Streptomyces shaanxiensis sp. nov., a blue pigment-producing streptomycete from sewage irrigation soil

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A novel isolate belonging to the genus *Streptomyces*, strain CCNWHQ 0031^T, was isolated from soil in a sewage irrigation area in Shaanxi province, China. The isolate produced light greyish-blue aerial mycelium and dark blue diffusible pigment on Gause's synthetic agar. Strain CCNWHQ 0031^T formed *Spirales*-type chains with spiny spores. Chemotaxonomic data confirmed that strain CCNWHQ 0031^T belonged to the genus *Streptomyces*. Analysis of the almost complete 16S rRNA gene sequence placed strain CCNWHQ 0031^T in the genus *Streptomyces* where it formed a distinct phyletic line with recognized *Streptomyces* species. Strain CCNWHQ 0031^T exhibited highest sequence similarities to *Streptomyces caeruleatus* GIMN4.002^T (99.3%), *Streptomyces coeruleorubidus* NBRC 12855^T (98.9%), *Streptomyces curacoi* NBRC 12761^T (98.8%) and *Streptomyces lincolnensis* NBRC 13054^T (98.0%). DNA–DNA hybridization studies of strain CCNWHQ 0031^T with these four closest relatives showed relatedness values of 56.6 ± 0.4, 50.3 ± 0.6, 49.8 ± 0.7 and 36.9 ± 0.9%, respectively. On the basis of its molecular and physiological properties, it is evident that strain CCNWHQ 0031^T represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces shaanxiensis* sp. nov. is proposed. The type strain is CCNWHQ 0031^T (=CCNWTJ 0031^T=JCM 16925^T=ACCC 41873^T).

The genus *Streptomyces* was proposed by Waksman & Henrici (1943). *Streptomyces* species are abundant in terrestrial environments and can be easily isolated under laboratory conditions. Members of the family *Streptomycetaceae* have attracted great attention because they produce various natural products with considerable potential commercial value (Bérdy, 2005). Some *Streptomyces* species producing blue spores have been reported previously by Trejo & Bennett (1963). Actinorhodin-related blue pigments have been reportedly produced by *Streptomyces coelicolor* A3(2) (Bystrykh *et al.*, 1996); blue pigment production has been studied most in *S. coelicolor*. In addition, two *Streptomyces* species able to produce blue or violet–blue diffusible pigment have been reported by Zhu *et al.* (2007, 2011). Meyers *et al.* (2003, 2004) reported two other *Streptomyces* species with blue substrate and aerial mycelia. Microbes producing blue pigments are rare (Margalith, 1992) and these natural pigments could be used in the food industry due to their

solubility, colouring, stability, safety and commercial potential (Pszczolla, 1998).

During our research into new actinobacterial resources, morphological, physiological, chemical and phylogenetic data were determined for isolate CCNWHQ 0031^T, which was shown to represent a novel species of the genus *Streptomyces*.

Strain CCNWHQ 0031^T was isolated using a dilution plating method from a soil sample in a sewage irrigation area collected from Xi'an city (34° 33' N 109° 12' E; altitude of 425 m), Shaanxi province, north-western China, in September 2007. Gause's synthetic agar medium (Atlas, 1993) was used for strain isolation.

Morphological properties were examined by light microscopy (Olympus CX31) and scanning electron microscopy (JSM 6360LV) of cultures grown on Gause's synthetic agar medium for 14 days. Electron micrograph preparation was carried out as described by Eguchi *et al.* (1993). Cultural characteristics were determined after 2 weeks' incubation at 28 °C on various cultivation media, according to the methods of the International *Streptomyces* Project (ISP; Shirling & Gottlieb, 1966). Colours were determined as described by Kelly (1964).

The diaminopimelic acid (DAP) isomer and whole-cell sugar compositions were analysed using TLC according to

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

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CCNWHQ 0031^T is FJ465151.

Two supplementary figures are available with the online version of this paper.

Fig. 1. Scanning electron micrograph of the spiral spore chains and spiny spores of strain CCNWHQ 0031^T after 12 days at 28 °C on Gause's synthetic agar medium. Bar, 1 µm.

the procedures described by Lechevalier & Lechevalier (1980). Menaquinones were extracted and determined by HPLC using the methods of Collins (1985). Phospholipid fatty acids were extracted and the constituents were separated by two-dimensional TLC and identified using the procedure of Minnikin *et al.* (1977). Fatty acids were extracted as described by Kämpfer & Kroppenstedt (1996). Fatty acid methyl esters were analysed by GC following the MIS operation manual (MIDI), and identified and qualified by the Sherlock MIS software (version 4.5). The DNA G+C content was determined using the thermal melting protocol (Marmur & Doty, 1962), with *Escherichia coli* K-12 as the standard. DNA–DNA relatedness was determined in triplicate by the initial renaturation rate method (De Ley *et al.*, 1970).

Utilization of carbon and nitrogen sources was determined by using the methods of Gordon & Mihm (1962) and Tsukamura (1966). Other phenotypic characteristics were tested by using standard procedures (Goodfellow, 1971; Williams *et al.*, 1983). Tolerances to temperature (20, 28, 37 and 45 °C) and pH (5–12, incubated at 28 °C) were determined on Bennett's agar plates after 2 weeks of incubation.

Genomic DNA extraction and PCR amplification of the 16S rRNA gene were carried out following the procedures

of Chun & Goodfellow (1995). Universal bacterial 16S rRNA gene primers [forward primer P1 (5'-CGGG-ATCCAGAGTTTGATCCTGGCTCAGAACGAACGCT-3') and reverse primer P6 (5'-CGGGATCCTACGGCTACCTTGTACGACTTCACCCC-3')] were used for 16S rRNA gene amplification. The PCR product was purified and sequenced directly using an automated DNA sequencing system (ABI 3730XL). The 16S rRNA gene sequence of the strain was aligned using CLUSTAL_X version 1.8 (Thompson *et al.*, 1997) with the sequences of type strains of recognized species of the genus *Streptomyces* obtained from the EzTaxon server (<http://www.eztaxon.org>) (Chun *et al.*, 2007). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1993) and maximum-parsimony (Fitch, 1971) methods in the TREECON software package version 1.3b (Van de Peer & De Wachter, 1994) and the MEGA3.1 software package (Kumar *et al.*, 2004). The genetic distance matrices were estimated by the Kimura two-parameter model (Kimura, 1980). Tree topology was evaluated by bootstrap analysis based on 1000 replicates (Felsenstein, 1985).

The isolate formed a highly branched substrate mycelium and aerial hyphae which differentiated into spiral spore chains with spiny spores (Fig. 1). Strain CCNWHQ 0031^T grew well on ISP 2, ISP 3, ISP 4, Bennett's agar and Gause's synthetic agar medium. It exhibited moderate growth on nutrient agar and Czapek agar media, but poor growth on ISP 5. Different coloured diffusible pigments were produced on the various test media listed in Table 1. Noticeably, the strain produced large quantities of dark blue diffusible pigment on Gause's synthetic agar medium.

Strain CCNWHQ 0031^T mainly contained mannose, arabinose and small quantities of xylose in its whole cell hydrolysate. The whole cell hydrolysate also contained LL-DAP, traces of *meso*-DAP and glycine, indicating that the strain had cell wall type I (Lechevalier & Lechevalier, 1970). Fatty acid analysis showed that strain CCNWHQ 0031^T contained straight-chain, and iso- and anteiso-branched components and a high proportion of saturated components, with unsaturated fatty acids present in small amounts: C_{16:0} (21.34 %), iso-C_{16:0} (19.01 %), anteiso-C_{15:0} (18.14 %), iso-C_{15:0} (7.36 %), anteiso-C_{17:0} (6.97 %), iso-C_{14:0} (6.18 %), C_{15:0} (5.20 %), C_{17:0} (3.25 %), iso-C_{17:0} (2.81 %) and C_{14:0}

Table 1. Cultural characteristics of strain CCNWHQ 0031^T on various media (incubated for 7 days at 28 °C)

Characteristic	ISP 2	ISP 3	ISP 4	ISP 5	Nutrient agar	Czapek agar	Bennett's agar
Growth	Good	Good	Good	Poor	Moderate	Moderate	Good
Sporulation	None	Good	Good	Poor	Moderate	Moderate	Good
Colour of:							
Aerial mycelium	Waxy	Greyish-blue	White	White	White	Greyish-white	White
Substrate mycelium	Brown	Brown	Brown	Greyish-white	Dark brown	Light brown	Brown
Diffusible pigment	None	Light blue	Light navy blue	None	Light brown	Light navy blue	None

Table 2. Fatty acid composition (%) of strain CCNWHQ 0031^T and *S. caeruleatus* GIMN4.002^TStrains: 1, CCNWHQ 0031^T; 2, GIMN4.002^T.

Fatty acid	1	2
iso-C _{14:0}	6.18	7.31
C _{14:0}	1.10	4.37
iso-C _{15:0}	7.36	3.90
anteiso-C _{15:0}	18.14	10.46
C _{15:0}	5.20	3.67
iso-C _{16:0}	19.01	18.67
C _{16:0}	21.34	25.19
iso-C _{17:0}	2.81	0.89
anteiso-C _{17:0}	6.97	2.56
C _{17:0}	3.25	0.85

(1.10 %) (Table 2). The predominant menaquinones were MK-9(H₈) (46.17 %), MK-9(H₂) (11.92 %), MK-9(H₄) (11.34 %) and MK-9(H₆) (4.98 %), and MK-10(H₂) (16.02 %). Phospholipid profiles mainly contained phosphatidylethanolamine, which belonged to phospholipid type II (Lechevalier *et al.*, 1977) (Fig. S1). The DNA G + C content of strain CCNWHQ 0031^T was 70.7 mol%.

The morphological and physiological characteristics of strain CCNWHQ 0031^T, including its cell-wall type, whole cell sugar pattern and fatty acid profile (Table 2), were also

consistent with those of members of the genus *Streptomyces* (Williams *et al.*, 1989).

To determine its phylogenetic position, the 16S rRNA gene sequence (1419 nt) of strain CCNWHQ 0031^T was aligned with closely related *Streptomyces* species. The isolate was most closely related to *Streptomyces caeruleatus* GIMN4.002^T, *Streptomyces coeruleorubidus* NBRC 12855^T, *Streptomyces curacoi* NBRC 12761^T and *Streptomyces lincolnensis* NBRC 13054^T, with sequence similarities of 99.3, 98.9, 98.8 and 98.0 %, respectively (Fig. 2).

Furthermore, DNA–DNA relatedness studies were carried out between strain CCNWHQ 0031^T and these four most closely related type strains; DNA–DNA relatedness values were 56.6 ± 0.4, 50.3 ± 0.6, 49.8 ± 0.7 and 36.9 ± 0.9 %, respectively, which were significantly lower than the 70 % cut-off point recommended for the delineation of genomic species (Wayne *et al.*, 1987).

Phenotypic characterization of strain CCNWHQ 0031^T clearly showed that it differed from the most closely related species *S. caeruleatus* GIMN4.002^T, *S. coeruleorubidus* NBRC 12855^T, *S. curacoi* NBRC 12761^T and *S. lincolnensis* NBRC 13054^T (Table 3). Moreover, a combination of physiological and biochemical characteristics enabled strain CCNWHQ 0031^T to be distinguished from its closest phylogenetic neighbours.

Strain CCNWHQ 0031^T grew well in Gause's synthetic liquid medium supplemented with 5.5 mM Pb²⁺ [Pb(NO₃)₂] and

Fig. 2. Unrooted neighbour-joining phylogenetic tree based on almost complete 16S rRNA gene sequences, showing the relationships between strain CCNWHQ 0031^T and related species in the genus *Streptomyces*. E and P indicate the branches that were also found when the minimum-evolution and maximum-parsimony methods, respectively, were used; asterisks indicate branches that were recovered by all three methods. Accession numbers are given in parentheses. Bootstrap percentages (based on 1000 replications) are shown at the branch points; only values of 50 % or above are given. Bar, 0.05 substitutions per nucleotide position.

Table 3. Phenotypic characteristics of strain CCNWHQ 0031^T and members of the most closely related *Streptomyces* species

Strains: 1, CCNWHQ 0031^T; 2, *S. caeruleatus* GIMN4.002^T; 3, *S. coeruleorubidus* NBRC 12855^T; 4, *S. curaco* NBRC 12761^T; 5, *S. lincolnensis* NBRC 13054^T. All strains are positive for assimilation of starch, D-galactose, glycerol, glucose and mannitol as sole carbon sources and for assimilation of L-phenylalanine, asparagine, alanine, L-methionine and L-threonine as sole nitrogen sources. All strains degrade Tween 80. All strains grow with 2 % NaCl, but not with 5 % NaCl. All strains grow at 28–37 °C and pH 6–11. +, Positive; –, negative; (+), weakly positive. All data were determined in the present study except data for strain *S. caeruleatus* GIMN4.002^T, which were from Zhu *et al.* (2011).

Characteristic	1	2	3	4	5
Culture characteristics on Gause's synthetic agar:					
Aerial mycelium colour	Light greyish-blue	White	Light grey	Greyish-green	White
Substrate mycelium colour	Dark blue	Reddish-brown	Light yellow	Dark grey	Light saffron
Spore chain morphology	Spiral	Spiral	Spiral	Spiral	Straight or flexuous
Diffusible pigment production	Dark blue	Dark blue	None	None	None
Assimilation of sole carbon sources (1.0 %, w/v):					
D-Fructose	+	+	+	–	–
Lactose	+	+	(+)	–	+
Raffinose	(+)	–	(+)	–	+
Ribose	–	–	(+)	–	–
Sucrose	+	–	+	(+)	+
Assimilation of sole nitrogen sources (1.0 %, w/v):					
Arginine	(+)	–	(+)	–	(+)
Histidine	–	–	–	–	(+)
Tween 20 decomposition	+	+	+	(+)	–
Growth at/with:					
20 °C	+	(+)	+	+	+
45 °C	–	–	+	+	–
pH 5	+	–	–	+	–
pH 12	–	+	–	+	+
3 % NaCl	+	+	+	+	–
4 % NaCl	–	–	+	+	–
Gelatin liquefaction	–	–	–	+	+
Milk coagulation	–	–	–	–	+
Milk peptonization	+	+	+	+	–
H ₂ S production	(+)	+	+	–	+
Melanin production	(+)	–	–	(+)	+
Starch hydrolysis	(+)	+	+	+	+
Nitrate reduction	(+)	–	+	+	–
Heavy metal resistance:					
Zn ²⁺ (20 mM)	+	–	–	–	–
Pb ²⁺ (5.5 mM)	+	–	+	–	(+)

20 mM Zn²⁺ (ZnCl₂). In contrast, *S. lincolnensis* NBRC 13054^T grew poorly in the presence of 5.5 mM Pb²⁺, and *S. caeruleatus* GIMN4.002^T and *S. curaco* NBRC 12761^T could not grow in the presence of 5.5 mM Pb²⁺.

Taken together, all the genotypic and phenotypic data suggest that strain CCNWHQ 0031^T represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces shaanxiensis* sp. nov. is proposed.

Description of *Streptomyces shaanxiensis* sp. nov.

Streptomyces shaanxiensis (sha.an.xi.en'sis, N.L. masc. adj. *shaanxiensis* pertaining to Shaanxi, a province of north-western

China, the geographical location from where the type strain was isolated).

Aerobic, Gram-positive, forms light greyish-blue aerial mycelium, dark blue substrate mycelium and dark blue diffusible pigment on Gause's synthetic agar (Fig. S2). Produces *Spirales* spore chains with spiny, light blue spores. Growth occurs at 20–37 °C, at pH 5.0–11.0 and in the presence of 0–3 % (w/v) NaCl. Optimum growth occurs at 28 °C, at pH 7.5 and in the presence of 1 % (w/v) NaCl. Utilizes starch, mannitol, lactose, D-galactose, sucrose, glycerol and D-fructose as carbon sources. L-Phenylalanine, alanine, L-threonine and asparagine are utilized as nitrogen sources. Cell wall contains LL-DAP, traces of meso-DAP and glycine (cell wall type I). Whole-cell hydrolysates contain the

diagnostic sugars mannose and arabinose, with small quantities of xylose. Cell membranes contain phosphatidylethanolamine and the phospholipid pattern is type II. The major cellular fatty acids (>5 %) are C_{16:0}, iso-C_{16:0}, anteiso-C_{15:0}, iso-C_{15:0}, anteiso-C_{17:0}, iso-C_{14:0} and C_{15:0}. Other physiological and biochemical properties are listed in Table 3.

The type strain, CCNWHQ 0031^T (=CCNWTJ 0031^T=JCM 16925^T=ACCC 41873^T), was isolated from a sewage irrigation area from Shaanxi province, north-western China. The DNA G+C content of the type strain is 70.7 mol%.

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