Actinoplanes sichuanensis sp. nov. and *Actinoplanes xinjiangensis* sp. nov.

Wei Sun,^{1†} Guo-Xia Dong,^{1†} Yu-Qin Zhang,¹ Yu-Zhen Wei,¹ Qiu-Ping Li,¹ Li-Yan Yu,¹ Hans-Peter Klenk² and Yue-Qin Zhang¹

Correspondence

Yu-Qin Zhang
zhyuqin@126.com
Yue-Qin Zhang
zyq_0525@yahoo.com.cn

¹Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, PR China

²DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstraße 7b, 38124 Braunschweig, Germany

Two motile actinomycetes, designated strains 03-723^T and 03-8772^T, which had potent inhibitory activity against *Enterococcus faecium* peptide deformylase and several clinical Gram-positive, antibiotic-resistant strains, were isolated from two soil samples collected from Sichuan Province and Xinjiang Uyghur Autonomous Region in China, respectively. The taxonomic status of these two organisms was established by using a polyphasic approach. The taxonomic data were consistent with the assignment of the strains to the genus *Actinoplanes*. The neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showed that the two isolates formed a branch with the type strains of *Actinoplanes lobatus*, *Actinoplanes auranticolor*, *Actinoplanes capillaceus*, *Actinoplanes campanulatus* and *Actinoplanes philippinensis* in the clade of *Actinoplanes* species. This branching pattern was also supported by the tree constructed with the maximum-parsimony method. Levels of 16S rRNA gene sequence similarity between strains 03-723^T and 03-8772^T and their phylogenetic neighbours ranged from 98.0 to 98.8% and 97.4 to 98.1%, respectively. However, the two strains shared low levels of DNA–DNA relatedness with the type strains of closely related *Actinoplanes* species and were readily distinguished by using a combination of phenotypic properties. Therefore, it is proposed that strains 03-723^T and 03-8772^T represent two novel species of the genus *Actinoplanes*, for which the names *Actinoplanes sichuanensis* sp. nov. (type strain 03-723^T=KCTC 19460^T=CCM 7526^T) and *Actinoplanes xinjiangensis* sp. nov. (type strain 03-8772^T=KCTC 19461^T=CCM 7527^T) are proposed.

The genus *Actinoplanes* was proposed by Couch (1950). Members of the genus are characterized by the presence of spherical, subspherical, cylindrical or very irregular sporangia. The sporangiospores are motile by means of tufts of polar or peritrichous flagella. Aerial hyphae are scant (Goodfellow *et al.*, 1990). Many species of the genus produce bioactive compounds, some of which have been extensively utilized clinically, such as those from *Actinoplanes teichomyceticus*, *Actinoplanes liguriensis*, *Actinoplanes capillaceus* and *Actinoplanes ferrugineus* (Wink *et al.*, 2006; Matsumoto *et al.*, 2000; Fukami *et al.*, 2000; Palleroni, 1979). The type species of the genus is

Actinoplanes philippinensis (Couch, 1950) and, at the time of writing, the genus comprised 25 recognized species.

During the course of screening for new inhibitors of peptide deformylase, two strains, designated 03-723^T and 03-8772^T, were found to exhibit potent inhibitory activity against *Enterococcus faecium* peptide deformylase and good activity against several clinical, Gram-positive, antibiotic-resistant strains, including vancomycin-resistant *Enterococcus* and meticillin-resistant *Staphylococcus aureus*. Based on data from the present polyphasic study, these two bioactive strains are shown to represent two novel *Actinoplanes* species.

Strains 03-723^T and 03-8772^T were isolated on humic acid-vitamin agar (Hayakawa & Nonomura, 1987) and oatmeal agar (ISP 3; Shirling & Gottlieb, 1966), respectively, after being seeded with soil suspension and incubated at 28 °C for about 3 weeks. The soil samples were collected from Sichuan Province and Xinjiang Uyghur Autonomous Region in China, respectively. Pure cultures were maintained on yeast extract-malt extract agar (ISP 2; Shirling & Gottlieb, 1966) slants at 4 °C and as 20% (v/v) glycerol

†These authors contributed equally to this work.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 03-723^T and 03-8772^T are EU531458 and EU531457, respectively.

Tables giving the cellular fatty acid contents of strains 03-723^T and 03-8772^T and the type strains of related *Actinoplanes* species, and levels of DNA–DNA hybridization between strains 03-723^T and 03-8772^T and related *Actinoplanes* species are available as supplementary material with the online version of this paper.

suspensions at -80°C . Biomass for chemotaxonomic and molecular genetic studies was obtained after incubation in shake flasks of trypticase soy broth medium (Difco) at 28°C for 10–14 days (shaking at about 160 r.p.m.).

The arrangement of hyphae and sporangia of strains 03-723^T and 03-8772^T was observed on inorganic salts-starch medium (ISP 4; Shirling & Gottlieb, 1966) by using light microscopy (model BH 2; Olympus) after 21 and 28 days, respectively, at 28°C . Morphology of the sporangia and surface ornamentation of the sporangiospores were observed by examining gold-coated dehydrated specimens with a model FEI QUANTA scanning electron microscope. Cultural characteristics were studied on standard media ISP 2, ISP 3, ISP 4, ISP 5, ISP 6 and ISP 7 (Shirling & Gottlieb, 1966), yeast extract-starch agar, nutrient agar (Difco), Czapek solution agar (Waksman, 1961) and modified Bennett's agar (Jones, 1949) following incubation at 28°C for 7, 14, 21 and 28 days. The two novel strains were examined for a range of phenotypic properties by using established procedures as described by Goodfellow *et al.* (1990) and Williams *et al.* (1983). Tolerance of pH, temperature and NaCl were determined on both ISP 2 agar and yeast extract-starch agar plates incubated for up to 28 days. Growth was tested at 4, 10, 15, 20, 28–37 (at intervals of 1.0°C), 40 and 45°C . The two media were adjusted to initial pH (4.5–11.0) (at intervals of 0.5 pH units) by using citric acid (0.1 M) – Na_2HPO_4 (0.2 M) and glycine (0.05 M) – NaOH (0.05 M) buffer systems to test the ability of the strains to grow at different pH. Growth at different NaCl concentrations was determined at 1 and 3–10% (at intervals of 1.0%) NaCl (w/v). Carbon source utilization tests and qualitative enzyme tests were determined by using API 50 CH and API ZYM test kits, according to the manufacturer's instructions (bioMérieux). Hydrolysis of urea was determined on peptone-glucose agar, comprising (per litre distilled water): 1 g peptone, 1 g glucose, 5 g NaCl and 2 g KH_2PO_4 , with 2% (w/v) urea and 0.001% (w/v) phenol red. Hydrolysis of starch, casein, gelatin and urea were determined following the methods described by Yuan *et al.* (2008). The incubation period for hydrolysis of urea, starch and casein was 4–10 days at 28°C . Gelatin hydrolysis was determined by incubation for 3 weeks at 28°C on peptone-gelatin medium, comprising (per litre distilled water) 5 g peptone and 120 g gelatin. Milk coagulation and peptonization were determined by using 20% (w/v) skimmed milk as the medium following incubation for 3 weeks at 28°C . Resistance to antibiotics was examined as described by Sun *et al.* (2007).

The isomers of diaminopimelic acid and whole-cell sugars were identified according to the procedures developed by Hasegawa *et al.* (1983) and Lechevalier & Lechevalier (1980). Polar lipids were extracted and identified according to the method of Minnikin *et al.* (1984). Menaquinones were estimated by using the methods of Collins (1985).

Acid methanolysis was used for extraction and analysis of mycolic acids (Minnikin *et al.*, 1975). Fatty acids were

extracted, methylated and estimated by GC by using the standard Sherlock MIDI (Microbial Identification) system (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). The G + C content of the genomic DNA was determined via the thermal denaturation method (Marmur & Doty, 1962) with *Escherichia coli* K-12 as a control.

Preparation of genomic DNA and PCR amplification of the 16S rRNA gene were carried out following Chun & Goodfellow (1995) by using an ABI PRISM 3730XL automatic sequencer. The resultant sequences were first aligned via the BLAST search program in NCBI. Corresponding sequences of representative species were then subjected to phylogenetic analysis by using the software package MEGA (molecular evolutionary genetics analysis) version 3.1 (Kumar *et al.*, 2004) and PHYLIP version 3.5c (Felsenstein, 1993). Phylogenetic trees were constructed according to the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) algorithms. Evolutionary distance matrices were generated following Kimura (1980). Bootstrap values were determined based on 1000 replications. Levels of DNA–DNA relatedness between strains 03-723^T and 03-8772^T and the type strains of related species were determined by using the fluorometric microplate method of Ezaki *et al.* (1989). Hybridization experiments were carried out under stringent conditions in 50% formamide at 51°C . Fluorescence intensities were measured by using a Fluostar Optima microplate reader (BMG LABTECH) at a wavelength of 360 nm for excitation and 460 nm for emission.

Strains 03-723^T and 03-8772^T grew well on ISP 2, ISP 3, ISP 4, ISP 5, ISP 7, yeast extract-starch agar, nutrient agar, Czapek solution agar and modified Bennett's agar, but with absence of aerial mycelium on these media. On ISP 6 agar good growth was observed for strain 03-723^T but only moderate growth for strain 03-8772^T (strain 03-8772^T produced melanin on this medium). Substrate mycelium of strain 03-723^T was cream to reddish orange on all media tested. The surface of the spores was smooth. Substrate mycelium of strain 03-8772^T was white to orange on all media tested. No soluble pigments were produced from either of the novel strains. The morphological characteristics of the two strains are shown in Fig. 1. Fragmentation of substrate mycelium was not observed.

Whole-cell hydrolysates of the two isolates contained meso-diaminopimelic acid and xylose and arabinose (wall chemotype II; Lechevalier & Lechevalier, 1970). The two strains contained tetrahydrogenated menaquinones with nine isoprene units [MK-9(H_4)] as the predominant isoprenologue and a polar lipid profile including phosphatidylethanolamine (phospholipid pattern 2; Lechevalier *et al.*, 1977). Mycolic acids were absent. The major cellular fatty acids (>10% of the total) of strains 03-723^T and 03-8772^T were iso- $\text{C}_{15:0}$ (22.2 and 10.8% of the total, respectively), iso- $\text{C}_{16:0}$ (15.3 and 16.0%) and $\text{C}_{17:1\omega 9c}$ (11.1 and 21.5%). These data supported the classification of the two strains as members of the genus *Actinoplanes*.

Fig. 1. Scanning electron micrographs of cells of strains 03-723^T and 03-8772^T grown on inorganic salts-starch (ISP 4) medium for 19 days at 28 °C. Strain 03-723^T: (a) sporophore and sporangia; (b) sporangium releasing motile spores. Strain 03-8772^T: (c) sporangium before rupture of the sporangial wall – internal spores are visible; (d) motile spores. Bars, (a) 5 μm, (b) 10 μm, (c) 10 μm, (d) 20 μm.

(Tamura & Hatano, 2001). However, strains 03-723^T and 03-8772^T could be differentiated from related *Actinoplanes* species based on cellular fatty acid profiles (see Supplementary Table S1, available in IJSEM Online).

Strains 03-723^T and 03-8772^T shared 98.5 % 16S rRNA gene sequence similarity. Levels of 16S rRNA gene sequence similarity between strain 03-723^T and *Actinoplanes lobatus* DSM 43150^T, *Actinoplanes auranticolor* DSM 43031^T, *A. capillaceus* DSM 44859^T, *Actinoplanes campanulatus* DSM 43148^T and *A. philippinensis* DSM 43019^T were 98.8, 98.7, 98.3, 98.0 and 98.1 %, respectively; levels of similarity between strain 03-8772^T and the above taxa were 98.1, 98.0, 97.5, 97.4 and 97.7 %, respectively. A distance matrix dendrogram based on 16S rRNA gene sequences is shown in Fig. 2. It was evident from the dendrogram that the two new isolates formed a distinct branch with *A. lobatus*, *A.*

auranticolor, *A. capillaceus*, *A. campanulatus* and *A. philippinensis*. This was also supported at a high bootstrap value in the maximum-parsimony tree. Levels of DNA–DNA relatedness between strains 03-723^T, 03-8772^T and the type strains of the above *Actinoplanes* species were all below 70 % (Supplementary Table S2, in IJSEM Online), the threshold value generally recognized for delineation of genomic species (Wayne *et al.*, 1987). Strains 03-723^T and 03-8772^T could also be distinguished from the type strains of their most closely related species based on differences in several phenotypic properties (Table 1).

It is clear from the genotypic and phenotypic data presented herein that strains 03-723^T and 03-8772^T represent two novel *Actinoplanes* species, for which the names *Actinoplanes sichuanensis* sp. nov. and *Actinoplanes xinjiangensis* sp. nov. are proposed, respectively.

Fig. 2. Neighbour-joining tree derived from aligned 16S rRNA gene sequences, showing the positions of strains 03-723^T and 03-8772^T among their nearest neighbours. Numbers at nodes indicate percentage bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values >50% are indicated. Asterisks represent clades that were also recovered with the maximum-parsimony algorithm. Bar, 0.01 substitutions per nucleotide position.

Description of *Actinoplanes sichuanensis* sp. nov.

Actinoplanes sichuanensis (si.chu.a.nen'sis. N.L. masc. adj. *sichuanensis* pertaining to Sichuan Province, south-west China, soil of which was the source of the type strain).

Cells are aerobic and Gram-positive. Spherical sporangia are formed (Fig. 1). Substrate mycelium is cream to reddish orange on ISP 2, ISP 3, ISP 4, ISP 5, ISP 6, ISP 7, yeast extract-starch agar, nutrient agar, Czapek solution agar and modified Bennett's agar. Aerial mycelium is absent. Soluble pigment and melanin are not formed. Sporangiospores are motile and have a smooth surface. Aesculin is hydrolysed and nitrate is reduced to nitrite. Amylase and gelatinase are produced. H₂S is not produced. Grows in the presence of 0–4% NaCl, but not in 5–10% NaCl. Growth occurs at an initial pH of 6.5–10.5 and between 10 and 37 °C, but not at pH 4.5–6.0 or at 40 °C. Sensitive to filter-paper discs soaked in (1 ml; µg ml⁻¹) erythromycin (15), gentamicin (10) and kanamycin (30), but resistant to ciprofloxacin (5),

novobiocin (5), oxacillin (1), streptomycin (10) and tobramycin (10). Peptidoglycan contains *meso*-diaminopimelic acid; diagnostic sugars are xylose and arabinose; mycolic acids are absent; MK-9(H₄) (94.4%) is the predominant menaquinone with MK-9(H₆) (3.7%) and MK-10(H₆) (1.9%) found as minor components. Major phospholipids are phosphatidylethanolamine and phosphatidylinositol. Predominant cellular fatty acids (>10%) are iso-C_{15:0}, iso-C_{16:0} and C_{17:1ω9c} (11.1%); C_{14:0}, C_{16:0} and C_{18:1ω9c} are present as minor components (>5%). Utilizes acetate, adonitol, citrate, D-ribose, fructose, galactose, gluconate, glucosamine, glucose, L-arabinose, L-tartrate, maltose, melampyrin, sodium malonate, sorbitol, sucrose, turanose and xylose as sole carbon sources for energy and growth, but not cellobiose, dextrin, D-mannitol, melibiose, raffinose, erythritol, inositol, lactose, L-phenylalanine, mannose, methyl α-D-glucoside, salicin, sodium acetate (0.1%, w/v), trehalose or α-L-rhamnose. Produces acid phosphatase, alkaline phosphatase, N-acetylglucosaminidase, β-galactosidase and

Table 1. Differential phenotypic properties between strains 03-723^T and 03-8772^T and the type strains of related *Actinoplanes* species

Strains: 1, 03-723^T (*A. sichuanensis* sp. nov.); 2, 03-8772^T (*A. xinjiangensis* sp. nov.); 3, *A. lobatus* DSM 43150^T; 4, *A. auranticolor* DSM 43031^T; 5, *A. capillaceus* DSM 44859^T; 6, *A. campanulatus* DSM 43148^T; 7, *A. philippinensis* DSM 43019^T. All are negative for decomposition of adenine (0.4 %, w/v), elastin (0.3 %, w/v), guanine (0.4 %, w/v), hypoxanthine (0.4 %, w/v) and xanthine (0.4 %, w/v), but positive for hydrolysis of aesculin. +, Positive; -, negative; w, weakly positive. All data are from the present study.

Characteristic	1	2	3	4	5	6	7
Nitrate reduction	+	+	+	-	+	-	+
Amylase	+	-	+	+	+	+	+
Gelatinase	+	+	-	-	-	-	-
H ₂ S production	-	+	-	-	-	-	-
Decomposition of (% w/v):							
Tyrosine (0.5)	+	-	+	+	+	-	+
Urease (0.5)	+	+	-	-	-	+	+
Casein (0.5)	-	-	-	-	+	-	-
Growth on carbon sources							
α -L-Rhamnose	-	-	-	-	+	-	-
Cellobiose	-	-	-	+	-	+	-
Dextrin	-	-	-	+	-	-	+
D-Galactose	+	-	-	-	+	-	-
D-Glucose	+	+	+	-	+	+	-
Maltose	+	-	+	+	+	+	+
D-Mannitol	-	-	+	+	+	+	-
D-Mannose	-	+	-	+	+	+	-
Raffinose	-	-	+	-	-	-	-
D-Salicin	-	-	+	-	-	-	+
Lactose	-	-	+	+	-	-	-
L-Tartrate (0.1 %, w/v))	+	-	+	+	+	+	+
Sodium citrate (0.1 %, w/v)	+	+	-	-	w	w	-
Sodium malonate (0.1 %, w/v))	+	-	+	-	-	-	-

β -glucosaccharase. Additional phenotypic properties are given in Table 1. The G + C content of the genomic DNA of the type strain is 70.4 mol%.

The type strain, 03-723^T (=KCTC 19460^T=CCM 7526^T), was isolated from a soil sample collected from Sichuan Province, south-west China.

Description of *Actinoplanes xinjiangensis* sp. nov.

Actinoplanes xinjiangensis (xin.ji.an.gen'sis. N.L. masc. adj. *xinjiangensis* pertaining to Xinjiang Uyghur Autonomous Region, north-west China, soil of which was the source of the type strain).

Cells are aerobic and Gram-positive. Spherical to oval sporangia are formed (Fig. 1). Aerial mycelium is absent. Grows well on ISP 2, ISP 3, ISP 4, ISP 5, ISP 7, yeast extract-starch agar, nutrient agar, Czapek solution agar and

modified Bennett's agar and moderately well on ISP 6 agar. Soluble pigment is not formed but melanin is produced on ISP 6 agar. Sporangiospores are motile and the surface is almost smooth. Aesculin is hydrolysed and nitrate is reduced to nitrite. Amylase is not produced but gelatinase is produced. H₂S is produced. Grows in the presence of 0–7 % NaCl, but not in 8–10 % NaCl. Growth occurs at an initial pH of 6.5–8.5 and between 10 and 37 °C, but not at pH 4.5–6.0 or pH 9.0–11, or at 4 or 40 °C. Sensitive to (1 ml; μ g ml⁻¹) kanamycin (30) but resistant to ciprofloxacin (5), erythromycin (15), gentamicin (10), novobiocin (5), oxacillin (1), streptomycin (10) and tobramycin (10). The peptidoglycan contains *meso*-diaminopimelic acid; diagnostic sugars are xylose and arabinose; mycolic acids are absent; MK-9(H₄) (96.1 %) is the predominant menaquinone and MK-10(H₆) (3.9 %) is found as a minor component. The major phospholipid is phosphatidylethanolamine. Predominant cellular fatty acids (>10 %) are iso-C_{15:0}, iso-C_{16:0} and C_{17:1} ω 9c; C_{15:0}, C_{16:0} and C_{16:1} ω 9c are present as minor components (>5 %). Utilizes acetate, adonitol, citrate, D-ribose, fructose, gluconate, glucosamine, glucose, L-arabinose, mannose, melampyrin, sorbitol, sucrose, turanose and xylose as sole carbon sources for energy and growth, but not cellobiose, dextrin, maltose, D-mannitol, melibiose, raffinose, erythritol, galactose, inositol, lactose, L-phenylalanine, L-tartrate, methyl α -D-glucoside, rhamnose, salicin, sodium acetate or sodium malonate. Produces acid phosphatase, α -glucosaccharase, alkaline phosphatase, *N*-acetylglucosaminidase, β -galactosidase and β -glucosaccharase. Additional phenotypic properties are given in Table 1. The G + C content of the genomic DNA of the type strain is 71.0 mol%.

The type strain, 03-8772^T (=KCTC 19461^T=CCM 7527^T), was isolated from a soil sample collected from Xinjiang Uyghur Autonomous Region, north-west China.

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