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

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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 MS405 T. Isolate MS405 T 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 MS405 T 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 MS405 T was shown to belong to the Streptomyces albidoflavus ‘supercluster’, being related to Streptomyces aureus DSM 41785 T (99.59 % similarity) and Streptomyces kanamyceticus DSM 40500 T (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 MS405 T from its closest phylogenetic neighbours. Therefore, strain MS405 T represents a novel species, for which the name Streptomyces durmitorensis sp. nov. is proposed, with the type strain MS405 T (=DSM 41863 T =CIP 108995 T).

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 MS405 T within the genus Streptomyces. The DNA G+C content of strain MS405 T 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 MS405 T was shown to belong to the Streptomyces albidoflavus ‘supercluster’, being related to Streptomyces aureus DSM 41785 T (99.59 % similarity) and Streptomyces kanamyceticus DSM 40500 T (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 MS405 T from its closest phylogenetic neighbours. Therefore, strain MS405 T represents a novel species, for which the name Streptomyces durmitorensis sp. nov. is proposed, with the type strain MS405 T (=DSM 41863 T =CIP 108995 T).

Actinomycete strain MS405 T was isolated by a serial dilution method (http://www.bio.com/protocolstools/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 MS405 T was investigated using a combination of phenotypic and molecular systematic means, which were indispensable in placing strain MS405 T within the genus Streptomyces. Polyphasic study of strain MS405 T 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; Sembirin 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\(^T\), 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.*, 1995) 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*\(_{\text{auc}}\) 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 (Felsenstein, 1993), the BIONJ (Gascuel, 1997) (Fig. 1). The 16S rRNA gene sequence similarity values were 99.59 and 99.32% between strain MS405\(^T\) and *S. aureus* DSM 41785\(^T\) and *S. kanamyceticus* DSM 40500\(^T\), over 1448 and 1477 nucleotides compared, respectively.

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\(^T\) was identified with the highest probability (0.969) as the closest matching sequence to MS405\(^T\). 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 scouleri* IMSNU 21266\(^T\), *S. aureus* DSM 41785\(^T\), *Streptomyces kanamyceticus* DSM 40500\(^T\) and strain MS405\(^T\). Within this subclade, strain MS405\(^T\) 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\(^T\) and *S. aureus* DSM 41785\(^T\) and *S. kanamyceticus* DSM 40500\(^T\), 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 *Streptomycies albidoflavus* strains (Hain *et al.*, 1997). Sequencing of the 16S–23S ITS region of strain MS405\(^T\), 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 MS405<sup>T</sup> 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 MS405<sup>T</sup>. The branching of MS405<sup>T</sup> 16S–87.79 (clone 79) (Fig. 2), showing the limited usefulness of

clearly indicate that isolate MS405T does not belong to the

region sequences. Bootstrap values indicated at branching points were based on neighbour-joining analyses of 1000 resampled datasets (only values above 600 are shown). Asterisks indicate branches that were recovered using the maximum-parsimony algorithm. Accession numbers are given in parentheses. Bar, 0.02 nucleotide substitutions per position.

**Fig. 2.** Unrooted neighbour-joining tree based on 16S–23S ITS region sequences. Bootstrap values indicated at branching points were based on neighbour-joining analyses of 1000 resampled datasets (only values above 600 are shown). Asterisks indicate branches that were recovered using the maximum-parsimony algorithm. Accession numbers are given in parentheses. Bar, 0.02 nucleotide substitutions per position.

S. *bikiniensis* DGGE band 2F-2 (AY956502)

S. *durmitorenensis* MS405<sup>T</sup> ITS8 (DQ667295)

S. *durmitorenensis* MS405<sup>T</sup> ITS5 (DQ667292)

S. *durmitorenensis* MS405<sup>T</sup> ITS3 (DQ667290)

S. *durmitorenensis* MS405<sup>T</sup> ITS1 (DQ667288)

S. *durmitorenensis* MS405<sup>T</sup> ITS4 (DQ667291)

S. *durmitorenensis* MS405<sup>T</sup> ITS2 (DQ667289)

S. *durmitorenensis* MS405<sup>T</sup> ITS7 (DQ667294)

S. *durmitorenensis* MS405<sup>T</sup> ITS6 (DQ667293)

S. *scabiei* isolate 87.79 clone 79 (AB042781)

S. *setonii* DSM 40395<sup>T</sup> (AF363495)

S. *griseus* subsp. *griseus* DSM 40236<sup>T</sup> (AF363492)

S. *albidoflavus* DSM 40455<sup>T</sup> clone 3 (Z77334)

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S. *durmitorenensis* MS405<sup>T</sup> ITS3 (DQ667290)

S. *durmitorenensis* MS405<sup>T</sup> ITS1 (DQ667288)

S. *durmitorenensis* MS405<sup>T</sup> ITS4 (DQ667291)

S. *durmitorenensis* MS405<sup>T</sup> ITS2 (DQ667289)

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**Description of *Streptomyces durmitorenensis* sp. nov.**

*Streptomyces durmitorenensis* (dur.mi.tor.en’sis. N.L. masc. adj. *durmitorenensis* pertaining to Durmitor, Serbia and Montenegro, where the type strain was isolated).
Table 1. Phenotypic characteristics that differentiate strain MS405T from its closest neighbours

<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></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISP4</td>
<td>Dark grey</td>
<td>Reddish orange</td>
<td>Faint brown</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>

Strains: 1, MS405T (S. diemortorenis sp. nov.); 2, S. aureus DSM 41785T (data from Atalan et al., 2000; Manfio et al., 2003); 3, S. kanamycticus DSM 40500T (Umezawa et al., 1957); 4, S. seoulensis IMSNU 21266T (Chun et al., 1997). All strains are positive for growth on 1% (w/v) D-(+)-mannitol.

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 oatmeal, yeast extract-malt extract or glycerol-asparagine agars. Soluble pigments are yellowish-grey and a greenish-grey substrate mycelium and a Gram-positive, non-acid-fast streptomycete that produces a 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 Sarccharonycies cerevisiae FAV 20, but not against Bacilus subtilis NCIMB 3610T, Candida albicans CBS 562, Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Sarccharonycies 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.

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References


