Streptomyces zinciresistens sp. nov., a zinc-resistant actinomycete isolated from soil from a copper and zinc mine

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A filamentous actinomycete, designated strain CCNWNQ 0016^T, was isolated from soil from a zinc and copper mine in Shaanxi province, north-western China. A polyphasic taxonomic study was carried out to establish the status of strain CCNWNQ 0016^T. Chemical and morphological properties of the isolate were similar to those of species of the genus *Streptomyces*. Analysis of the almost complete 16S rRNA gene sequence placed strain CCNWNQ 0016^T in the genus *Streptomyces* where it formed a distinct phyletic line with recognized species of this genus. The strain exhibited highest sequence similarities to *Streptomyces niveoruber* NBRC 15428^T (98.8%), *Streptomyces griseoviridis* NBRC 12874^T (98.7%) and *Streptomyces aurantiogriseus* NBRC 12842^T (98.7%). Furthermore, DNA–DNA hybridization studies between the novel isolate and these three strains showed relatedness values of 49.6 ± 0.6, 47.2 ± 0.9 and 46.9 ± 1.1%, respectively. A number of phenotypic properties also enabled the isolate to be differentiated from related species of the genus *Streptomyces*. Therefore, it is proposed that strain CCNWNQ 0016^T (=K42^T =ACCC 41871^T =HAMBI 3107^T) should be classified as the type strain of a novel species in the genus *Streptomyces*, *Streptomyces zinciresistens* sp. nov. The maximum level of resistance to Zn²⁺ for strain CCNWNQ 0016^T is 35 mM.

Heavy metal contamination is a serious problem world-wide. Increasing numbers of environmental micro-organisms are being studied as possible remediation agents for heavy metal contamination (Thompson & Watling, 1987; Smith *et al.*, 1994; Poopal & Laxman, 2009). The effects of heavy metals on a range of species belonging to the genus *Streptomyces* have been reported by Abbas & Edwards (1989). Despite being reported to be susceptible to heavy metals (Lugauskas *et al.*, 2005), little is known about the presence of actinomycetes in soils contaminated with heavy metals, especially zinc. Zinc is an essential metal for the normal growth and development of organisms since it is a constituent of many enzymes and proteins. Although it is an essential trace element for animals, plants and micro-organisms, zinc is toxic to organisms at millimolar concentrations (Barceloux, 1999). Strain CCNWNQ 0016^T exhibited a higher degree of resistance to Zn²⁺ than previously reported strains (Wani *et al.*, 2007, 2008; Li *et al.*, 2010). The strain will be used for further investigation into the bioremediation of zinc-contaminated environments.

Strain CCNWNQ 0016^T was isolated using the dilution plating method from a sample of zinc- and copper-polluted soil collected from Ningqiang (106° 10' 37.9" E 33° 01' 13.5" N and an altitude of 669 m), Shaanxi province, north-western China, in July 2006. The zinc concentration of the soil sample was 1216.7 mg kg⁻¹. The isolation medium used was modified Gause's synthetic agar medium (16.0 g soluble starch, 4.0 g D-glucose, 1.0 g potassium nitrate, 0.5 g potassium phosphate dibasic trihydrate, 0.5 g magnesium sulfate heptahydrate, 0.001 g ferrous sulfate heptahydrate, 0.5 g sodium chloride, 20 g agar powder, 0.5 mg vitamin B compound and 1000 ml distilled water, pH 7.2). The vitamin B compound contained 0.5 mg nicotinic acid, 0.5 mg vitamin B₆, 0.5 mg lactoflavin, 0.5 mg inositol and 0.25 mg biotin (Guo *et al.*, 2009). The isolate was maintained on modified Gause's synthetic agar slopes and as glycerol suspensions (20%, v/v) at -20 °C. Biomass for most of the chemotaxonomic and molecular systematic studies was harvested after incubation in shake flasks of modified Gause's broth at 28 °C for 4–7 days.

Morphological properties were examined by light microscopy (Olympus CX31) and scanning electron microscopy (JSM 6360LV). Colours were determined by the methods described by Kelly (1964).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CCNWNQ 0016^T is GU225938.

A supplementary table is available with the online version of this paper.

The diaminopimelic acid isomer and the whole-cell sugar composition were analysed using TLC according to procedures described by Lechevalier & Lechevalier (1980). Menaquinones were extracted and determined by HPLC using the methods of Collins (1985). Phospholipid analysis was carried out according to the method of Lechevalier *et al.* (1981). Fatty acids were extracted as described by Kämpfer & Kroppenstedt (1996). 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).

Strain CCNWNQ 0016^T was examined for a range of chemotaxonomic and physiological properties following the methods of Williams *et al.* (1983). International *Streptomyces* Project (ISP) media were used to determine the colour of diffusible pigments and the utilization of sole carbon and nitrogen sources (Shirling & Gottlieb, 1966). Temperature (20, 28, 37 and 45 °C) and pH (5–12, incubated at 28 °C) tolerances were determined on Bennett's agar plates after incubation for 2 weeks.

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'-CGGGATC-CAGAGTTTGATCCTGGCTCAGAACGAACGCT-3') and reverse primer P6 (5'-CGGGATCCTACGGCTACCTTGT-TACGACTTCACCCC-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 almost complete 16S rRNA gene sequences of type strains of recognized species of the genus *Streptomyces* obtained from GenBank/EMBL/DBJ. A phylogenetic tree was 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). 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 chemotaxonomic and morphological properties of strain CCNWNQ 0016^T were consistent with those of members of the genus *Streptomyces* (Williams *et al.*, 1989). The isolate formed a highly branched substrate mycelium and aerial hyphae which differentiated into spiral spore chains with spiny spores (Fig. 1). Growth of the organism was moderate on ISP media 2, 4 and 5, and nutrient agar, but poor on ISP medium 3. The colour of the aerial mycelium was light grey–white to greenish-grey and the substrate mycelium was light grey–white to light yellow. Diffusible pigments were not produced on any of the

Fig. 1. Scanning electron micrograph of the spiral spore chains and spiny spores of strain CCNWNQ 0016^T after 15 days at 28 °C on modified Gause's synthetic agar medium. Bar, 2 µm.

media tested (Supplementary Table S1, available in IJSEM Online).

The whole cell hydrolysate contained LL-diaminopimelic acid. Hexa- and octa-hydrogenated menaquinones with nine isoprene units [MK-9(H₆,H₈)] were the predominant menaquinones and diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides were the major polar lipids (phospholipid

Fig. 2. Unrooted neighbour-joining phylogenetic tree based on almost complete 16S rRNA gene sequences showing the relationship between strain CCNWNQ 0016^T and related species in the genus *Streptomyces*. Accession numbers are given in parentheses. Bootstrap percentages (based on 1000 replications) are shown at the branch points; only values above 50 % are given. Bar, 0.05 substitutions per nucleotide position.

Table 1. Phenotypic characteristics of strain CCNWNQ 0016^T and the most closely related species of the genus *Streptomyces*

Strains: 1, *Streptomyces zinciresistens* sp. nov. CCNWNQ 0016^T; 2, *S. niveoruber* NBRC 15428^T; 3, *S. griseoviridis* NBRC 12874^T; 4, *S. aurantiogriseus* NBRC 12842^T. All strains are positive for assimilation of D-galactose and D-glucose as sole carbon sources and for assimilation of L-phenylalanine, L-valine and L-tyrosine as sole nitrogen sources. All strains degrade Tweens 40, 60 and 80 and grow with 4 % NaCl, but not with 8 % NaCl. All strains grow at 28–37 °C and pH 7–9. All strains are positive for nitrate reduction and starch hydrolysis. +, Positive; –, negative; (+), weakly positive. All data were determined in the present study.

Characteristics	1	2	3	4
Culture characteristics:				
Aerial mycelium colour on Gause's synthetic agar	Grey to greenish-grey	Ivory	Pale greyish-purple	Flaxen
Substrate mycelium colour on Gause's synthetic agar	Greyish-yellow	Pale yellow	Purplish-red	Apricot yellow
Spore chain morphology	Spiral	Straight	Rectiflexibles	Rectiflexibles/spiral
Production of diffusible pigments	None	Brown	None	None
Assimilation of sole carbon sources (1.0 %, w/v, except where marked):				
D-Sorbitol	+	(+)	+	–
Lactose	+	(+)	+	+
myo-Inositol	+	(+)	+	+
Sucrose	+	+	+	(+)
Maltose	+	+	+	(+)
L-Rhamnose	+	(+)	+	+
D-Fructose	–	+	+	+
L-Arabinose	(+)	+	(+)	+
D-Xylose	–	+	+	+
Trehalose	+	(+)	+	+
Dextrin	–	+	+	+
Sodium acetate (0.1 %)	–	+	+	+
Assimilation of sole nitrogen sources (1.0 %, w/v):				
L-Isoleucine	–	(+)	–	(+)
L-Cysteine	–	–	+	(+)
L-Aspartic acid	+	–	–	–
Decomposition of:				
Adenine	–	–	+	+
Xanthine	–	–	+	+
Tween 20	–	+	+	+
Growth at:				
45 °C	–	–	+	+
pH 5	+	–	+	–
pH 12	–	–	+	+
Growth with:				
7 % NaCl	+	–	+	+
0.1 % Phenol	–	+	+	+
0.01 % NaNO ₃	(+)	–	(+)	–
Gelatin liquefaction	+	(+)	+	+
Milk coagulation	+	+	–	–
Milk peptonization	–	–	+	+
H ₂ S production	+	–	–	–
Melanin production	(+)	+	–	–
Heavy metal resistance				
Zn ²⁺ (20 mM)	+	–	–	–
Zn ²⁺ (35 mM)	+	–	–	–
Cu ²⁺ (0.2 mM)	+	–	–	–
Cd ²⁺ (0.5 mM)	+	–	–	–

type II *sensu* Lechevalier *et al.*, 1977). There were no characteristic whole-cell sugars or mycolic acids. Analysis of the major fatty acids showed that the organism mainly contained saturated fatty acids such as anteiso-C_{15:0} (20.76 %), C_{16:0} (19.25 %) and iso-C_{16:0} (10.92 %), with a small proportion of unsaturated fatty acids.

The 16S rRNA gene sequence (1460 nt) of strain CCNWNQ 0016^T was aligned with 16S rRNA gene sequences of other species of the genus *Streptomyces*. The isolate was found to be most closely related to *Streptomyces niveoruber* NBRC 15428^T, *Streptomyces griseoviridis* NBRC 12874^T and *Streptomyces aurantiogriseus* NBRC 12842^T with sequence similarities of 98.8, 98.7 and 98.7 %, respectively (Fig. 2).

DNA–DNA relatedness studies were carried out between strain CCNWNQ 0016^T and the most closely related type strains, i.e. *S. niveoruber* NBRC 15428^T, *S. griseoviridis* NBRC 12874^T and *S. aurantiogriseus* NBRC 12842^T. DNA–DNA relatedness values were 49.6 ± 0.6, 47.2 ± 0.9 and 46.9 ± 1.1 %, 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 CCNWNQ 0016^T showed clearly that it differed from the three most closely related species (Table 1). Moreover, a combination of physiological and biochemical characteristics enabled strain CCNWNQ 0016^T to be distinguished from its closest phylogenetic neighbours.

Strain CCNWNQ 0016^T grew well in SC liquid medium (10 g soluble starch, 1.0 g casein, 0.5 g K₂HPO₄, 15.0 g agar and 1000 ml distilled water, pH 7.2) supplemented with 20 mM Zn²⁺ (ZnCl₂) and visible growth was observed in the presence of 35 mM Zn²⁺. In contrast, *S. niveoruber* NBRC 15428^T, *S. griseoviridis* NBRC 12874^T and *S. aurantiogriseus* NBRC 12842^T could not grow in the presence of 20 mM Zn²⁺. The maximum level of resistance to Zn²⁺ for strain CCNWNQ 0016^T was 35 mM.

Taken together, all the genotypic and phenotypic data suggest that strain CCNWNQ 0016^T represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces zinciresistens* sp. nov. is proposed.

Description of *Streptomyces zinciresistens* sp. nov.

Streptomyces zinciresistens (zin.ci.re.sis'tens. L. n. *zincum* zinc; L. part. adj. *resistens* resisting; N.L. part. adj. *zinciresistens* zinc-resisting, referring to the organism's ability to resist zinc).

Aerobic, Gram-positive, zinc-resistant actinomycete that develops a well-branched grey–white to greenish-grey aerial mycelium and a greyish-yellow to light brown substrate mycelium on Gause's synthetic agar and ISP medium 2. Produces spiral spore chains with spiny, greyish-green spores. No diffusible pigment is formed on any of the tested media. Growth occurs at 20–37 °C, at pH 5.0–11.0 and in

the presence of 4–7 % (w/v) NaCl. Optimum growth occurs at 28 °C, at pH 7.5 and in the presence of 4 % (w/v) NaCl. Physiological and biochemical profiles, including those relating to carbon source utilization, biodegradation and growth, are listed in Table 1. Chemotaxonomic properties are typical of the genus *Streptomyces*.

The type strain is CCNWNQ 0016^T (=K42^T =ACCC 41871^T =HAMBI 3107^T), isolated from a zinc-contaminated soil sample collected from Shaanxi province, north-western China. The DNA G + C content of the type strain is 70.3 mol%.

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