Streptomyces plumbiresistens sp. nov., a lead-resistant actinomycete isolated from lead-polluted soil in north-west China

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The taxonomic position of an actinomycete isolated from a lead-polluted soil in Gansu province, north-west China, was determined by using a polyphasic approach. Chemical and morphological properties of the isolate, designated strain CCNWHX 13-160^T, were similar to those of streptomycetes. Analysis of the almost complete 16S rRNA gene sequence placed strain CCNWHX 13-160^T in the genus *Streptomyces* where it formed a distinct phyletic line with recognized *Streptomyces* species. The strain was most similar to *Streptomyces pseudovenezuelae* NBRC 12904^T (98.9%) and *Streptomyces resistomycificus* NBRC 12814^T (98.8%). Furthermore, DNA–DNA hybridization studies between the novel isolate and these two strains showed relatedness values of 49.7 ± 0.8 and 43.2 ± 1.1 %, respectively. It is proposed that strain CCNWHX 13-160^T (=ACCC 41207^T=HAMBI 2991^T) be classified as the type strain of a novel species in the genus *Streptomyces*, *Streptomyces plumbiresistens* sp. nov. The MIC of Pb²⁺ for growth of strain CCNWHX 13-160^T was 4.0 mM.

Pollution of soils with heavy metals is becoming one of the most severe environmental hazards. Elevated levels of heavy metals not only decrease soil microbial activity and crop production, but also threaten human health through the food chain (McLaughlin *et al.*, 1999). Selection pressure in polluted habitats has led to adaptation, resulting in micro-organisms possessing special resistance mechanisms as a consequence of their permanent exposure to heavy metals. Increasing numbers of environmental micro-organisms have been studied as part of investigations into methods to remediate heavy metal contamination (Thompson & Watling, 1987; Smith *et al.*, 1994). Some micro-organisms, e.g. members of the genera *Citrobacter* and *Pseudomonas*, both Gram-negative, rod-shaped bacteria growing in heavy metal-contaminated sites, have been found with the ability to resist and accumulate the heavy metal lead (Aickin & Dean, 1979; Macaskie & Dean, 1987). There have been previous reports on the effects of heavy metals (except for

lead) on a range of *Streptomyces* species (Abbas & Edwards, 1989). Since actinomycetes are susceptible to heavy metals (Lugauskas *et al.*, 2005), little is known about their presence in soils contaminated with heavy metals, especially lead.

The novel strain, CCNWHX 13-160^T, was isolated by the dilution plating method from a sample of lead-polluted soil collected from Huixian (33° 54' 10.3" N 106° 07' 44.3" E; altitude of 1049 m), Gansu province, north-west China, in July 2006. The medium used was modified Gause's synthetic agar (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 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 (CX31; Olympus) and scanning electron microscopy (6360LV; JSM) after cultivation for 2 weeks on modified Gause's synthetic agar medium at 28 °C. Colours were determined using the methods described by Kelly (1964).

Analysis of the diaminopimelic acid isomers and whole-cell sugars in whole-cell hydrolysates was performed according

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CCNWHX 13-160^T is EU526954.

The growth and culture characteristics of strain CCNWHX 13-160^T on different media (Table S1), the fatty acid composition of strain CCNWHX 13-160^T (Table S2), growth characteristics of strain CCNWHX 13-160^T and related strains in the presence of various lead concentrations (Table S3) and a graph showing the effects of lead concentration on survival of strain CCNWHX 13-160^T (Fig. S1) are available with the online version of this paper.

to Lechevalier & Lechevalier (1980). Fatty acids were extracted as described by Kämpfer & Kroppenstedt (1996) and estimated by GC using the standard Sherlock MIDI (Microbial Identification) system. Menaquinones were examined by the method of Collins (1985) and phospholipids were determined following the procedure of Lechevalier *et al.* (1981).

Strain CCNWHX 13-160^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 colours of diffusible pigments and the utilization of sole carbon and nitrogen sources (Shirling & Gottlieb, 1966). Temperature and pH tolerances were determined on Bennett's agar plates after incubation for 2 weeks. Resistance to antibiotics was carried out according to Al-Tai *et al.* (1999).

Genomic DNA extraction and 16S rRNA gene PCR amplification were carried out according to Chun & Goodfellow (1995). The universal bacterial 16S rRNA gene primers [forward primer P1 (5'-CGGGATCCAGAGTTTG-ATCCTGGCTCAGAACGAACGCT-3') and reverse primer P6 (5'-CGGGATCCTACGGCTACCTTGTACGACTT-CACCCC-3')] were used. The PCR product was purified and sequenced directly by an automated DNA sequencing system (ABI 3730XL). The 16S rRNA gene sequence of the strain was aligned manually by CLUSTAL_X version 1.8 (Thompson *et al.*, 1997) with the 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 constructed 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). The topology of the tree was evaluated in a bootstrap analysis based on 1000 replicates (Felsenstein, 1985). 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 by the initial renaturation rate method in triplicate (De Ley *et al.*, 1970).

The methods of Amoroso *et al.* (1998) were used to determine the lead resistance of strain CCNWHX 13-160^T. Spore suspensions (1×10^9 c.f.u. ml⁻¹) of the strain were prepared as described by Kieser *et al.* (2000). Samples of liquid 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 and 1000 ml distilled water; pH 6.0) containing different concentrations of Pb(NO₃)₂ (0.5–5.0 mM) and each inoculated with 100 µl spore suspension were incubated with shaking (150 r.p.m.) at 28 °C for 4 days. After being centrifuged (5000 g, 10 min), the cell pellets were washed

three times with 25 mM Tris-EDTA buffer (pH 8.0). To analyse the weight changes of the culture, the pellets were dried to constant weight at 105 °C. Residual lead in the supernatants was determined by atomic absorption spectrophotometry (180-80; Hitachi). All samples were analysed in triplicate and mean values were determined.

The chemotaxonomic and morphological properties of strain CCNWHX 13-160^T were consistent with those of members of the genus *Streptomyces* (Williams *et al.*, 1989). The strain formed a highly branched substrate mycelium and aerial hyphae which differentiated into *Rectiflexibiles* spore chains with smooth, greenish white spores (Fig. 1). The growth of the organism was moderate on ISP3, ISP4, ISP5 and ISP6 agars, but poor on nutrient agar. The colour of the aerial hyphal mass was white to greyish white and the substrate mycelium was light yellow to brownish red on ISP6, Bennett's agar and ISP3. A brown diffusible pigment was produced on ISP4, ISP5, Bennett's agar and Gause's synthetic agar (Table 1 and Supplementary Table S1, available in IJSEM Online).

The whole cell hydrolysates contained LL-diaminopimelic acid, but no diagnostic sugars (cell wall type I) (Lechevalier & Lechevalier, 1980). The major menaquinones of the novel strain were hexahydrogenated, octahydrogenated and tetrahydrogenated with nine isoprene units [MK-9(H6, H8 and H4)]. The major polar lipids were phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides (phospholipid type II) (Lechevalier *et al.*, 1981). Analysis of the major fatty acid components showed that the organism contained mainly iso- and anteiso-branched-chain fatty acids and a small proportion of unsaturated fatty acids (Supplementary Table S2, available in IJSEM Online).

The almost complete 16S rRNA gene sequence (1429 nt) of strain CCNWHX 13-160^T was aligned with 16S rRNA gene sequences of other *Streptomyces* species and a distinct phyletic line was formed with *Streptomyces pseudovenezuelae* NBRC 12904^T. The strain was closely related to *S.*

Fig. 1. Scanning electron micrograph of the *Rectiflexibiles* spore chains and smooth-surfaced spores of strain CCNWHX 13-160^T after cultivation for 2 weeks at 28 °C on modified Gause's synthetic agar medium. Bar, 2 µm.

Table 1. Phenotypic characteristics that differentiate strain CCNWHX 13-160^T from its closest phylogenetic relatives, *S. pseudovenezuelae* NBRC 12904^T and *S. resistomycificus* NBRC 12814^T

Strains: 1, CCNWHX 13-160^T; 2, *S. pseudovenezuelae* NBRC 12904^T; 3, *S. resistomycificus* NBRC 12814^T. Data for reference type strains are taken from Fujie *et al.* (1993) and Omura *et al.* (1978). All strains are positive for utilization of L-arabinose, D-fructose, glucose, *myo*-inositol, D-mannitol, D-mannose, raffinose, L-rhamnose and sucrose. +, Positive; –, negative; (+), weakly positive.

Characteristic	1	2	3
Aerial spore mass colour	Greenish white	Pale, yellowish	Grey
Spore chain morphology	<i>Rectiflexibiles</i>	<i>Rectiflexibiles</i>	Spiral, <i>Rectiflexibiles</i>
Production of diffusible pigments	Brown	None	Brown, reddish brown
Melanin production	–	–	+
Utilization as sole carbon source (1.0 %, w/v):			
Glycerin	+	–	–
D-Ribose	–	–	+
Trehalose	–	–	+
D-Xylose	+	(+)	–
Utilization as sole nitrogen source (1.0 %, w/v):			
L-Phenylalanine	–	+	+
L-Tyrosine	–	+	–
Degradation of:			
Cellulose	–	+	–
Xanthine	+	+	–
Growth at/in:			
45 °C	+	+	–
NaCl (8 %)	–	+	–
pH 12	+	+	(+)
H ₂ S production	+	+	–
Gelatin liquefaction	–	–	+
Milk coagulation	+	–	+
Milk peptonization	+	–	+
Nitrate reduction	+	–	+

pseudovenezuelae NBRC 12904^T and *Streptomyces resistomycificus* NBRC 12814^T with sequence similarities of 98.9 and 98.8 %, respectively (Fig. 2).

DNA–DNA relatedness studies were carried out between strain CCNWHX 13-160^T and the most closely related type strains (*S. pseudovenezuelae* NBRC 12904^T and *S. resistomycificus* NBRC 12814^T); DNA–DNA relatedness values were 49.7 ± 0.8 and 43.2 ± 1.1 %, respectively, and both values were significantly lower than the 70 % cut-off point recommended for the delineation of genomic species (Wayne *et al.*, 1987).

It is clear that strain CCNWHX 13-160^T differs from its most closely related phylogenetic neighbours *S. pseudovenezuelae* NBRC 12904^T and *S. resistomycificus* NBRC 12814^T (Table 1). Physiological characteristics, degradation of organic compounds and production of diffusible pigments enabled strain CCNWHX 13-160^T to be distinguished from closely related type strains.

Strain CCNWHX13-160^T grew well in liquid medium supplemented with 0.5 mM Pb²⁺ and visible growth was observed in the presence of 2.0 mM Pb²⁺. *S. pseudovenezuelae* NBRC 12904^T and *S. resistomycificus* NBRC 12814^T could not grow in the presence of 2.0 mM Pb²⁺ (see

Supplementary Table S3, available in IJSEM Online). The MIC of Pb²⁺ for strain CCNWHX 13-160^T was 4.0 mM. The MIC was determined by the intersection of the relative survival curve with the horizontal axis. The relative survival curve was produced using weight changes of cultures that were supplemented with different concentrations of lead compared with non-lead-supplemented controls under the same conditions (see Supplementary Fig. S1, available in IJSEM Online). Several strains of the genus *Frankia* are known to be resistant to Pb²⁺ (MIC 5–8 mM) (Richards *et al.*, 2002). However, *Escherichia coli* W3110 (MIC 2.0 mM), *Bacillus subtilis* 168 (MIC 3.0 mM) and *Micromonospora echinospora* ATCC 15836^T (MIC 3.0 mM) are less resistant to lead (Richards *et al.*, 2002).

Based on a combination of phenotypic and genotypic studies, strain CCNWHX 13-160^T represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces plumbiresistens* sp. nov. is proposed.

Description of *Streptomyces plumbiresistens* sp. nov.

Streptomyces plumbiresistens (plum.bi.re.sis'tens. L. n. *plumbum* lead; L. part adj. *resistens* resisting; N.L. part

Fig. 2. Neighbour-joining tree based on almost complete 16S rRNA gene sequences (1429 nt) showing relationships between strain CCNWHX 13-160^T and related type strains of the genus *Streptomyces*. Asterisks indicate branches that were also found using the minimum-evolution and maximum-parsimony tree-making algorithms. Numbers at branching nodes indicate bootstrap values (based on 1000 replications). Bar, 0.02 substitutions per nucleotide position.

adj. *plumbiresistens* lead-resisting, referring to the organism's ability to resist lead).

Aerobic, Gram-positive, lead-resistant actinomycete that forms a well-branched white to greyish white aerial mycelium and light yellow to brownish red substrate mycelium on ISP6, Bennett's agar and ISP3. Produces *Rectiflexibiles* spore chains with smooth, greenish white spores. A brown diffusible pigment is produced on ISP4, ISP5, Bennett's agar and Gause's synthetic agar. Melanin production is negative on ISP6 and ISP7. Growth occurs at 10–45 °C, pH 5.0–12.0 and in the presence of 5–7% (w/v) NaCl, but not at 0 or 50 °C, pH 4.0 or 13.0, or in 8% (w/v) NaCl. Milk coagulation and peptonization, nitrate reduction and H₂S production are positive. Gelatin liquefaction and degradation of cellulose are negative. L-Arabinose, D-fructose, galactose, glucose, glycerin, *myo*-inositol, lactose, maltose, mannose, raffinose, rhamnose, starch, sucrose and D-xylose are utilized as sole carbon sources, but not ribose or trehalose. The peptidoglycan contains LL-diaminopimelic acid (cell wall type I). The predominant menaquinones are MK-9(H₆, H₈ and H₄). The major fatty acids are iso-C_{14:0}, C_{14:0}, iso-C_{15:0}, anteiso-C_{15:0}, C_{15:0}, iso-C_{16:0}, C_{16:0} and anteiso-C_{17:0}.

The type strain, CCNWHX 13-160^T (=ACCC 41207^T=HAMBI 2991^T), was isolated from a lead-contaminated soil sample collected from Gansu Province, north-west China. The genomic DNA G+C content of the type strain is 70.9 mol%.

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