# *Streptomyces samsunensis* sp. nov., a member of the *Streptomyces violaceusniger* clade isolated from the rhizosphere of *Robinia pseudoacacia*

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The taxonomic position of a *Streptomyces* isolate, strain M1463$^T$, recovered from the rhizosphere of *Robinia pseudoacacia* was established in a polyphasic study. The organism had chemical and morphological markers that were consistent with its classification in the *Streptomyces violaceusniger* clade. This assignment was confirmed by 16S rRNA gene sequence data, which also showed that the strain formed a distinct subclade together with *Streptomyces malaysiensis* DSM 41697$^T$. However, the two strains were readily distinguished on the basis of DNA relatedness and phenotypic data. The combined genotypic and phenotypic data show that strain M1463$^T$ should be recognized as a representative of a novel species in the *Streptomyces violaceusniger* clade, for which the name *Streptomyces samsunensis* sp. nov. is proposed. The type strain of *S. samsunensis* is M1463$^T$ (=DSM 42010$^T$=NRRL B-24803$^T$).

Taxonomic relationships within the genus *Streptomyces* have been clarified and extended by the application of genotypic and phenotypic methods to representatives of species with validly published names and putatively novel species (Goodfellow *et al.*, 1992; Manfio *et al.*, 1995; Anderson & Wellington, 2001; Kumar & Goodfellow, 2008). It is now apparent that the type strains of many species of the genus *Streptomyces* can be assigned to distinct multimembered species groups, as exemplified by species classified in the *Streptomyces albidoflavus* (Lanoot *et al.*, 2005), *Streptomyces griseus* (Liu *et al.*, 2005), *Streptomyces violaceoruber* (Duangmal *et al.*, 2005), *Streptomyces violaceusniger* (Sembiring *et al.*, 2000) and *Streptomyces yeochonensis* (Xu *et al.*, 2006) 16S rRNA gene clades. Members of the *S. violaceusniger* clade typically form a greyish aerial spore mass and a greyish yellow substrate mycelium on oatmeal agar, produce aerial hyphae that differentiate into spiral chains of rugose ornamented spores (Sembiring *et al.*, 2000; Goodfellow *et al.*, 2007; Kumar & Goodfellow, 2008), give a characteristic amplification product with taxon-specific primers (Kumar *et al.*, 2007), and produce the metabolites elaiophylin, geldanamycin, nigericin and an uncharacterized polyene (Ward & Goodfellow, 2004).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Streptomyces samsunensis* M1463$^T$ is EU077190.

The *S. violaceusniger* clade currently encompasses species with validly published names and that are mainly circumscribed using a combination of DNA–DNA relatedness and phenotypic data (Labeda & Lyons, 1991; Sembiring *et al.*, 2000; Goodfellow *et al.*, 2007; Kumar & Goodfellow, 2008). Members of the clade have been isolated from geographically diverse soils (Al-Tai *et al.*, 1999; Saintpierre *et al.*, 2003; Hayakawa *et al.*, 2004) and from rhizosphere and non-rhizosphere soils (Sembiring *et al.*, 2000). PCR amplification of DNA extracted from marine and terrestrial samples using *S. violaceusniger* clade-specific primers has provided evidence for the widespread distribution of novel members of the clade in natural habitats (Kumar *et al.*, 2007).

In the course of a screening programme designed to recover novel streptomycetes from rhizosphere soil, a strain, designated M1463$^T$, was shown to have activity against several microbial targets and to have colonial and morphological features suggesting that it may be a member of the *S. violaceusniger* clade. The aim of the present study was to determine the taxonomic status of the isolate using a polyphasic taxonomic approach. The resultant data indicate that the organism should be classified as a representative of a novel species of the genus *Streptomyces*.

Strain M1463$^T$ was isolated after 14 days of incubation at 28 °C from the rhizosphere of *Robinia pseudoacacia* by plating soil suspensions onto starch-casein agar (Küster,

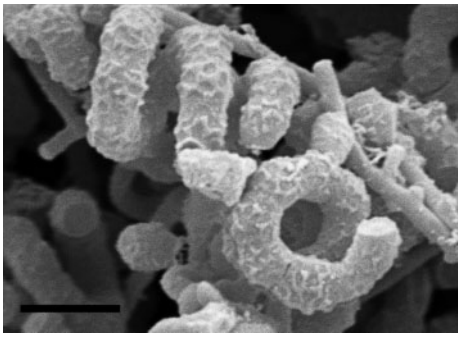

**Fig. 1.** Scanning electron micrograph showing spiral chains of rugose ornamented spores of strain M1463$^T$ grown on inorganic salts-starch agar (ISP 4 medium) at 28 °C for 14 days. Bar, 2 μm.

1959) supplemented with filter-sterilized cycloheximide (50 μg ml$^{-1}$), nystatin (50 μg ml$^{-1}$) and rifampicin (0.5 μg ml$^{-1}$). The organism was maintained on slopes of oatmeal agar (DSMZ, 1998) and as mycelial fragments and spores in glycerol (20 %, v/v) at −20 °C. Biomass for chemotaxonomic and molecular systematic studies was grown in shake flasks of tryptic soy broth at 160 r.p.m. at 28 °C for 14 days, checked for purity and harvested by centrifugation. Cells for the chemotaxonomic tests were washed twice in distilled water and freeze-dried; cells for molecular systematic procedures were washed in NaCl/EDTA buffer (0.1 M EDTA, 0.1 M NaCl; pH 8.0) and stored at −20 °C until required.

The isolate was grown on oatmeal agar (ISP 3; Shirling & Gottlieb, 1966) and peptone-yeast extract-iron agar (ISP 6; Shirling & Gottlieb, 1966) plates at 28 °C for 14 and 4 days, respectively. The oatmeal agar plates were examined by eye to determine aerial spore mass colour, substrate mycelium pigmentation and the colour of any diffusible pigment using National Bureau of Standards Color Name Charts (Kelly, 1958; National Bureau of Standards, 1964). The peptone-yeast extract-iron agar plates were examined to see whether the strain produced melanin pigments. Spore-chain morphology and spore-surface ornamentation were determined by examining gold-coated dehydrated specimens using a Cambridge Stereoscan 240 instrument. The strain was also probed using the *S. violaceusniger* clade-specific oligonucleotide primers described by Kumar *et al.* (2007). The organism formed a greyish aerial spore mass, which later turned black, a greyish yellow substrate mycelium and no diffusible pigment on oatmeal agar; it did not produce melanin pigments on peptone-yeast extract-iron agar. It formed spiral chains of rugose ornamented spores (Fig. 1) and gave a PCR amplification product characteristic of members of the *S. violaceusniger* clade. All of these properties indicated that isolate M1463$^T$ is a *bona fide* member of the *S. violaceusniger* clade (Goodfellow *et al.*, 2007; Kumar & Goodfellow, 2008).

Genomic DNA extraction and PCR-amplification of the 16S rRNA gene of strain M1463$^T$ were carried out with minor modifications of the procedure used by Pitcher *et al.* (1989), as described by Sembiring *et al.* (2000). The amplified fragments were purified with QIAquick purifica-

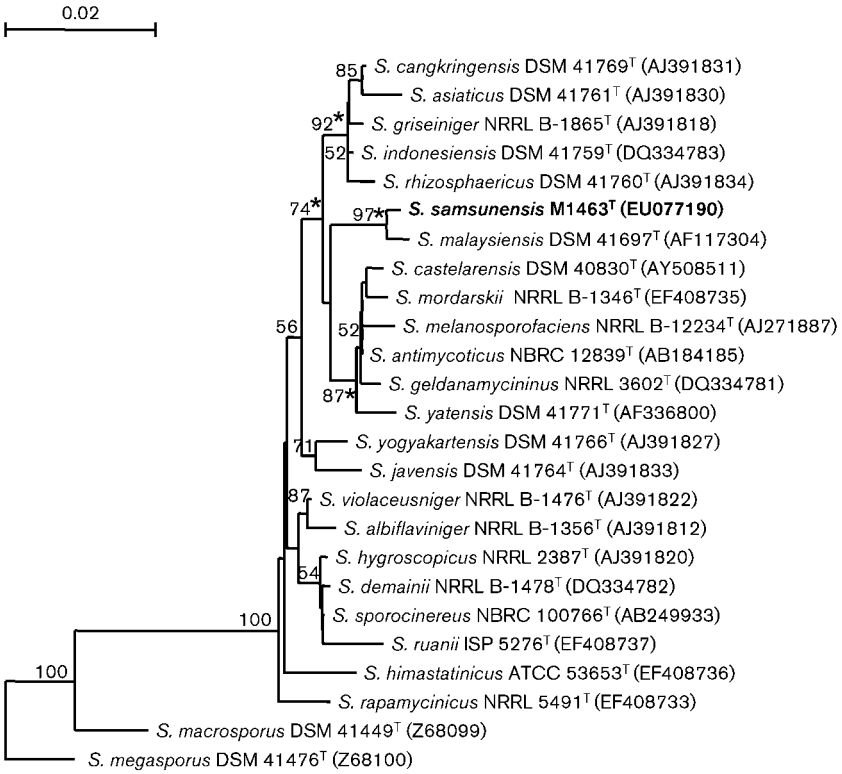


**Fig. 2.** Neighbour-joining tree based on 1438 bp of 16S rRNA gene sequences showing the position of strain M1463$^T$ on the *S. violaceusniger* gene tree. The asterisks denote branches that are conserved when using the least-squares, maximum-likelihood and maximum-parsimony tree-making algorithms. The numbers at the nodes indicate levels of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets; only values >50 % are cited. GenBank accession numbers are given in parentheses. Bar, 0.02 substitutions per site.

tion kits (Qiagen) and sequenced directly using ABI Prism BigDye Terminator v2.0 Cycle Sequencing kits (Applied Biosystems) and standard oligonucleotide primers (Lane, 1991; Chun & Goodfellow, 1995). Sequencing gel electrophoresis was performed and the nucleotide sequences were obtained automatically by using an Applied Biosystems DNA sequencer (ABI PRISM 310) and software provided by the manufacturer. The resultant 16S rRNA gene sequence was aligned against corresponding sequences of members of the *S. violaceusniger* clade retrieved from GenBank/EMBL/DDBJ using the PHYDIT program (Chun, 1995). Unrooted phylogenetic trees were inferred using the least-squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1993) and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms drawn from the PHYLIP 3.5c suite of programs (Felsenstein, 1993). Evolutionary distance matrices were generated for the neighbour-joining and least-squares methods, as described by Jukes & Cantor (1969). The resultant tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) with 1000 resamplings from the neighbour-joining dataset using the SEQBOOT and CONSENSE programs from the PHYLIP package (Felsenstein, 1993).

Comparison of the almost complete 16S rRNA nucleotide sequence obtained for strain M1463$^T$ (1478 nt) with corresponding sequences of type strains of species classified in the *S. violaceusniger* clade showed that the isolate formed a well-delineated subclade with *S. malaysiensis* DSM 41697$^T$; the taxonomic status of the subclade was supported by all of the tree-making algorithms and by a bootstrap value of 97 % in the neighbour-joining analysis (Fig. 2). Identification of the closest phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were provided using the EzTaxon server (http://www.eztaxon.org/; Chun *et al.*, 2007). The two strains shared a 16S rRNA gene similarity of 99.5 %, a value which corresponded to 7 nt differences at 1438 locations. Strain M1463$^T$ was also relatively closely related to *Streptomyces indonesiensis* DSM 41759$^T$ (98.8 % similarity, 17 nt differences), *Streptomyces griseiniger* NRRL B-1865$^T$ (98.7 % similarity, 18 nt differences) and *Streptomyces cangkringensis* DSM 41769$^T$ (98.6 % similarity, 19 nt differences). All of these similarity values were below those recorded between the type strains of several species classified in the *S. violaceusniger* clade (Goodfellow *et al.*, 2007; Kumar & Goodfellow, 2008). Consequently, these results were in line with strain M1463$^T$ being recognized as the type strain of a novel species.

The level of DNA–DNA relatedness between M1463$^T$ and *S. malaysiensis* DSM 41697$^T$ was determined by measuring the divergence between the thermal denaturation midpoints of homologous and heterologous DNA preparations ($\Delta T_m$) following the procedure developed by Gonzalez & Saiz-Jimenez (2005). In addition, the DNA G+C content of strain M1463$^T$ was determined following the procedure used by these workers. The $\Delta T_m$ value found between the DNA preparations of the two strains was 7.2 °C, a result well above the cut-off point recommended for the delineation of genomic species ($\Delta T_m$ >5 °C; Wayne *et al.*, 1987) and one equivalent to a DNA relatedness value of just above 50 %. $\Delta T_m$ values well below 7.2 °C, but above 5 °C, have been recorded between type strains of species assigned to the *S. violaceusniger* clade. The DNA G+C content of strain M1463$^T$ was 71.8 mol%.

Strain M1463$^T$ was also examined for a range of chemotaxonomic, colonial and other phenotypic features, the latter by using modified Bennett's agar (Jones, 1949) and standard ISP media (Shirling & Gottlieb, 1966). All phenotypic features were examined using media and methods described by Williams *et al.* (1983). The dominant

**Table 1.** Phenotypic characteristics that distinguish strain M1463$^T$ from related species of the genus *Streptomyces*

Strains: 1, M1463$^T$; 2, *S. malaysiensis* DSM 41697$^T$ (data from Al-Tai *et al.*, 1999); 3, *S. indonesiensis* DSM 41759$^T$ (Sembiring, 2000); 4, *S. griseiniger* DSM 41895$^T$ (Sembiring, 2000). All strains were positive for urea hydrolysis, nitrate reduction, degradation of casein, growth at 37 °C and pH 5–10, utilization of fructose, galactose, glucose, mannitol, trehalose, melibiose and rhamnose as sole carbon sources, and utilization of L-alanine as sole carbon and nitrogen source. In contrast, they did not grow at 4 or 45 °C, hydrolyse allantoin, degrade chitin, guanine or xanthine, use sucrose (1 %, w/v), sodium citrate or sodium acetate as a sole carbon source (0.1 %, w/v), or grow in the presence of neomycin sulphate (30 μg ml$^{-1}$) or gentamicin sulphate (10 μg ml$^{-1}$). +, Positive or utilized; −, negative or not utilized.

| Characteristic | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Degradation tests (%, w/v) | | | | |
| Aesculin hydrolysis (0.1) | − | + | + | + |
| Arbutin hydrolysis (0.1) | + | − | + | + |
| Gelatin (0.4) | − | + | + | + |
| Hypoxanthine (0.4) | − | + | − | + |
| L-Tyrosine (0.4) | − | + | + | + |
| RNA | − | − | + | − |
| Tween 80 (1.0) | + | + | − | − |
| Growth on sole carbon sources (%, w/v) | | | | |
| Adonitol (1.0) | − | + | + | + |
| (+)-L-Arabinose (1.0) | + | + | + | − |
| Cellobiose (1.0) | + | + | + | − |
| Dextrin (1.0) | + | + | + | − |
| Lactose (1.0) | + | − | + | + |
| Salicin (1.0) | − | + | + | + |
| *myo*-Inositol (1.0) | − | + | + | + |
| Raffinose (1.0) | − | + | + | + |
| Xylose (1.0) | − | + | − | − |
| Sodium propionate (0.1) | − | + | − | + |
| Growth on sole nitrogen sources (0.1 %, w/v) | | | | |
| L-Isoleucine | + | + | − | + |
| L-Methionine | + | − | + | + |
| L-Serine | + | + | − | + |
| Growth at pH 4.0 | − | + | + | + |
| Growth in 8 μg chloramphenicol ml$^{-1}$ | + | − | + | − |

diaminopimelic acid isomer was determined using the TLC system of Staneck & Roberts (1974), albeit using a modified solvent system (methanol/$H_2O$/10 M HCl/pyridine, 85 : 15 : 5 : 10, by vol.); menaquinones were extracted and purified following Collins (1985) and analysed by HPLC (Wu *et al.*, 1989). Fatty acids were extracted, methylated and analysed by GC using the MIDI (Microbial ID) system (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). The organism contained LL-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan, octahydrogenated menaquinones with nine isoprene units as the dominant isoprenologue and had a fatty acid profile rich in iso- and anteiso-fatty acids (iso-$C_{15:0}$, 33.4 %; anteiso-$C_{15:0}$, 15.3 %; $C_{16:0}$, 10.9 %), properties that are typical of streptomycetes (Manfio *et al.*, 1995; Anderson & Wellington, 2001). It can be seen from Table 1 that while strain M1463$^T$ and *S. malaysiensis* DSM 14697$^T$ had properties in common, they can be readily distinguished by using a combination of biochemical and physiological features, i.e. strain M1463$^T$, unlike *S. malaysiensis* DSM 14697$^T$, hydrolyses arbutin but does not hydrolyse aesculin, degrade gelatin, hypoxanthine or L-tyrosine, or grow on a range of sole carbon sources for energy and growth. The two strains can also be separated based on the colour of their aerial spore mass and substrate mycelia, and their ability to produce diffusible pigments on a range of organic media (Table 2).

Isolate M1463$^T$ and the most related species of the genus *Streptomyces* were examined for a range of phenotypic markers using established procedures (Williams *et al.*, 1983). A comparison of the phenotypic characteristics of

**Table 2.** Comparative growth and cultural characteristics of strain M1463$^T$ and related species of the genus *Streptomyces*

Taxa: 1, M1463$^T$; 2, *S. malaysiensis* DSM 41697$^T$ (data from Al-Tai *et al.*,1999); 3, *S. indonesiensis* DSM 41759$^T$ (Sembiring, 2000); 4, *S. griseiniger* DSM 41895$^T$ (Sembiring, 2000). + + +, Abundant growth; + +, moderate growth; +, poor growth.

| Media | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Modified Bennett's agar | | | | |
| Growth | + + + | + + | + + + | + |
| Aerial mycelium | Grey | White | White | White |
| Reverse colour | Yellow-green | Brown | Brown | Colourless |
| Soluble pigment | None | Light brown | Brown | None |
| Glucose-yeast extract-malt extract agar | | | | |
| Growth | + + + | + + + | + | + + + |
| Aerial mycelium | Grey | Dark grey | Grey–white | White–grey |
| Reverse colour | Yellow–brown | Brown–grey | Brown | Yellow |
| Soluble pigment | None | Brown | Yellow | None |
| Glycerol-asparagine agar (ISP 5) | | | | |
| Growth | + + | + | + + | + + |
| Aerial mycelium | Grey | White–grey | White | White |
| Reverse colour | Brown | Pale yellow–grey | Light yellow | Colourless |
| Soluble pigment | None | None | None | None |
| Oatmeal agar (ISP 3) | | | | |
| Growth | + + + | + + + | + + + | + + + |
| Aerial mycelium | Grey | Smoky black | Grey | Grey |
| Reverse colour | Greyish yellow | Yellow–brown | Greyish yellow | Greyish yellow |
| Soluble pigment | None | None | Yellow | None |
| Inorganic salts-starch agar (ISP 4) | | | | |
| Growth | + + + | + + + | + + + | + |
| Aerial mycelium | Grey | Smoky black | Brownish grey | Grey–white–black |
| Reverse colour | Grey–green | Grey | Yellow | Yellow–orange |
| Soluble pigment | None | Yellow | None | Orange |
| Tyrosine agar (ISP 7) | | | | |
| Growth | + + + | + + + | + + | + + |
| Aerial mycelium | Grey | Grey | Dark grey | White–grey |
| Reverse colour | Brown | Brown | Brown | Brown |
| Soluble pigment | None | Dark brown | None | None |
| Yeast extract-malt extract agar (ISP 2) | | | | |
| Growth | + + + | + + + | + | + + + |
| Aerial mycelium | Grey | Dark grey | White | White |
| Reverse colour | Brown | Brown–grey | Brown | Yellow |
| Soluble pigment | None | Brown | Light brown | Yellow |

strain M1463$^T$ and the most closely related *Streptomyces* species is shown in Table 1. It is clear from these comparisons that strain M1463$^T$ differs phenotypically from the type strains of the three recognized *Streptomyces* species.

The genotypic and phenotypic data show that strain M1463$^T$ merits recognition as a representative of a novel species of the *S. violaceusniger* clade. The name proposed for this taxon is *Streptomyces samsunensis* sp. nov.

### Description of *Streptomyces samsunensis* sp. nov.

*Streptomyces samsunensis* (sam.sun.en′sis. N.L. masc. adj. *samsunensis* from Samsun, Turkey, the source of the organism).

Spore chains are spirals; the spore surface is rugose. Grows well on modified Bennett's, glucose-yeast extract-malt extract, glycerol-asparagine, inorganic salts-starch, tyrosine, yeast extract-malt extract, oatmeal and peptone-yeast extract-iron agars. On oatmeal agar, the aerial spore mass colour is grey, becoming black and moist when mature, the substrate mycelium is greyish yellow and diffusible pigments are not produced. Melanin pigments are not produced on either peptone-yeast extract-iron or tyrosine agars. Growth occurs between 15 and 37 °C, but not at 4 or 45 °C, and from pH 5.0 to 10.0, but not at pH 4.0 or 11.0. Degrades arbutin, casein, starch, and Tweens 20 and 80, but not adenine, aesculin, chitin, DNA, elastin, gelatin, guanine, hypoxanthine, L-tyrosine, RNA, xanthine or xylan. Utilizes cellobiose, (−)-D-fructose, (+)-D-galactose, (+)-D-mannose, melezitose, trehalose, dextrin, maltose, D-mannitol, (+)-L-arabinose, (+)-L-rhamnose, L-glutamate and melibiose as sole carbon sources, but not adonitol, (+)-D-arabinose, (−)-D-sorbitol, inulin, (−)-L-sorbose, sucrose, *myo*-inositol, raffinose, sucrose, salicin, sodium propionate, xylitol or xylose. Utilizes glycine, L-alanine, L-arginine, L-histidine, L-hydroxyproline, L-proline, L-serine, L-threonine and L-isoleucine as sole nitrogen sources but not L-cysteine, L-methionine, L-valine, DL-phenylalanine or L-phenylalanine. Sensitive to streptomycin sulphate (32 μg ml$^{-1}$). Antimicrobial activity is shown against *Aspergillus niger* (isolated strain), *Bacillus licheniformis* NRRL B-1001, *Bacillus subtilis* NRRL B-209, *Candida albicans* ATCC 10231, *Listeria monocytogenes* (medical isolate), *Micrococcus luteus* NRRL B-287$^T$, *Pseudomonas aeruginosa* NRRL B-2679, *Staphylococcus aureus* ATCC 25923 and *Streptomyces murinus* ISP 5091, but not against *Aspergillus flavus* NRRL 1957, *Escherichia coli* ATCC 25922, *Saccharomyces cerevisiae* ATCC 9763 or *Proteus vulgaris* NRRL B-123. Additional phenotypic properties are shown in Tables 1 and 2. The predominant fatty acids in whole-organism methanolysates are iso-$C_{15:0}$, anteiso-$C_{15:0}$ and $C_{16:0}$.

The type strain is M1463$^T$ (=DSM 42010$^T$=NRRL B-24803$^T$), isolated from the rhizosphere of *Robinia pseudoacacia*. The DNA G+C content of the type strain is 71.8 mol%. The species description is based on a single strain and hence serves as the type strain description.

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