Streptomyces durmitorensis sp. nov., a producer of an FK506-like immunosuppressant

Miloje Savic,† Ivana Bratic‡ and Branka Vasiljevic

Institute of Molecular Genetics and Genetic Engineering, Vojvode Stepe 444a, PO Box 23, 11 010 Belgrade, Serbia

Screening of soil samples from the Durmitor National Park, Serbia and Montenegro, for strains producing immunosuppressants with a similar mechanism of action to FK506 resulted in the isolation of the actinomycete strain MS405T. Isolate MS405T was found to have morphological and phenotypic properties that were consistent with its classification as a Streptomyces strain. The DNA G+C content of strain MS405T was 72 mol%. 16S rRNA gene sequence data confirmed the taxonomic position of the strain, following the generation of phylogenetic trees by using various treeing algorithms. On the basis of 16S rRNA gene sequence similarity, strain MS405T was shown to belong to the Streptomyces albidoflavus ‘supercluster’, being related to Streptomyces aureus DSM 41785T (99.59 % similarity) and Streptomyces kanamyceticus DSM 40500T (99.32 %). The 16S–23S rRNA internally transcribed spacer (ITS) region exhibited variations in length and sequence composition, showing limited usefulness in phylogenetic analyses. However, DNA relatedness values support the classification of this isolate within a novel species. A number of physiological and biochemical tests distinguished strain MS405T from its closest phylogenetic neighbours. Therefore, strain MS405T represents a novel species, for which the name Streptomyces durmitorensis sp. nov. is proposed, with the type strain MS405T (= DSM 41863T = CIP 108995T).

Actinomycetes are distributed in terrestrial environments and have been a source of useful bioactive molecules. By conventional isolation methods, members of the genus Streptomyces comprise more than 95 % of the filamentous actinomycete population of soil (Elander, 1987). Therefore, an organized taxonomic system to identify novel strains is needed in order to preclude reisolation of already known species. The 16S rRNA gene sequence is highly conserved within living cells and has been widely used for evolutionary studies in bacteria (Woese, 1987), placing strain MS405T within the genus Streptomyces. The DNA G+C content of strain MS405T was 72 mol%. 16S rRNA gene sequence data confirmed the taxonomic position of the strain, following the generation of phylogenetic trees by using various treeing algorithms. On the basis of 16S rRNA gene sequence similarity, strain MS405T was shown to belong to the Streptomyces albidoflavus ‘supercluster’, being related to Streptomyces aureus DSM 41785T (99.59 % similarity) and Streptomyces kanamyceticus DSM 40500T (99.32 %). The 16S–23S rRNA internally transcribed spacer (ITS) region exhibited variations in length and sequence composition, showing limited usefulness in phylogenetic analyses. However, DNA relatedness values support the classification of this isolate within a novel species. A number of physiological and biochemical tests distinguished strain MS405T from its closest phylogenetic neighbours. Therefore, strain MS405T represents a novel species, for which the name Streptomyces durmitorensis sp. nov. is proposed, with the type strain MS405T (= DSM 41863T = CIP 108995T).

Actinomycete strain MS405T was isolated by a serial dilution method (http://www.bio.com/protocoltools/protocol.jhtml?id=p2181) from soil samples collected at the Durmitor National Park, Serbia and Montenegro, as a producer of a secondary metabolite exhibiting an FK506-like immunosuppressant mechanism of action (Skoko et al., 2005). The taxonomic status of strain MS405T was investigated using a combination of phenotypic and molecular systematic means, which were indispensable in placing strain MS405T within the genus Streptomyces. Polyphasic study of strain MS405T showed that this strain sequences may be insufficient to define phylogenetic relationships among closely related species and among strains belonging to a species because of evolutionary conservation of the 16S rRNA gene (Woese, 1987). It has been suggested that the 16S–23S rRNA internally transcribed spacer (ITS) region is a powerful tool for phylogenetic analysis of Gram-negative bacteria, but not of Gram-positive bacteria, especially Streptomyces species (Gurtler & Stanisich, 1996; Hain et al., 1997; Song et al., 2004). To overcome these problems, it has now become common practice to delineate novel Streptomyces species using a combination of genotypic and phenotypic data (Kim et al., 1998, 2000; Sembiring et al., 2000) in a so-called polyphasic taxonomic study, which is expected to lead to well-described species and a stable nomenclature (Goodfellow et al., 1997).
should be formally recognized as representing a novel species of the genus *Streptomyces*.

To investigate the phylogenetic relationships of strain MS405<sup>T</sup>, its almost-complete 16S rRNA gene sequence (1517 nt) was determined. Bacterial DNA was extracted by a method described previously (Hopwood et al., 1985). The extracted DNA was subjected to PCR amplification with the bacteria-specific 16S rRNA primers 27f (Lane, 1991) and 1392rev (Marchesi et al., 1998). PCR amplification was performed as described by Marchesi et al. (1998). The 16S–23S ITS region, including the 3′ end of the 16S rRNA gene, was amplified by PCR using primers AM45 (Mehling et al., 1993) and L1 (Jensen et al., 1993). PCR products were excised from the gel and purified using a QIAEXII gel extraction kit (Qiagen) according to the manufacturer’s instructions. The purified product was then ligated to pMOSBlue vector according to the manufacturer’s instructions (Amersham Pharmacia Biotech). Recombinant plasmid constructs were isolated using Qiagen minicolumns (QIAPrep Spin Miniprep kit) and sequenced on an ALF Express sequencer using a Cy5-AutoRead kit (Amersham Biosciences) and the universal sequencing primers M13f and M13r.

16S rRNA gene sequence analysis was conducted using the BLAST network services provided by the NCBI (Altschul et al., 1997) and the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu) (Maidak et al., 2001). Alignment was performed with the CLUSTAL W program (Thompson et al., 1994). 16S rRNA gene sequences and 16S–23S ITS sequences were aligned against published sequences available in the DDBJ/GenBank/EMBL databases by the K<sub>nuc</sub> value of Kimura (1980), and phylogenetic trees were constructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971; Felsenstein, 1993) algorithms contained in the PHYLIP package (version 3.5c; http://www.pasteur.fr). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates. 16S rRNA gene sequence similarities were calculated using the EMBOSS:needle (global) method available at http://www.ebi.ac.uk.

Phylogenetic analysis, at the RDP (Maidak et al., 2001), placed the strain within the evolutionary radiation encompassed by the genus *Streptomyces*, where the 16S rRNA gene sequence of *Streptomyces aureus* DSM 41785<sup>T</sup> was identified with the highest probability (0.969) as the closest matching sequence to MS405<sup>T</sup>. The analysis was supported by the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1993) methods. Comparing *Streptomyces* 16S rRNA gene sequences by phylogenetic tree algorithms, it was obvious that a subclade was formed (Fig. 1) containing sequences of *Streptomyces soculensis* IMSNU 21266<sup>T</sup>, *S. aureus* DSM 41785<sup>T</sup>, *Streptomyces kanamyceticus* DSM 40500<sup>T</sup> and strain MS405<sup>T</sup>. Within this subclade, strain MS405<sup>T</sup> formed a monophyletic line. This relationship was evident in evolutionary trees based on different treeing algorithms: neighbour-joining and DNADIST contained in the PHYLIP package (Felsenstein, 1993), the maximum-parsimony algorithm (Fitch, 1971; Felsenstein, 1993), the maximum-likelihood (Felsenstein, 1993) and BIONJ (Gascuel, 1997) (Fig. 1). The 16S rRNA gene sequence similarity values were 99.59 and 99.32% between strain MS405<sup>T</sup> and *S. aureus* DSM 41785<sup>T</sup> and *S. kanamyceticus* DSM 40500<sup>T</sup>, over 1448 and 1477 nucleotides compared, respectively.

The high degree of sequence divergence of the 16S–23S ITS region among streptomycetes has been shown to be of limited usefulness during phylogenetic positioning of strains (Song et al., 2004), though the utility of this region for inferring phylogenetic relationships has been demonstrated for *Streptomyces albidoflavus* strains (Hain et al., 1997). Sequencing of the 16S–23S ITS region of strain MS405<sup>T</sup>, from eight randomly selected clones, revealed length and sequence composition heterogeneity. The analysed sequences were 283 to 303 nt in length. Within the sequenced spacer regions, five variable regions and six conserved regions were identified. Highly conserved regions C1 to C6 were respectively 14, 99, 12, 11, 9 and 53 nucleotides long. For each of the variable regions V1–V5, more than one sequence was found (see Supplementary Table S1 in IJSEM Online). Consistent with previous observations for other streptomycetes, no tRNA-like sequences were found in any of the 16S–23S ITS regions.
Phylogenetic analyses based on the 16S–23S ITS regions placed strain MS405T close to Streptomyces scabiei isolate 87.79 (clone 79) (Fig. 2), showing the limited usefulness of the 16S–23S ITS region in inferring the phylogenetic position of strain MS405T. The branching of MS405T 16S–23S ITS sequences in two closely related clades is probably due to indel events contained within sequences ITS2, ITS4, ITS6 and ITS7 that are absent in the other four sequences.

DNA relatedness analysis was performed with strain MS405T and the type strains S. aureus DSM 41785T and S. kanamyceticus DSM 40500T by the method of De Ley et al. (1970), under consideration of the modifications described by Huß et al. (1983), using a Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermo-statted 6 × 6 multicell changer and a temperature controller with in situ temperature probe (Varian). Levels of DNA relatedness between strain MS405T and the type strains of two closely related species (based on the phylogenetic data), S. aureus DSM 41785T and S. kanamyceticus DSM 40500T, were respectively 15.5 and 13.3 %. These values clearly indicate that isolate MS405T does not belong to the species S. aureus or S. kanamyceticus, as these values are well below the threshold value of 70 % for definition of bacterial species according to Wayne et al. (1987). Additionally, DNA relatedness values below 80 % have been recommended for the recognition of novel genomic species of Streptomyces (Labeda, 1993, 1996, 1998).

The morphological characteristics of strain MS405T were assessed by transmission (model CM12; Philips) and scanning (model JSM-6460; JEOL) electron microscopy of 14-day-old cultures grown on NE medium at 30 °C (Skeggs et al., 1985), following the procedure of O’Donnell et al. (1993). Cultural characteristics of strain MS405T were recorded after 14 days incubation according to the International Streptomyces Project (ISP) methods (Shirling & Gottlieb, 1966). Colours were described according to the NBS-ISCC Color System (http://www.anthus.com).

Morphological observation of 14-day-old cultures grown on NE agar revealed aerial mycelium which consisted of straight chains of 10 or more rod-shaped, smooth-surfaced spores (0.5–0.9 × 1.0–1.5 μm) (Supplementary Fig. S1). The colour of the substrate mycelium was greenish grey on glycerol-asparagine agar (ISP5), where no diffusible pigment was detected, and medium grey on inorganic salts-starch agar (ISP4). Melanin was not produced on peptone-yeast extract-inositol agar (ISP6) or tyrosine agar (ISP7) plates. Soluble dark-grey and dark-olive-green pigments were observed on ISP4 and NE, respectively. The phenotypic characteristics of strain MS405T and phylogenetically related Streptomyces species are shown in Table 1. Biochemical, physiological and polymer degradation tests were performed as described previously (Seeley & VanDenMark 1981; Hopwood et al., 1985; Williams et al., 1983).

Sensitivity to antibiotics was tested by growing colonies on NE plates with linear antibiotic gradients ranging from 0 to 100 μg ml⁻¹ for 7 days at 30 °C. Detailed physiological and biochemical characteristics of strain MS405T are given in the species description.

The isomer type of dianimonopelic acid (DAP) in the peptidoglycan layer was determined by hydrolysing cells with 6 M HCl at 100 °C for 18 h and analysed by TLC as described previously (Rhuuland et al., 1955; Becker et al. 1965; Hasegawa et al., 1983), followed by ninhydrin staining (0.1 % w/v ninhydrin in aceton). Analysis showed that the cell wall contains LL-DAP, thereby indicating that strain MS405T exhibits cell-wall chemotype I (Lechevalier & Lechevalier, 1970). Utilization of sugars or sugar alcohols as sole carbon sources was monitored in minimal medium containing M9 salts (6 g Na₂HPO₄, 3 g K₂HPO₄, 0.5 g NaCl, 1 g NH₄Cl per litre, filter-sterilized) supplemented with 1 ml 1 M MgSO₄, 1 ml 0.1 M CaCl₂ and 1 % carbon source (w/v) (Miller, 1992). Plates were incubated for 7 days at 30 °C. All media were adjusted to pH 7 prior to seeding.

The chemical, morphological and phylogenetic data suggest that strain MS405T represents a novel species when compared with type strains of species with validly published names within the genus Streptomyces. Thus, on the basis of this polyphasic taxonomic study, strain MS405T merits classification as the type strain of a novel species within the genus Streptomyces, and the name Streptomyces durmitorensis sp. nov. is proposed.

**Description of Streptomyces durmitorensis sp. nov.**

*Streptomyces durmitorensis* (dur.mi.tor.en’sis. N.L. masc. adj. *durmitorensis* pertaining to Durmitor, Serbia and Montenegro, where the type strain was isolated).
L-lysine, ornithine hydrochloride, L-phenylalanine and D-(+)-arabinose are not formed on peptone/yeast extract/iron or tyrosine formed on inorganic salts-starch agar. Melanoid pigments are not formed on oatmeal, yeast extract-malt extract or greenish-yellow aerial spore mass on yeast extract-malt yellowish-grey and a greenish-grey substrate mycelium and a Gram-positive, non-acid-fast streptomycete that produces a

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour of aerial spore mass on ISP5</td>
<td>Greenish yellow</td>
<td>Grey</td>
<td>Colourless to yellow</td>
<td>Grey</td>
</tr>
<tr>
<td>Colour of soluble pigment on:</td>
<td>Dark grey</td>
<td>Reddish orange</td>
<td>Faint brown</td>
<td>Grey</td>
</tr>
<tr>
<td>ISP4</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ISP3</td>
<td>–</td>
<td>Golden</td>
<td>Yellowish pink</td>
<td>–</td>
</tr>
<tr>
<td>Growth on sole carbon sources (1 % w/v):</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Dextran</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>myo-Inositol</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>d-Lactose</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Growth on sole nitrogen sources (0.1 % w/v):</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Hydroxyproline</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Degradation of xanthine</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Maximum NaCl concentration for growth (% w/v)</td>
<td>9</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Growth in the presence of thallous acetate (0.001 % w/v)</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Antibiosis against Micrococcus luteus NCIMB 196</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

Gram-positive, non-acid-fast streptomycete that produces a yellowish-grey and a greenish-grey substrate mycelium and a greenish-yellow aerial spore mass on yeast extract-malt extract and glycerol-asparagine agars. Soluble pigments are not formed on oatmeal, yeast extract-malt extract or glycerol-asparagine agars, while dark-grey pigment is formed on inorganic salts-starch agar. Melanoid pigments are not formed on peptone/yeast extract/iron or tyrosine agars. Spore chains are rectiflexibles, with 10 or more rod-shaped, smooth-surfaced spores (0.5–0.9 × 1.0–1.5 μm) per chain. Temperature range for growth is 10–37 °C with an optimum between 28 and 32 °C. The cell wall contains LL-DAP. Cellobiose, dextran, D-(−)-fructose, D-(−)-galactose, D-(−)-glucose, glycerol, D-(−)-mannit, D-(−)-mannose, α-melibiose, D-(−)-raffinose, L-(−)-rhamnose, sucrose, z-xetralose and D-(−)-xylose are utilized for growth, but L-(−)-arabinose, myo-inositol, α-lactose, β-lactose, maltose and d-sorbitol are not utilized. Acid is produced from glucose. L-Alanine, L-arginine, L-cysteine, L-glycine, L-histidine, L-hydroxyproline, L-methionine, L-proline and L-valine are utilized as sole nitrogen sources, but L-asparagine, L-lysine, ornithine hydrochloride, L-phenylalanine and thiamine are not utilized. Nitrate is reduced to nitrite, gelatin is liquefied, xanthine is degraded, starch is not hydrolysed and ascinulin and DNA are not degraded. Positive for nitrate reduction, catalase, extracellular protease, hae-molysin (β-haemolysis) and urease. Negative for lecithinase and H₂S and indole are not produced. Grows in the presence of NaCl (9 %, w/v) and thallous acetate (0.001 %, w/v), but not sodium azide (0.01 %, w/v), phenol (0.1 %, w/v) or potassium tellurite (0.001 %, w/v). Susceptible to apramycin (10 μg ml⁻¹), kanamycin (5 μg ml⁻¹), gentamycin (5 μg ml⁻¹), tetracycline (10 μg ml⁻¹), thiostrpton (10 μg ml⁻¹), chloramphenicol (35 μg ml⁻¹) and spectinomycin (90 μg ml⁻¹), but resistant to ampicillin (100 μg ml⁻¹), erythromycin (100 μg ml⁻¹) and FK506 (100 μg ml⁻¹). The type strain, MS405ᵀ, produces an immunosuppressant with an FK506-like mechanism of action. The G+C content of the DNA is 72 mol%. The type strain shows antimicrobial activity against Micrococcus luteus NCIMB 196 and Saccharomyces cerevisiae FAV 20, but not against Bacillus subtilis NCIMB 3610ᵀ, Candida albicans CBS 562, Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Saccharomyces cerevisiae FAS 20 or Staphylococcus aureus ATCC 25923.

The type strain, MS405ᵀ (=DSM 41863ᵀ =CIP 108995ᵀ), was isolated from a soil sample taken at the Durmitor National Park, Serbia and Montenegro.

Acknowledgements

This work was supported by grant 143056 from the Ministry of Science of the Republic of Serbia. We would like to thank Dr Graeme Conn for carefully reading and correcting the manuscript, Dr Aleksandra Korac for help with electron microscopy and Mr Dusan Misic and Lidija Djokic for help with physiological tests. We thank the DSMZ staff for their technical assistance with DNA relatedness analysis.

References


