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NOTE

Streptomyces tsukubensis sp. nov., a producer of the immunosuppressant tacrolimus

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Streptomyces is well-known as a good producer of pharmaceutically important chemical compounds, not only antibiotics but also physiologically active compounds. Tacrolimus was found as a strong immunosuppressant from a culture broth of an actinomycete strain, '*Streptomyces tsukubaensis*' 9993^T, which was isolated from a soil sample collected from Mount Tsukuba, Ibaraki, Japan, in 1984. The first report of this compound was published in 1987,[1] and the drug was launched in 1993 in Japan as an immunosuppressive agent for the prevention of graft rejection after liver transplantation. To date, it has been sold in about 90 countries in several therapeutic areas, including organ transplantation, myasthenia gravis, articular rheumatism, lupus nephritis, ulcerative colitis and atopic dermatitis, among others.

After initial discovery of the compound, several taxonomically diverse streptomycete strains were reported as tacrolimus producers, including *S. tacrolimicus* ATCC 55098^T,[2] *S. clavuligerus* CKD 1119[3] and *S. kanamyceticus* KCC S-0433^T.[4] Further, biosynthetic gene clusters of tacrolimus in some strains have been reported.[5–7] To date, however, no effective taxonomic description of the species '*S. tsukubaensis*' has been available. Clarification of the taxonomic position of strain 9993^T is important not only for streptomycete taxonomy, but also for evolutionary studies of secondary metabolite biosynthesis gene clusters. We previously revealed that strain 9993^T does not belong to any described species according to its 16S rRNA gene sequence phylogeny.[4] We therefore investigated its morphological and physiological characteristics and phylogeny to confirm its taxonomic position. Here, we report this taxonomic study of strain 9993^T and propose *S. tsukubensis* sp. nov.

Strain 9993^T, the original strain of '*S. tsukubaensis*', was isolated from a soil sample collected from Mount Tsukuba, Ibaraki, Japan in 1984,[1] and has been stored as a lyophilized ampule for 27 years. The suspension of lyophilized spores was inoculated on inorganic salts–starch agar (ISP medium 4) and incubated at 28 °C for 1–2 weeks. The culture was stored at 4 °C during this study. *S. clavuligerus* NBRC 13307^T was obtained from Biological Resource Center, National Institute of Technology and Evaluation, Japan. Media preparations, morphological observations and carbon sources utilization tests were performed by the methods of Shirling and Gottlieb.[8] For determination of morphological characteristics, the strain was incubated at 28 °C for 14 days, then morphologically examined using light and scanning electron microscopy (Hitachi S-2600N, Hitachi High-Technologies Corp., Tokyo, Japan). Color determinations were referenced against the Methuen Handbook of Color (Methuen London Ltd., London, UK). Growth temperature was determined on ISP medium 4 at 5, 13, 15, 20, 25, 28, 30, 32, 34, 35, 36, 37 and 38 °C, while tolerance to sodium chloride was determined on yeast extract-malt extract agar (ISP medium 2) with 0, 4, 7, 10 and 13% of sodium chloride.

Amino acids in cell wall hydrolysates, cellular fatty acids, menaquinones and G + C content of the genomic DNA and DNA–DNA reassociation value were determined at TechnoSuruga Laboratory Co., Ltd. (Shizuoka, Japan). Amino acids, menaquinones and G + C content were analyzed by high-performance liquid chromatography. Cellular fatty acids were analyzed using the standard MIDI system. Whole-cell sugar composition was determined by Thin Layer Chromatography, following the method of Hasegawa *et al.*[9] Polar lipids were analyzed by two-dimensional Thin Layer Chromatography method.[10]

The 16S rRNA gene sequence of the strain was determined in our previous study (Accession no.; AB217600).[4] Homology search was performed using the FASTA program.[11] DNA sequence data was downloaded from the NCBI web site (http://www.ncbi.nlm.nih.gov/). BLAST search[12] was performed at the DDBJ web site (http://www.ddbj.nig.ac.jp/). Validity of bacterial names was referenced against the List of Prokaryotic Names with Standing in Nomenclature (http://www.bacterio.cict.fr/). The phylogenetic trees were constructed with 68 closest valid species using the neighbor-joining[13] or maximum-parsimony methods[14] in CLUSTAL X[15] or MEGA package.[16]

Strain 9993^T grew well on ISP medium 4 and ISP medium 2. The color of the substrate mycelium was pinkish white to grayish orange. The strain produced a grayish aerial mycelium abundantly on ISP medium 4, and moderately on ISP medium 2. It formed flexuous

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spore chains composed of >10 smooth surface spores (Figure 1). The shape of spores was cylindrical, 0.5–0.7 μm in diameter and 0.7–0.8 μm in length. It showed weak or poor growth on oatmeal agar (ISP medium 3), glycerol–asparagine agar (ISP medium 5), peptone–yeast extract-iron agar (ISP medium 6) and tyrosine agar (ISP medium 7). No or very few aerial mycelia were observed on these media.

Strain 9993^T did not produce melanin or any other soluble pigments in the media tested. Growth occurred at 15–35 °C and optimum growth was observed at 28 °C. No aerial mycelia were observed at 35 °C. Strain 9993^T showed weak growth and different morphology on ISP medium 2 with 4% of sodium chloride from that with 0%. After 1-week incubation, the strain showed green colonies with a colorless edge, without aerial mycelium. Further, the colonies turned black in color after one more week of incubation, forming *Micromonospora*-like colonies. No growth was observed with 7%. Other physiological characteristics are given in Table 1 and in the species description.

LL-Diaminopimelic acid, glycine, glutamic acid and alanine were detected in cell wall hydrolysates, and glucose was detected as whole-cell sugar but mannose, ribose, rhamnose, galactose, arabinose, xylose and madurose were not. These results are typical of cell wall chemotype I.[17] The major cellular fatty acids were iso-$C_{16:0}$ (21.67%), iso-$C_{15:0}$ (13.16%), $C_{16:1}$ cis-9 (11.96%) and $C_{16:0}$ (10.57%). Phosphatidylethanolamine and diphosphatidylglycerol were detected as major phospholipids. The major menaquinones were MK-9 (H_8) (86.6%) and MK-9 (H_6) (8.0%). The DNA G+C content of strain 9993^T was calculated to be 72.4 mol%. These chemotaxonomic characteristics of the strain agreed with those of the genus *Streptomyces*.

FASTA homology search result revealed that the closest species to strain 9993^T was *S. clavuligerus* NBRC 13307^T (Accession no.; AB184343). The 200 highest-scored sequences of the FASTA search were examined to exclude invalid species and redundancies. Finally, 45 species with similarity values to strain 9993^T ranging from 98.38 to 97.16% were selected. Thirty-three species with similarity values ranging from 97.93 to 96.62% were selected from the 200 highest-scored sequences of the BLAST search using same procedure. Ten species were common between the FASTA and BLAST results, so 68 sequences of valid species of the genus *Streptomyces* were used for further phylogenetic analysis. The neighbor-joining and maximum-parsimony phylogenetic trees on the basis of almost-complete 16S rRNA gene sequences of strain 9993^T and the selected 68 valid species indicated that strain 9993^T did not form a reliable cluster with any valid species (Figure 2). The similarity value between strain 9993^T and *S. clavuligerus* NBRC 13307^T was 98.38%. The average value of DNA–DNA reassociation between strain 9993^T and NBRC 13307^T was 12%, which is consistent with Stackebrandt's theory.[18] This result thus strongly indicates that strain 9993^T does not belong to any known species. This conclusion was supported by differences in some phenotypic characters between strain 9993^T and *S. clavuligerus*.[19] *S. clavuligerus* had a club-shaped side-branching chain, which is uncommon in the genus *Streptomyces*, while strain 9993^T had a branchless flexuous chain. Gelatin liquefaction was positive for strain 9993^T. These characteristics did not agree with those of *S. clavuligerus*. Utilization of dexran, D-fructose, galactose, lactose, D-melibiose and sodium propionate differed between strain 9993^T and *S. clavuligerus* NBRC 13307^T (Table 1).

Although '*S. tsukubaensis*' NRRL 18488 is a patent strain, we consider that it is worthwhile to mention the relationship between strain 9993^T and NRRL 18488 because the biosynthetic gene cluster for tacrolimus of NRRL 18488 is already available,[6] and the genome sequence project of NRRL 18488 is now ongoing.[20] Strain 9993^T was deposited on 19 October 1985 with the Fermentation Research Institute, Agency of Industrial Science and Technology (Japan) under the deposit number FERM BP-927 for patent application.[21] According to another patent description,[22] the strain FERM BP-927 was redeposited on 27 April 1989 with the Agricultural Research

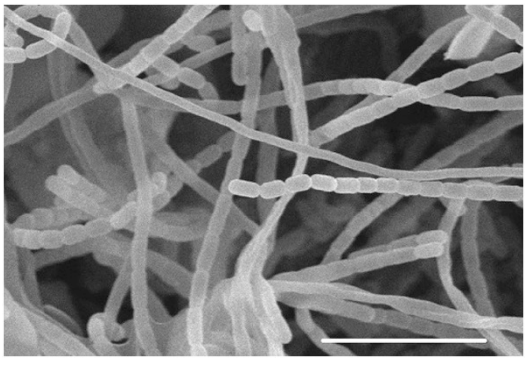

Figure 1 Scanning electron micrograph of strain 9993^T. Bar, 5 μm.

Table 1 Carbon source utilization of strain 9993^T and *S. clavuligerus* NBRC 13307^T

Carbon source	*9993^T*	*S. clavuligerus NBRC 13307^T*
L-arabinose	−	−
D-xylose	w	w
D-glucose	+	+
D-fructose	−	+
Sucrose	−	−
L-rhamnose	−	−
Raffinose	−	−
Inositol	w	−
D-Mannitol	−	−
Ribitol	−	−
D-cellobiose	+	+
Dextran	+	−
Dextrin	+	+
Dulcitol	−	−
Galactose	−	+
Glycerol	+	+
Inulin	−	w
Lactose	−	+
Mannose	w	+
Maltose	w	+
D-Melezitose	−	−
D-Melibiose	−	+
Salicin	+	+
Sorbitol	−	−
Starch	+	+
Sorbose	−	−
Trehalose	w	+
Xylitol	−	−
Sodium acetate	w	+
Sodium citrate	w	w
Sodium malonate	−	−
Sodium propionate	−	+
Sodium pyruvate	w	w

Abbreviations: +, positive; −, negative; w, weakly positive.

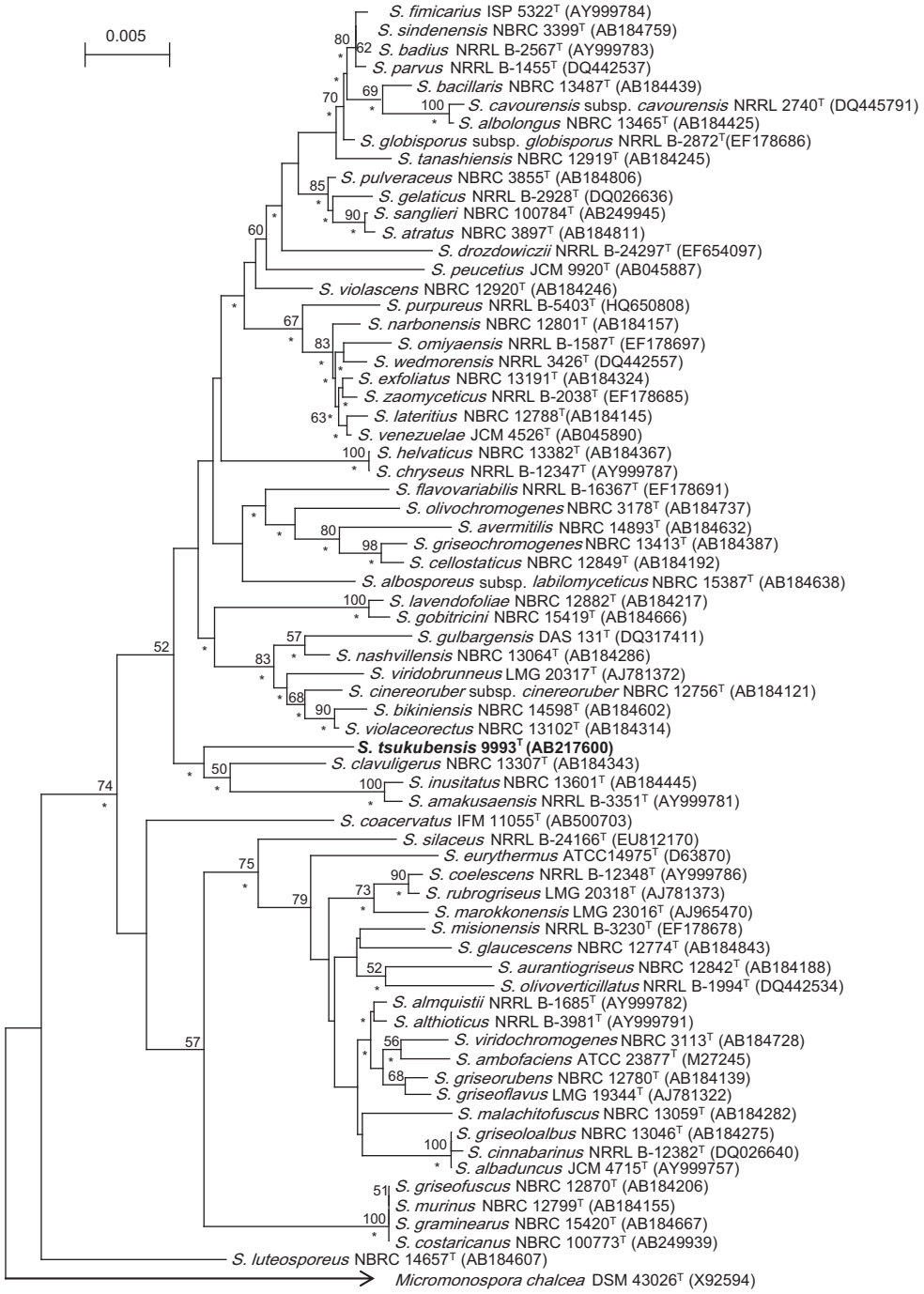

Figure 2 Neighbor-joining phylogenetic tree on the basis of almost-complete 16S rRNA gene sequences showing the relationship between strain 9993^T and closely related valid species of the genus *Streptomyces*. Bootstrap value (>50%) on the basis of 1000 replicates are shown at branch nodes. Asterisks indicate branches on the tree that were also recovered with the maximum-parsimony method. Bar, 0.005 substitutions per nucleotide position.

Culture Collection International Depository (USA) under the deposit number NRRL 18488. Strain NRRL 18488 was therefore considered to be identical to strain 9993^T.

Description of *Streptomyces tsukubensis* sp. nov.

Streptomyces tsukubensis (tsu.ku.b.en'sis. N.L. masc. adj. *tsukubensis* pertaining to Mt. Tsukuba, Ibaraki, Japan, the origin of the soil sample from which the type strain was isolated).

A Gram-positive, aerobic actinomycete that forms extensively branched substrate hyphae. Vegetative hyphae are finely branched and do not fragment. Monopodially or dichotomously branching aerial mycelia develop abundantly. Grayish orange colonies are formed on ISP 2. Temperature range for growth is 15–35 °C, with optimum growth at 28 °C. D-Glucose, D-cellobiose, dextran, dextrin, glycerol, salicin and starch are used as sole carbon sources. Milk peptonization and gelatin liquefaction are positive, milk coagulation

and cellulose decomposition are negative. Major menaquinones are MK-9 (H_8) and MK-9 (H_6). Major phospholipids are phosphatidylethanolamine and diphosphatidylglycerol. Major cellular fatty acids are iso-$C_{16:0}$, iso-$C_{15:0}$, $C_{16:1}$ cis-9 and $C_{16:0}$. The cell wall contains LL-A_2pm, glycine, glutamic acid and alanine. Contains glucose as a whole-cell sugar. The DNA G + C content of the type strain is 72.4 mol%. The type strain, 9993^T (= NBRC 108819^T), was isolated from soil from Mount Tsukuba, Japan, and produces the immunosuppressant tacrolimus.

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