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Streptomyces tacrolimicus sp. nov., a low producer of the immunosuppressant tacrolimus (FK506)

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The number of tacrolimus (FK506)-producing isolates has increased remarkably because of the clinical importance of tacrolimus as an immunosuppressant. However, the taxonomy of several of these isolates has not been studied. The taxonomic status of strain ATCC 55098^T, a tacrolimus-producing strain, was determined in this study. The genotypic, phenotypic and chemotaxonomic properties were consistent with the inclusion of strain ATCC 55098^T in the genus *Streptomyces*. The 16S rRNA gene sequence of strain ATCC 55098^T was determined and used to generate phylogenetic trees with corresponding sequences of the most closely related type strains ($\geqslant$98 % 16S rRNA gene sequence similarity) of species of the genus *Streptomyces*. Strain ATCC 55098^T formed a distinct phylogenetic branch adjacent to a cluster comprising *Streptomyces fulvissimus* NBRC 13482^T and *Streptomyces flavofungini* NBRC 13371^T. However, morphological and physiological tests and DNA–DNA relatedness distinguished strain ATCC 55098^T from its closest phylogenetic neighbours. On the basis of these results, strain ATCC 55098^T represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces tacrolimicus* sp. nov. is proposed. The type strain is ATCC 55098^T (=CECT 7664^T).

Species of the genus *Streptomyces* have been an excellent source of clinically and industrially relevant compounds because their genomes contain a large number of gene clusters that encode secondary metabolites (Bentley *et al.*, 2002; Ikeda *et al.*, 2003; Ohnishi *et al.*, 2008). The macrolide tacrolimus (FK506), discovered by Kino *et al.* (1987), was the first of a new family of immunosuppressants that are used in the treatment of graft rejection after organ transplantation. Initially, tacrolimus was detected in a fermentation broth of '*Streptomyces tsukubaensis*' and other tacrolimus-producing strains of the genus *Streptomyces* have been discovered since then, for example strain ATCC 55098^T (Dumont *et al.*, 1992), strain ATCC 53770 (Garrity *et al.*, 1993), strain MA 6949 (Sigmund *et al.*, 2003) and *Streptomyces clavuligerus* CKD1119 (Kim & Park, 2008). Phylogenetic studies have been performed for some of the strains that produce tacrolimus and related compounds (Garrity *et al.*, 1993, Muramatsu *et al.*, 2005) and have shown an apparent lack of genetic relationships between them, despite sharing the same cluster of tacrolimus-biosynthesis genes. Part of the gene cluster for the biosynthesis of tacrolimus has been reported in strain ATCC 55098^T, but there is no effective taxonomic description of this strain.

There are more than 500 recognized species and subspecies in the genus *Streptomyces* (Euzéby, 1997), and the large number of strains has made the taxonomy of this genus difficult. In 1964, the International *Streptomyces* Project (ISP) was established to classify authentic type strains of species of the genus using a relatively small number of traditional tests. This system defined the basis of *Streptomyces* systematics but showed some limitations, which have been solved by a polyphasic approach. The combination of genotypic and phenotypic features provides useful information for the classification of this complex group of bacteria. The present study was designed to determine the taxonomic position of the tacrolimus-producing strain ATCC 55098^T. Strain ATCC 55098^T was obtained from the American Type Culture Collection. This strain was isolated from dung of white-tailed deer in Poverty Creek Drainage, Montgomery County, VA, USA (Dumont *et al.*, 1992).

Abbreviations: DAP, diaminopimelic acid; ISP, International *Streptomyces* Project.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ATCC 55098^T is FN429653.

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Morphological characterization of strain ATCC 55098^T was performed following the standard ISP protocol (Shirling & Gottlieb, 1966). Sporulation, aerial spore-mass colour, substrate mycelium pigmentation and production of diffusible pigments were observed on different agar media following incubation at 28 °C for 14 days. Colours were described according to the ISCC–NBS colour charts (Kelly, 1964). Microscopic observation of hyphal and spore chain arrangements was performed by scanning electron microscopy (JEOL 6100) of 14-day-old cultures grown on ISP 2 medium at 28 °C.

Physiological characteristics were determined following the methods described by Shirling & Gottlieb (1966) and Williams *et al.* (1983) and were recorded after 14 days of incubation at 28 °C, except where specified. Growth with 1, 2, 3, 5, 7 and 10 % (w/v) NaCl was tested on 172 medium [containing (l^{-1}) 10 g glucose, 20 g glucidex-20, 5 g yeast extract, 5 g casein peptone and 1.8 g agar, adjusted to pH 6.8 and then supplemented with 8 g $CaCO_3$]. Antibiotic sensitivity of strain ATCC 55098^T and its closest phylogenetic neighbours was tested on modified Bennett's agar (MBA; Jones, 1949) with different antibiotic concentrations at 28 °C for 7 days.

Fatty acid analysis was carried out using mycelium grown on solid medium. Strain ATCC 55098^T was cultured on 172 medium at 28 °C for 7 days and then on 172A agar [containing (l^{-1}) 10 g glucose, 20 g glucidex-20, 5 g yeast extract, 5 g casein peptone, 2.5 g MOPS and 1.8 g agar; pH 7.2] at 28 °C for another 7 days. Analysis of the fatty acid composition was performed with an Agilent 5898A gas chromatograph using an Ultra2 capillary column as described by Miller (1982) with the modifications of Miller & Berger (1985). The whole-cell sugar composition was determined by TLC following the procedure described by Hasegawa *et al.* (1983). Analysis of the isomer of diaminopimelic acid (DAP) was performed by TLC with ninhydrin staining by the following method. Liquid culture (2 ml) was centrifuged at 3000 r.p.m. for 10 min. The pellet was washed twice by resuspension in 2 ml 8 % SDS, sonication for 10 min in a sonicator bath and centrifugation and then washed with 8 ml distilled water and centrifuged for 5 min. The suspension was hydrolysed with sulfuric acid following the indications of Becker *et al.* (1964) and neutralized with a saturated solution of $Ba(OH)_2$. The supernatant fluid was evaporated and the residue was redissolved in 0.3 ml distilled water. TLC was performed as described by Staneck & Roberts (1974). The G+C content of the genomic DNA was determined by HPLC (Agilent 1100) following the procedure described by Tamaoka & Komagata (1984).

For 16S rRNA gene sequence analysis, genomic DNA of strain ATCC 55098^T was isolated by the salting-out method (Kieser *et al.*, 2000) and the 16S rRNA gene was amplified using primers that corresponded to conserved regions of the gene sequence in other *Streptomyces* species and which were designed to incorporate restriction sites (16S-*Cla*I, 5′-ACATCGATGACGCTGTGAAC-3′; 16S-*Xba*I, 5′-GTG-ATCTAGACGCACCTTCC-3′). PCR was performed with a high-fidelity *Pfx* polymerase (Invitrogen), with the following conditions: 96 °C for 30 s, 67 °C for 30 s and 72 °C for 100 s. The amplification products were separated using gel electrophoresis, excised from the gel and purified using the GFX PCR DNA and Gel Band Purification kit (GE Healthcare). The purified fragments were cloned into the pBluescript SK$^+$ plasmid (Stratagene). The 16S rRNA gene was sequenced using a ABI Prism 3130 Genetic Analyzer (Applied Biosystems). A preliminary sequence analysis was performed using the BLAST network service provided by the European Bioinformatic Institute (EBI) and showed similarity between strain ATCC 55098^T and many members of the genus *Streptomyces*. For phylogenetic analysis, type strains of species of the genus *Streptomyces* that showed ≥98 % 16S rRNA gene sequence similarity with strain ATCC 55098^T were selected. Sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994). Phylogenetic trees were generated according to neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods in PHYLIP 3.65 (Felsenstein, 2005) and MEGA4 (Tamura *et al.*, 2007), respectively. The evolutionary distance matrix for the neighbour-joining algorithm was calculated with Kimura's two-parameter correction model (Kimura, 1980). The topologies of the resultant trees were evaluated by bootstrap analysis (Felsenstein, 1985) with 1000 replicates.

DNA–DNA relatedness was determined between strain ATCC 55098^T and its two closest phylogenetic neighbours. Genomic DNA was isolated using a French pressure cell (Thermo Spectronic) and purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out following the procedure of De Ley *et al.* (1970) with the modifications described by Huß *et al.* (1983) using a 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).

The phenotypic characteristics of strain ATCC 55098^T are compared with those of its closest phylogenetic neighbours in Table 1. The morphological and chemical properties of strain ATCC 55098^T are in concordance with its inclusion in the genus *Streptomyces*, although there were differences in morphological features between the isolate and its closest phylogenetic neighbours. Strain ATCC 55098^T grew well on yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salts-starch agar (ISP 4) and glycerol-asparagine agar (ISP 5). Sporulation was produced on all ISP media after 14 days of incubation, but diffusible pigments were not produced. Melanin was not produced on peptone-yeast extract-iron agar (ISP 6) or tyrosine agar (ISP 7). Morphological observation of 14-day-old cultures grown on ISP 2 medium revealed a branched mycelium without verticils. The aerial mycelium consisted of flexuous chains of short rod-shaped and warty-surfaced spores (Fig. 1).

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Table 1. Phenotypic characteristics that differentiate strain ATCC 55098^T from its closest phylogenetic neighbours

Strains: 1, *Streptomyces tacrolimus* sp. nov. ATCC 55098^T; 2, *S. fulvissimus* DSM 40593^T; 3, *S. flavofungini* DSM 40366^T. Data were taken from this study. All of the strains utilized D-glucose, D-mannitol and D-mannose (1 % w/v) as sole carbon sources, assimilated L-valine, L-cysteine, L-histidine, L-phenylalanine and L-threonine (0.1 % w/v) as sole nitrogen sources, grew at pH 5–9 and 16–30 °C and were resistant to (μg ml^{-1}) penicillin G (100), ampicillin (100) and tacrolimus (100). None of the strains produced melanin on ISP 7, used DL-2-aminobutyric acid as a sole nitrogen source, grew at 4 °C or was resistant to (μg ml^{-1}) kanamycin sulfate (10), apramycin (10) or gentamicin sulfate (10).

Characteristic	1	2	3
Sporulation on:			
ISP 2	Good	Good	Moderate
ISP 3	Good	Poor	Poor
ISP 4	Good	Good	Moderate
ISP 5	Good	Good	Absent
Colour of aerial spore/substrate mycelium on:*			
ISP 2	YG/LO	LP–VO/SO	BYG/LYG
ISP 3	YW/YW	None/MR	None/YW
ISP 4	YW/LO	VO–YP–W/MO	W/PYG
ISP 5	YW/YW	PO–YP/DO	None/PYG
Melanin production on ISP 6	−	+	−
Assimilation of sole carbon sources (1 %, w/v)†			
L-Arabinose	−	+	±
D-Fructose	±	+	±
Lactose	±	−	±
Maltose	−	±	±
D-Rhamnose	+	−	−
Sucrose	−	±	−
Assimilation of L-hydroxyproline as sole nitrogen source‡	−	+	+
Maximum NaCl concentration for growth (%)	10	7	7
Growth at 37 °C	−	+	+
Resistance to (μg ml^{-1}):			
Novobiocin (100)	+	−	−
Thiostrepton (20)	−	+	+
Rifampicin (20)	−	+	−
Streptomycin (20)	−	+	−
Chloramphenicol (20)	−	+	+
Chlortetracycline (20)	−	+	+
Tetracycline (20)	−	+	+

*BYG, Brilliant yellow green; DO, deep orange; LO, light olive; LP, light pink; LYG, light yellow green; MO, moderate orange; MR, moderate red; PO, pale orange; PYG, pale yellowish green; SO, strong orange; VO, vivid orange; W, white; YG, yellowish grey; YP, yellowish pink; YW, yellowish white.
†+, Positive, growth with carbon source is significantly better than without, but somewhat less than with, glucose; ±, doubtful, growth with carbon source is only slightly better than without, and significantly less than with, glucose; −, negative, growth with carbon source is similar to or less than in its absence.
‡L-Asparagine was used as the growth control, as recommended by Langham *et al.* (1989).

Chemotaxonomic analysis of strain ATCC 55098^T showed that the cell wall contained trace levels of LL-DAP but not the *meso* isomer. This trait, together with the lack of diagnostic sugars, is in accordance with the classification of the isolate to the cell-wall type I group (Lechevalier & Lechevalier, 1970). The most important fatty acids in the equivalent chain-length range of C_{12}–C_{18} were iso-$C_{14:0}$ (16.8 %), iso-$C_{16:0}$ (12.5 %), iso-$C_{15:0}$ (12.3 %), anteiso-$C_{15:0}$ (11.4 %), $C_{16:0}$ (3.4 %), $C_{12:0}$ (2.1 %), $C_{16:1}$ *cis*-9 (1.3 %), iso-$C_{17:0}$ (1.3 %), anteiso-$C_{17:0}$ (1.2 %), iso-$C_{16:1}$ (1.0 %) and $C_{17:0}$ cyclo (1.0 %). This composition is characteristic of the genus *Streptomyces*, where the most important fatty acids are of the iso- and anteiso-branched types as well as saturated straight-chain fatty acids (Locci, 1989). The fatty acid profile indicated that strain ATCC 55098^T is a member of the genus *Streptomyces*. The DNA G+C content of strain ATCC 55098^T was 71 mol%.

A 1629 bp fragment of the 16S rRNA gene sequence of strain ATCC 55098^T was obtained. The neighbour-joining phylogenetic tree based on almost-complete (1469 bp) 16S rRNA gene sequences of the isolate and closely related type strains of species of the genus *Streptomyces* indicated that strain ATCC 55098^T formed a separate branch adjacent to a cluster that comprised *Streptomyces fulvissimus* NBRC

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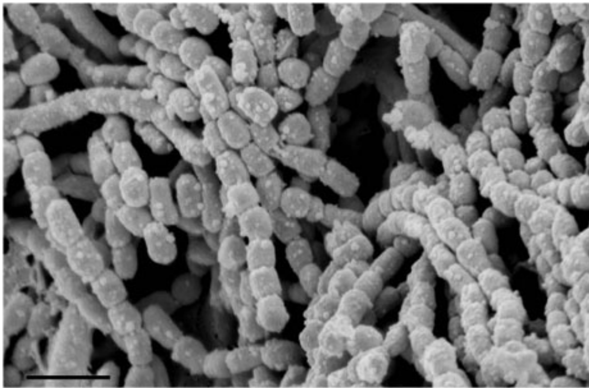

Fig. 1. Scanning electron micrograph of spores of strain ATCC 55098^T after growth on yeast extract/malt extract agar (ISP 2) for 14 days at 28 °C. Bar, 2 μm.

13482^T and *Streptomyces flavofungini* NBRC 13371^T (Fig. 2). In the maximum-likelihood tree, the isolate formed a distinct lineage that was not included in any group. The highest 16S rRNA gene sequence similarities were observed between strain ATCC 55098^T and *S. fulvissimus* NBRC 13482^T (98.6 %) and *S. flavofungini* NBRC 13371^T (98.4 %).

DNA–DNA relatedness between strain ATCC 55098^T and *S. fulvissimus* DSM 40593^T and *S. flavofungini* DSM 40366^T was 31.5 and 14.5 %, respectively. These values were well below the 70 % cut-off point recommended for the separation of genomic species (Wayne *et al.*, 1987) and indicated that strain ATCC 55098^T is a member of a novel species.

On the basis of phenotypic, phylogenetic and chemotaxonomic analysis, we propose that strain ATCC 55098^T represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces tacrolimicus* sp. nov. is proposed. Strain ATCC 55098^T is a less-efficient tacrolimus producer than '*S. tsukubaensis*' under the same fermentation conditions, as measured by HPLC with a method based on that of Shafiee *et al.* (1994).

Description of *Streptomyces tacrolimicus* sp. nov.

Streptomyces tacrolimicus (ta.cro.li′mi.cus. N.L. neut. n. *tacrolimun* tacrolimus; L. suff. *icus -a -um* related to; N.L. masc. adj. *tacrolimicus* related to tacrolimus, referring to the type strain's ability to produce tacrolimus).

Aerobic actinomycete that develops an extensively branched substrate mycelium and aerial hyphae, which differentiate into flexuous chains of short rod-shaped and warty-surfaced spores. Yellowish grey to yellowish white aerial spore mass and light olive to yellowish white substrate mycelium are formed on ISP media. Does not produce diffusible pigments or melanin pigments on peptone-yeast extract-iron agar or tyrosine agar. Growth occurs at 16–30 °C, at pH 5–9 and with 10 % (w/v) NaCl. As sole carbon sources for energy and growth, D-glucose, D-mannitol, D-mannose, D-fructose, lactose and D-rhamnose (each at 1 %, w/v) are used, but L-arabinose, maltose and sucrose are not. As sole nitrogen sources for energy and growth, L-valine, L-cysteine, L-histidine, L-phenylalanine, L-threonine and L-asparagine (each at 0.1 %, w/v) are used, but DL-2-aminobutyric acid and L-hydroxyproline are not. Additional phenotypic characteristics are shown in Table 1. The cell wall contains LL-DAP. Chemotaxonomic characteristics are typical of the genus *Streptomyces*. The DNA G + C content of the type strain is 71 mol%.

The type strain, ATCC 55098^T (=CECT 7664^T), was isolated from the dung of white-tailed deer in Virginia, USA, and produces a small amount of the immunosuppressant tacrolimus.

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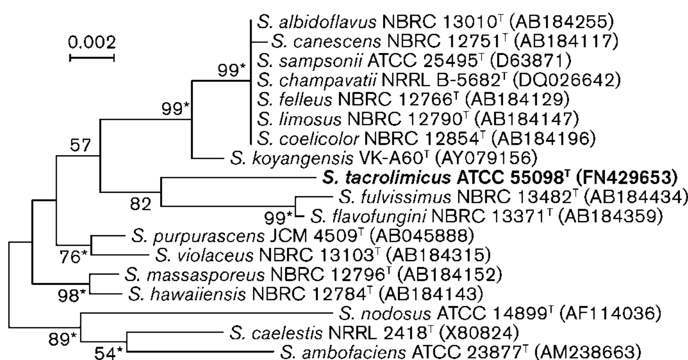

Fig. 2. Neighbour-joining phylogenetic tree based on almost-complete (1469 bp) 16S rRNA gene sequences showing the relationships between strain ATCC 55098^T and closely related type strains of species of the genus *Streptomyces*. Bootstrap values (>50 %) based on 1000 replicates are shown at branch nodes. Asterisks indicate that the corresponding nodes were also recovered in the tree generated using the maximum-likelihood algorithm. Bar, 0.002 substitutions per nucleotide position.

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