as described previously (Klatte *et al.*, 1994) using the standard Microbial Identification system (MIDI) for automated GC analysis (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 by the method of Tresner *et al.* (1968). Growth temperature range 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) incubated at 28 °C for 21 h. The antimicrobial activity of strain X4^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 (yeasts) or 48 h (moulds).

For 16S rRNA gene sequence analysis, cells of strain X4^T were cultivated for 2 days at 28 °C with agitation in 500 ml flasks containing 100 ml Hickey-Tresner medium (per litre: 1 g yeast extract, 1 g beef extract, 2 g NZamine A, 10 g Dextrin, 20 mg CoCl₂·6H₂O; 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 universal primers PA (5'-AGA-GTTTGATCCTGGCTCAG-3') and PH (5'-AAGGAGGT-GATCCAGCCGCA-3'). Amplification was carried out in 50 µl reaction volumes containing 1.5 U AmpliTaq Gold Taq polymerase (Applied Biosystems), dNTPs (0.25 mM each), 1 µM of 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 elongation step 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 the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). 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 the www.ncbi.nlm.nih.gov web server.

For DNA–DNA hybridization analysis, 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-thermostatted 6 × 6 multicell changer and a temperature controller with an *in situ* temperature probe (Varian), as described by Wayne *et al.* (1987). All DNA–DNA hybridizations were done in duplicate and the reported results give the means of the two experiments.

For phylogenetic analysis, 16S rRNA gene reference sequences for the type strains of closely related species of the genus *Streptomyces* were retrieved from GenBank, aligned with CLUSTAL W2 (Thompson *et al.*, 1994), 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 X4^T had morphological characteristics that were consistent with those of members of the genus *Streptomyces*. Light and electron microscopy revealed that strain X4^T had *Rectiflexibiles*-type spore chains with smooth spore surface ornamentation (Fig. 1). The chemotaxonomic characteristics of strain X4^T supported its classification as a member of the genus *Streptomyces*. LL-Diaminopimelic acid and glycine were detected in its peptidoglycan. As usually observed in streptomycetes, the fatty acid profile was composed mainly of fatty acids with a length of 14–18 carbon atoms (Lechevalier & Moss, 1977), in particular saturated iso- and anteiso-branched chain fatty acids [i-C_{16:0} (48.7 %), i-C_{16:1} (8.5 %), ai-C_{15:0} (7.8 %), i-C_{14:0} (6.8 %), ai-C_{17:0} (6.1 %), i-C_{15:0} (5.6 %), 9-methyl C_{16:0} (4.1 %), 10-methyl C_{17:0} (2.4 %)], with only a few unbranched fatty acids [C_{16:0} (0.9 %), C_{15:0} (0.3 %)]. Fatty acid profiles of the novel isolate and closely related strains

Fig. 1. Micrograph showing spore chains and spore shape of strain X4^T grown on ISP 2 for 14 days at 28 °C. Bar, 5 µm.

are shown in Supplementary Table S1, available in IJSEM Online.

A BLAST search of the EzTaxon database showed that the similarities between the 1518 bp 16S rRNA gene sequence of strain X4^T and 16S rRNA gene sequences of type strains of the most closely related members of the genus *Streptomyces* were less than 99%; highest similarities were observed with *Streptomyces coeruleus* NBRC 12758^T (98.97%), *Streptomyces lilacinus* NBRC 12884^T (98.78%), *Streptomyces abikoensis* NBRC 13860^T (98.76%), *Streptomyces ramulosus* NRRL B-2714^T (98.72%), *Streptomyces varsoviensis* NRRL B-3589^T (98.66%) and *Streptomyces kasugaensis* M338-M1^T (98.43%). DNA–DNA hybridizations were performed with *Streptomyces coeruleus* DSM 40146^T, the organism with the highest degree of 16S rRNA gene sequence similarity, with *Streptomyces ramulosus* DSM 40100^T and *Streptomyces kasugaensis* DSM 40819^T, the closest neighbours in the phylogenetic tree, and with *Streptomyces varsoviensis* NRRL B-3589^T, but not for the other top-scoring strains in the BLAST search (type strains of *Streptomyces lilacinus* and *Streptomyces abikoensis*), because these were not supported by the topology of the phylogenetic tree (Fig. 2). Low levels of DNA–DNA relatedness to strain X4^T were found for the four strains studied: *Streptomyces coeruleus* NBRC 12758^T ($28.5 \pm 0.5\%$), *Streptomyces ramulosus* NRRL B-2714^T ($11.4 \pm 4.8\%$), *Streptomyces kasugaensis* M338-M1^T ($24 \pm 4.4\%$) and *Streptomyces varsoviensis* NRRL B-3589^T ($5.4 \pm 4.0\%$). When

applying the recommended threshold of 70% DNA–DNA relatedness as proposed by Wayne *et al.* (1987), strain X4^T could be differentiated from its closest neighbours.

The phenotypic characteristics of strain X4^T and the most closely related strains (highest BLAST results) are shown in Table 1. It is clear from these comparisons that strain X4^T is phenotypically distinct from the most closely related species of the genus *Streptomyces*. Additional phenotypic properties of the isolate are given in the species description.

From the phenotypic and genotypic data obtained, it is proposed that strain X4^T represents a novel species within the genus *Streptomyces*. The name *Streptomyces youssoufiensis* sp. nov. is proposed, with strain X4^T as the type strain.

Description of *Streptomyces youssoufiensis* sp. nov.

Streptomyces youssoufiensis (yous.sou.fi.en'sis. N.L. masc. adj. *youssoufiensis* of or belonging to Youssoufia, named after the rock phosphate mining town in Morocco where the strain was isolated).

Hyphae are abundant and well-developed. No diffusible pigments are produced on any test media and cream substrate mycelium and white aerial mycelium are visible on yeast-malt extract agar (ISP 2). No melanin production

Fig. 2. Unrooted maximum-likelihood phylogenetic tree based on 1518 aligned positions of the 16S rRNA gene showing the phylogenetic relationships between strain X4^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 any of the maximum-parsimony, neighbour-joining and least-squares (FITCH) inferences. Bar, 0.005 substitutions per nucleotide position.

Table 1. Physiological characteristics of strain X4^T and its phylogenetic neighbours

Strains: 1, X4^T; 2, *Streptomyces varsoviensis* DSM 40346^T; 3, *Streptomyces coeruleus* DSM 40146^T; 4, *Streptomyces ramulosus* DSM 40100^T; 5, *Streptomyces kasugaensis* DSM 40819^T. +, Positive/well utilized; ±, weakly positive/poorly utilized; –, negative/not utilized. All strains were negative for degradation of aesculin. All data were determined in this study.

Characteristic	1	2	3	4	5
Colour of:					
Aerial mass	W	G	P-GR	Y-BR	BR-B
Substrate mycelium	C	Y-BR	BR-B	G	Y-BR
Reverse side pigment	B	Y	B	Y	B
Production of:					
Diffusible pigment	–	+	+	+	+
Melanin pigments	–	–	+	–	±
Utilization of:					
D-Arabinose	–	+	+	–	+
Lactose	+	–	+	–	–
myo-Inositol	–	–	+	+	+
Raffinose	–	–	+	–	–
Rhamnose	–	–	+	+	–
Sucrose	+	–	+	+	+
Xylose	–	+	–	+	–
Growth with 7 % NaCl	+	–	–	–	–

*W, White; C, cream; G, grey; Y-BR, yellow-brown; Y, yellow; B, beige; BR-B, brown-beige; P-GR, pale green.

is observed on peptone-yeast extract-iron agar (ISP 6) or tyrosine agar (ISP 7). Good growth is observed on ISP 2 agar. Starch, cellulose and pectin are degraded. Gelatin is not liquefied. Milk is coagulated and peptonized. H₂S is not produced. Nitrate is reduced. D-Fructose, D-glucose, lactose, D-mannitol, D-mannose and sucrose are utilized as sole carbon sources. Cellobiose and maltose are utilized weakly, whereas D-arabinose, D-galactose, myo-inositol, raffinose, L-rhamnose, D-sorbitol and D-xylose are not utilized as sole carbon sources. Growth occurs at 28–42 °C, at pH 5–10 and in the presence of 7 % (w/v) NaCl. Resistant to ampicillin (10 µg ml^{–1}), amoxicillin (10 µg ml^{–1}), nalidixic acid (30 µg ml^{–1}), penicillin G (10 µg ml^{–1}), sulfamide (25 µg ml^{–1}), gentamicin (10 µg ml^{–1}), cefalotin (30 µg ml^{–1}), bacitracin (10 µg ml^{–1}) and rifampicin (5 µg ml^{–1}), but sensitive to novobiocin (30 µg ml^{–1}), polymyxin B (300 U) and streptomycin (10 µg ml^{–1}). Active against moulds (*Fusarium oxysporum* f. sp. *albedinis*, *Pythium ultimum*, *Mucor ramanianus* and *Verticillium dahliae*), yeasts (*Candida albicans* and *Candida tropicalis*), bacteria (*Bacillus subtilis*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Micrococcus luteus*, *Rhodotorula rubra*, *Staphylococcus aureus* and *Streptomyces scabiei*).

The type strain is X4^T (=CCMM B709^T =DSM 41920^T), isolated from a phosphate mine in Youssoufia, 100 km north of Marrakesh, Morocco.

Acknowledgements

We are grateful to Professor Pr. Outzoughite A. (Scanning Microscopy Unit, Faculty of Science of Marrakesh) for his help with scanning electron microscopy. We would like to gratefully acknowledge the help of Bettina Streubler (DSMZ) for DNA–DNA hybridization.

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