Streptomyces caeruleatus sp. nov., with dark blue diffusible pigment

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An actinomycete, designated strain GIMN4.002^T, was isolated from a tomato rhizosphere soil sample in Guangzhou, China. The strain produces white aerial mycelium and dark blue diffusible pigment on Gause's synthetic agar, and microscopic observation revealed that it produces looped chains of spiny spores. Morphological and chemotaxonomic characteristics of the strain are typical of the genus *Streptomyces*. Melanin was produced and antibacterial activity was detected against Gram-positive micro-organisms, such as *Bacillus subtilis*, *Micrococcus luteus* and *Staphylococcus aureus*. The 16S rRNA gene sequence of strain GIMN4.002^T had highest similarity (99.4 %) to *Streptomyces lincolnensis* B91; however, DNA–DNA relatedness between strain GIMN4.002^T and *S. lincolnensis* NBRC 13054^T was only 32.17 %. Further, the morphological, physiological and biochemical characteristics of strain GIMN4.002^T are distinct from *S. lincolnensis* and other species of the genus *Streptomyces* with which this strain has high 16S rRNA gene sequence similarity (98–99 %). On the basis of the physiological and molecular properties observed, it is proposed that strain GIMN4.002^T represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces caeruleatus* sp. nov. is proposed, with GIMN4.002^T (=CCTCC M 208213^T =NRRL B-24802^T) as the type strain.

Actinomycetes are widespread Gram-positive bacteria with filamentous growth and high DNA G+C contents (60–78 mol%). Among the actinobacteria, members of the genus *Streptomyces* produce useful compounds, notably antibiotics, enzymes, enzyme inhibitors and pharmacologically active agents (Bérdy, 2005). The genus *Streptomyces* was first described by Waksman & Henrici (1943) and now consists of more than 540 species with validly published names (Euzéby, 2008). The systematics of the genus *Streptomyces* at the species level, however, is still in a state of confusion and the genus is believed to be overspeciated (Groth *et al.*, 1999). Strain GIMN4.002^T was isolated from tomato rhizosphere soil at Guangzhou, China, in April 2007. The soil sample was inoculated onto Gause's synthetic agar medium (Atlas, 1993) and incubated for 3–5 days at 28 °C; the strain produced large quantities of dark blue diffusible pigment on this medium.

International *Streptomyces* Project (ISP) media were prepared according to the methods of Shirling & Gottlieb

(1966). Morphological and physiological characteristics were determined as recommended by Williams *et al.* (1989). Morphological observations of spores and mycelia were conducted by using light microscopy (Leica DM RAR) and scanning electron microscopy (Phillip FEI-XL30). Physiological tests were carried out at 28 °C unless otherwise indicated. Melibiose, D-glucose, sucrose, D-fructose, D-xylose, L-rhamnose, L-arabinose, inositol and D-mannitol were tested as sole carbon source at concentrations of 0.1 % (w/v) and were filter-sterilized. Colour determinations were referenced against the Methuen Handbook of Colour (Kornerup & Wanscher, 1978).

Analysis of the isomer of diaminopimelic acid (DAP) and whole-cell sugar composition followed the procedure described by Hasegawa *et al.* (1983) except that dried cells were used instead of colonies from agar plates. Fatty acid methyl esters were prepared by the trimethylsulphonium hydroxide method (Butte, 1983) and analysed by GC (6890; Hewlett Packard) using the Microbial Identification software package (Sasser, 1990). Menaquinones were extracted according to the method of Collins *et al.* (1977) and analysed by HPLC (Kroppenstedt, 1985). The base composition of genomic DNA of strain GIMN4.002^T was determined in 0.1 × SSC by the method of Mandel & Marmur (1968). Genomic DNA was extracted (Cui *et al.*,

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

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain GIMN4.002^T is GQ329712.

2001) and the 16S rRNA gene amplified by PCR using universal bacterial 16S rRNA gene primers. Forward primer F27 (5'-AGAGTTTGGATCCTGGCTCAG-3') and reverse primer 1522R (5'-AAGGAGGTGATCCAGCCGCA-3') were adapted from primers pA and pH of Edwards *et al.* (1989). The 16S rRNA gene was sequenced with an automated capillary DNA sequencing system (ABI 3730) and a BigDye terminator cycle sequencing kit (Applied Biosystems).

DNA-DNA relatedness was determined by the fluorometric microdilution plate method (Ezaki *et al.*, 1988; Sawabe *et al.*, 1998). Levels of relatedness were expressed as percentage values. The fluorescence intensity was measured with a MicroFluor reader (Dynatech) at wavelengths of 360 nm for excitation and 450 nm for emission. Fluorescence intensity of a well of salmon sperm DNA was calculated as 0, and the intensity of the well which emitted the strongest fluorescence was calculated as 100 %.

Chemotaxonomic analyses showed that the cell wall contained LL-DAP, typical of cell wall type I (Lechevalier & Lechevalier, 1970). Whole-cell hydrolysates contained predominantly glucose and hence no diagnostic whole-cell sugars. The predominant menaquinones were MK-9(H₈) (45.53 %), MK-9(H₂) (8.25 %), MK-9(H₄) (5.78 %) and MK-9(H₆) (4.52 %), and MK-10(H₂) (29.11 %). Strain GIMN4.002^T contained straight-chain, iso- and anteiso-branched fatty acids and a proportion of unsaturated fatty acids. The major cellular fatty acids were C_{16:0} (25.19 %),

iso-C_{16:0} (18.67 %), anteiso-C_{15:0} (10.46 %), *cis*-9-C_{16:1} (7.51 %), iso-C_{14:0} (7.31 %), cyclo-C_{17:0} (4.80 %), C_{14:0} (4.37 %), iso-C_{15:0} (3.90 %), C_{15:0} (3.67 %), anteiso-C_{17:0} (2.56 %) and iso-C_{16:1} H (1.08 %). The G + C content of the genomic DNA was 70.7 mol %.

A 1397 bp 16S rRNA gene sequence was determined for strain GIMN4.002^T. A BLAST search (Altschul *et al.*, 1997) of the GenBank database using this sequence showed its similarity to many species of the genus *Streptomyces* and, in particular, it was 99.4 % similar to *Streptomyces lincolnensis* B91 (DQ462654.1), 99.2 % similar to '*Streptomyces viridochromogenes* subsp. *komabensis*' NBRC 13859 and 98–99 % similar to the other species in the genus *Streptomyces*.

A phylogenetic tree based on 16S rRNA gene sequences representing members of the genus *Streptomyces* was reconstructed using the neighbour-joining method of Saitou & Nei (1987) with CLUSTAL W (version 1.81) (Jeanmougin *et al.*, 1998; Thompson *et al.*, 1997) and MEGA (version 3.1, Kumar *et al.*, 2001) (Fig. 1). For the neighbour-joining analysis, a distance matrix was calculated according to Kimura's two-parameter correction model (Kimura, 1980). The minimum-evolution and maximum-parsimony methods were also used for tree reconstruction (Kumar *et al.*, 2004). Branches marked with an asterisk are conserved in all methods used. This tree shows the close phylogenetic association of strain GIMN4.002^T with certain members of other species in the genus *Streptomyces*.

Fig. 1. Unrooted neighbour-joining tree reconstructed from 16S rRNA gene sequences, showing the phylogenetic relationship between strain GIMN4.002^T and species of the genus *Streptomyces* belonging to the major, minor and single-member clusters defined by Williams *et al.* (1983). *Actinomadura glomerata* IMSNU 22179^T was used as the outgroup. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are given at nodes. Minimum-evolution and maximum-parsimony methods were also used for tree reconstruction. Branches marked with an asterisk are conserved in all methods used. Bar, 0.01 substitutions per nucleotide position.

Fig. 2. Scanning electron micrographs of strain GIMN4.002^T grown on inorganic salts/starch agar (ISP 4) at 28 °C for 14 days. Note looped spore chains of spiny spores. Bars, 5 µm (a) and 2 µm (b).

The morphological and physiological characteristics of strain GIMN4.002^T, including cell-wall type, whole-cell sugar pattern and fatty acid profile, were consistent with the characteristics of members of the genus *Streptomyces*.

Morphological features were observed on yeast extract/malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salts/starch agar (ISP 4) and glycerol-asparagine agar (ISP 5). Cultures were incubated for 2 weeks at 28 °C. Strain GIMN4.002^T had characteristics typical of the genus *Streptomyces* and microscopic observations revealed a branched mycelium without verticils. The aerial mycelium produced spiral chains of cylindrical and spiny-surfaced spores (Fig. 2). Strain GIMN4.002^T developed well on ISP 2, ISP 3, ISP 5, Czapek's agar (Atlas, 1993) and Gause's synthetic agar. It exhibited moderate growth on ISP 4. Diffusible pigments of different colours were produced on the various different media (Table 1), ranging from dark blue (20-E-5) to blackish-blue (20-F-8). Melanin was produced on tyrosine agar ISP 7.

Strain GIMN4.002^T produces a greyish-white aerial mycelium and a blue substrate mycelium on ISP 2, and looped spore chains without verticils and with spiny spores. Melanin is produced. Dark blue or blue diffusible pigments are produced. Pigment production was pH-sensitive. Comparison of the cultural characteristics of strain GIMN4.002^T and its closest phylogenetic neighbours (Table 2) revealed significant differences.

Streptomyces lincolnensis differs from strain GIMN4.002^T in that it produces long and flexuous spore chains with oval-shaped and smooth spores while *Streptomyces viridochromogenes* differs from strain GIMN4.002^T in that melanin is not produced on ISP 7 media and milk coagulation was negative. Despite the high 16S rRNA gene sequence similarity between GIMN4.002^T and *Streptomyces lincolnensis*, morphological and cultural characteristics and carbon-utilization patterns are different (Table 2), indicating that GIMN4.002^T is not a strain of *Streptomyces lincolnensis*. DNA–DNA relatedness between strain GIMN4.002^T and *Streptomyces lincolnensis* NBRC 13054^T was found to be 32.17 %, far below the 70 % threshold value proposed by Wayne *et al.* (1987) for indicating species status. Thus, DNA–DNA hybridization studies confirmed that strain GIMN4.002^T is unique and support the classification of strain GIMN4.002^T as a novel species of the genus *Streptomyces*, for which the name *Streptomyces caeruleatus* sp. nov. is proposed.

Description of *Streptomyces caeruleatus* sp. nov.

Streptomyces caeruleatus (ca.e.ru.le.a'tus. L. masc. adj. *caeruleatus* dark blue coloured, referring to the dark blue pigments produced).

Aerobic, Gram-positive, catalase-positive actinomycete that forms a greyish-white aerial mycelium and a blue substrate mycelium on ISP 2. The substrate mycelium does not fragment. Looped chains of spiny-surfaced spores are

Table 1. Cultural characteristics of strain GIMN4.002^T

ISP media (Shirling & Gottlieb, 1966) are described in the text. Diffusible pigment was observed on most of the media listed. Colour comparisons were taken from Kornerup & Wanscher (1978).

Characteristic	ISP 2	ISP 3	ISP 4	ISP 5	Czapek's agar	Gause's synthetic agar
Growth	Good	Good	Moderate	Good	Luxuriant	Good
Sporulation	Good	Good	Moderate	Good	Luxuriant	Good
Colour of:						
Aerial mycelium	Greyish-white	Greyish-white	Bright yellow	Greyish-white	Bright blue	White
Substrate mycelium	Blue	Reddish-brown	Reddish-brown	Blue	Blue	Reddish-brown
Colour of diffusible pigment	Dark blue	—	—	Blue	Dark blue	Blackish-blue

Table 2. Differential cultural characteristics of strain GIMN4.002^T and its phylogenetic neighbours

Strains: 1, strain GIMN4.002^T; 2, *Streptomyces lincolnensis* NBRC 13054^T; 3, *Streptomyces viridochromogenes* ISP 5110^T. Data for reference strains were taken from Shirling & Gottlieb (1968, 1969), Gause *et al.* (1983) and Williams *et al.* (1983). +, Positive; –, negative; w, weakly positive reaction; ND, no data available.

Characteristics	1	2	3
Colony colour on ISP 2	Greyish-white	Bright-yellow	Green
Spore shape	Spiny	Oval, smooth	Spiny
Spore chain morphology	Looped	Long, flexuous	Spiral
Production of diffusible pigment	Dark blue	Yellow	Green
Milk coagulation	+	w	–
Melanin production	+	+	–
Utilization of:			
Melibiose	+	ND	+
Sucrose	–	+	–
D-Fructose	+	+	–

produced. Diffusible pigments are produced on ISP 2, ISP 5, Czapek's agar and Gause's synthetic agar, but not in ISP 3 or ISP 4. Melanin pigment is produced on ISP 7. Although growth on ISP 4 is initially slow, very good growth with profuse sporulation is observed on this medium after 14 days. Develops well on ISP 2, ISP 3, ISP 5, Czapek's agar and Gause's synthetic agar. Moderate growth on ISP 4. Substrate mycelium is reddish-brown on ISP 3, ISP 4 and Gause's synthetic agar, but blue on ISP 2, ISP 5 and Czapek's agar. The cell wall contains LL-DAP (cell wall type I). The whole cell sugar pattern contains glucose. The predominant menaquinones are MK-9(H₈), MK-9(H₂), MK-9(H₄) and MK-9(H₆), and MK-10(H₂). The major cellular fatty acids are C_{16:0}, iso-C_{16:0}, anteiso-C_{15:0}, *cis*-9-C_{16:1}, iso-C_{14:0}, cyclo-C_{17:0}, C_{14:0}, iso-C_{15:0}, C_{15:0}, anteiso-C_{17:0} and iso-C_{16:1} H. Antibacterial activity against *Bacillus subtilis*, *Micrococcus luteus* and *Staphylococcus aureus*, but not against *Escherichia coli*, *Pseudomonas aeruginosa* or *Salmonella choleraesuis*. Grows with melibiose, D-glucose, D-fructose, D-xylose, L-rhamnose, L-arabinose, inositol and D-mannitol as sole carbon source. H₂S production is positive.

The type strain is GIMN4.002^T (=CCTCC M 208213^T =NRRL B-24802^T). The DNA G+C content of the type strain is 70.7 mol%.

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