Streptomyces lacrimifluminis sp. nov., a novel actinobacterium that produces antibacterial compounds isolated from soil from the Qinghai-Tibet Plateau

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Abstract

A novel actinobacterial strain, designated Z1027^T, was isolated from a soil sample collected near the Tuotuo river, Qinghai-Tibet Plateau (China). The strain exhibited antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. The strain Z1027^T identity was determined using a polyphasic taxonomic approach. The organism has chemotaxonomic and morphological properties consistent with its classification in the genus *Streptomyces* and forms a distinct phyletic line in the 16S rRNA gene tree, together with the type strains *Streptomyces turgidiscabies* ATCC 700248^T (99.19%), *Streptomyces graminilatus* JL-6^T (98.84%), and *Streptomyces reticuliscabiei* CFBP 4531^T (98.36%). The genomic DNA G+C content of strain Z1027^T was 74±1 mol%. The DNA–DNA relatedness values between strain Z1027^T and strain *Streptomyces turgidiscabies* ATCC 700248^T, and between strain Z1027^T and strain *Streptomyces reticuliscabiei* CFBP 4531^T were 38.5±0.4 mol% and 26.2±1.2 mol%, respectively, both of them significantly lower than 70%. Chemotaxonomic data revealed that strain Z1027^T possesses MK-9(H_6) and MK-9(H_8) as the major menaquinones, LL-diaminopimelic acid as the diagnostic diamino acid, and galactose as a whole cell sugar. Diphosphatidylglycerol, phosphatidylethanolamine, phosphatydilinositol and seven other unknown polar lipids were detected; iso-$C_{16:0}$, summed feature 3 ($C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c), $C_{16:0}$ were the major fatty acids. On the basis of these genotypic and phenotypic data, it is proposed that isolate Z1027^T (=CGMCC 4.7272^T =JCM 31054^T) should be classified in the genus *Streptomyces* as *Streptomyces lacrimifluminis* sp. nov.

The Qinghai-Tibet Plateau is the largest plateau in China and, with an average elevation exceeding 4,500 m, the highest plateau in the world. Low air temperatures, high UV radiation and low atmospheric oxygen content are stress conditions organisms must adapt to and consequently this environment is a potential source of genetic diversity. A survey of soil actinobacteria from this area underlined this diversity (Zhang *et al.*, 2016). The genus *Streptomyces* was first described by Waksman and Henrici (1943). They are typically Gram-positive, with a high DNA G+C content, the presence of LL-diaminopimelic acid and no diagnostic sugars in whole cell hydrolysates (Anderson & Wellington, 2001; Kämpfer, 2012). Species of the genus *Streptomyces* typically possess a wide range of metabolic pathways and produce many bioactive secondary metabolites, notably the majority of antibiotics used in medicine (Bérdy, 2012; Goodfellow & Fiedler, 2010). Isolation of novel streptomycetes from this environment is important in the search for new bioactive compounds, including new antibiotics. Here we report a novel streptomycete isolated from grassland soil collected near the Tuotuohe on the Qinghai-Tibet Plateau, China.

Strain Z1027^T was isolated from the soil sample using Gause's synthetic agar medium (l^{-1} distilled water: 20.0 g soluble starch, 1.0 g KNO_3, 0.5 g $K_2HPO_4.3H_2O$, 0.5 g $MgSO_4.7H_2O$, 0.001 g $FeSO_4$, 0.5 g NaCl, 20.0 agar, pH 7.2), supplemented with nalidixic acid (25 μg ml^{-1}) incubated for 7 days at 30°C. The strain was stored at -86°C in the presence of 20 % (v/v) glycerol.

Morphological observation of spores and mycelia were conducted by light microscopy (BH-2; Olympus) and scanning electron microscopy (QUANTA FEG-450) using cultures grown on ISP 4 medium for 20 days (Shirling & Gottlieb, 1966). Growth characteristics were also examined after culturing on standard media ISP 2-7 (Shirling & Gottlieb, 1966) , Czapek's agar and nutrient agar after incubation at 30°C for 14 days. The utilization of sole carbon and nitrogen sources, and decomposition of starch, cellulose or gelatin, were examined as described previously (Gordon *et al.*, 1974; Yokota *et al.*, 1993). Growth at various temperatures (4, 10, 20, 30, 37, 45 and 50) and NaCl concentrations (0-10%) was examined on yeast extract-malt extract (ISP 2). The pH range and the optimum pH were determined by incubating at 30 °C in ISP 2 broth, of which pH was adjusted to 4 − 12 by addition of KH_2PO4/HCl, KH_2PO_4/K_2HPO_4 and K_2HPO_4/NaOH (at intervals of 1.0 pH unit). Antibacterial activity of strain S10^T was determined using a cylinder plug antibacterial bioassay with *Escherichia coli* ATCC 25922^T and *Staphylococcus aureus*

ATCC 25923^T as indicator strains (Li *et al.*, 2007). The reference strains were *Streptomyces turgidiscabies* JCM 10429^T, *Streptomyces reticuliscabiei* DSM 41804^T and *Streptomyces graminilatus* NBRC 108882^T.

The morphological features of isolate Z1027^T were consistent with its classification in the genus *Streptomyces* (Williams *et al.*, 1983). After 20 days of incubation on ISP 4 medium, strain Z1027^T formed a white grey aerial mycelium, which differentiated into straight chains of smooth surfaced spores (Fig. 1). Aerial mycelia were not observed when the strain was grown on the ISP 6 and Nutrient agar media. Strain Z1027^T showed good growth on ISP 2, 3, 6, 7, Czapek agar and Nutrient agar, and moderate growth on ISP 4 and 5 media (Table S1). The physiological and biochemical properties of Z1027^T are given in Table 1, Table S1 and in the species description (see below). There were significant differences in the phenotypic properties of strain Z1027^T compared to the three related type species. Z1027^T could utilize a wider diversity of carbon sources. Whereas Z1027^T could not utilize L-histidine as a nitrogen source, the other reference strains could. The strain Z1027^T could metabolize Tween 20 whereas the references strains could not. The ability to sporulate on different media was also different. The colour of the aerial mycelium of strain Z1027^T grown on ISP 7 was grey, which was again different to the reference strains. There was no pigment produced by strain Z1027^T on any media, whereas *Streptomyces reticuliscabiei* DSM 41804^T produced a light brown pigment on ISP 4. The strain Z1027^T exhibited antibacterial activities against *Escherichia coli* ATCC 25922^T (zone size 13 ± 2 mm) and *Staphylococcus aureus* ATCC 25923^T (zone size 8 ± 2 mm), compared with zone sizes of 11± 2 mm and 9± 2 mm, respectively, resulting after application of 10 µl of a 50 mg/ml ampicillin solution to 10 mm sterile filter discs added to the indicator plates .

The genomic DNA of strain Z1027^T was extracted and the 16S rRNA was amplified by PCR using bacterial universal primers 27 F and 1492 R (Han *et al.*, 2015). The PCR product was purified and cloned into the vector pMD19-T (Takara) and sequenced (He *et al.*, 2014). The almost full-length 16S rRNA gene sequence of strain Z1027^T (1495 nt) was compared with the 16S rRNA sequences on the EzTaxon database (Kim *et al.*, 2012). This analysis indicated that the strain is closely related to *Streptomyces turgidiscabies* ATCC 700248^T. A phylogenetic tree was generated using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and

maximum-likelihood (Felsenstein, 1981) algorithms in MEGA5.0 (Tamura *et al.*, 2011). Evolutionary distances were calculated using the model of Jukes and Cantor (1969). Topologies of the resultant tree were evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 re-samplings. Strain Z1027^T formed an independent clade with the type strains *Streptomyces turgidiscabies* ATCC 700248^T (99.19%), *Streptomyces graminilatus* JL-6^T (98.84%), and *Streptomyces reticuliscabiei* CFBP 4531^T (98.36%) (Fig. 2).

Biomass for chemotaxonomic studies was prepared by growing the strain in TSB medium in flasks on a rotary shaker at 200 r.p.m for 10 days at 30°C. Biomass was harvested by centrifugation, washed twice in distilled water, re-centrifuged and freeze-dried. Analysis of the diaminopimelic acid isomers in the cell wall and whole-cell sugars were performed as described by Lechevalier & Lechevalier (Lechevalier, 1980; Lechevalier & Lechevalier, 1970) and Staneck and Roberts (1974), respectively. The menaquinones were extracted and purified using the method of Collins *et al.* (1977) and analysed by HPLC (Kroppenstedt, 1982). Polar lipids were extracted, separated by two-dimensional TLC and identified according to procedures outlined by Minnikin *et al.* (1984). Cellular fatty acids were extracted, methylated and separated by according to the standard protocol of the Sherlock Microbial identification (MIDI) system (Kampfer & Kroppenstedt, 1996; Sasser, 1990) and the fatty acid methyl ester peaks were quantified using the TSBA 5.0 database. DNA G+C content was examined by the thermal denaturation method (Marmur & Doty, 1962).

The chemotaxonomic features of strain Z1027^T were consistent with those of the genus *Streptomyces*. It contained LL-diaminopimelic acid, with galactose present in whole-organism hydrolysates. The predominant isoprenoid quinone compounds were MK-9 (H_6) (48.9%), MK-9 (H_8) (48.1%) and MK-8 (H_6) (3%). It accorded with the characteristics of *Streptomyces* describe by Collins *et al.* (1985). The polar lipids were diphosphatidylglycerol, phoshphatidylethanolamine, phosphatydilinositol, four unknown lipids, two unknown phospholipids and one unknown phosphatidylglycolipid. This pattern corresponds to polar lipid type II of Lechevalier *et al.* (1977). The major fatty acids found were iso-$C_{16:0}$, summed feature 3 ($C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c) and $C_{16:0}$ (Table 2). The major fatty acid features of strain Z1027^T and three reference strains accorded with features of the genus *Streptomyces*, but there were slightly different between them. For example, the type and relative abundance of the top three fatty acid in the four strain were different. The DNA

G+C content of strain Z1027^T was 74±1 mol%.

DNA-DNA hybridization was performed according to the method of Ezaki *et al.* (1989). The DNA-DNA relatedness value of strain Z1027^T with *Streptomyces turgidiscabies* ATCC 700248^T and *Streptomyces reticuliscabiei* CFBP 4531^T were 38.5±0.4 mol% and 26.2±1.2 mol%, respectively, and both values were significantly lower than 70%, the level considered to be the threshold value for the delineation of genomic species (Wayne *et al.*, 1987).

Based on the phenotypic and phylogenetic evidence, strain Z1027^T is clearly different from all other species of the genus *Streptomyces*, which supports its classification as a novel species within the genus *Streptomyces*, for which the name *Streptomyces lacrimifluminis* sp. nov. is proposed.

Description of *Streptomyces lacrimifluminis* sp. nov.

Streptomyces lacrimifluminis (la.cri.mi.flu'mi.nis. L. fem. n. lacrima tear; L. neut. n. flumen river; N.L. gen. n. lacrimifluminis of the River of Tears, the Tuotuo River, Qinghai-Tibet Plateau, China, where the type strain was isolated).

Aerobic, Gram-stain-positive, non-motile, actinobacterium. Produces long, straight chains of cylindrical and smooth-surfaced white-grey spores. Grows well on ISP medium 2, 3, 6, 7, Czapek and nutrient. agar medium. Diffusible pigments are not formed on any tested medium. Grows at 20-40°C (optimum, 30°C), at pH 6-11 (optimum, pH 8). The maximum NaCl concentration for growth is 4% (w/v) (optimum, 0-2 %). Positive for starch, cellulose, gelatin and tween 20 degradation and urease test. Negative for tween 80 degradation. Utilizes myo-inositol, L-arabinose, D-fructose, D-glucose, D-lactose, D-galactose, D-mannitol, D-raffinose, L-rhamnose, sucrose or D-xylose as sole carbon sources. Utilizes leucine or L-asparagine as sole nitrogen sources, but not L-alanine, L-cysteine or L-histidine. The cell wall contains LL-diaminopimelic acid. The whole-cell sugar pattern mainly consists of galactose. The predominant menaquinone are MK-9 (H_6), MK-9 (H_8) and MK-8 (H_6). The polar lipid profile contains diphosphatidylglycerol, phoshphatidylethanolamine, phosphatydilinositol, four unknown lipids, two unknown phospholipids and one unknown phosphatidylglycolipid.. The major cellular fatty acids are iso-$C_{16:0}$, summed feature 3 ($C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c) and $C_{16:0}$.

The type strain, Z1027^T (=CGMCC 4.7272^T =JCM 31054^T) was isolated from a soil sample

collected from near the Tuotuo river, Qinghai-Tibet Plateau, China. The G+C content of the genomic DNA of the type strain is 74±1 mol%.

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Figure legends

Fig. 1 Scanning electron micrograph of strain Z1027^T cultivated on ISP 4 at 30°C for 20 days showed straight spore chains and smooth spore surface. Bars, 5 μm.

Fig. 2 Maximum-likelihood phylogenetic tree, based on nearly complete 16S rRNA gene sequences, showing the relationships between strain Z1027^T and related species of the genus *Streptomyces*. Numbers at nodes are bootstrap values based on 1000 re-samplings (only values above 50% are shown). Asterisks (*****, #) indicate that the clades are recovered in neighbor-joining and maximum-parsimony trees, respectively.

Table 1. Phenotypic properties of strain Z1027^T and related type species

All data were obtained in this study. Abbreviations: +, positive; − , negative. All strains were positive for utilization of L-arabinose, D-fructose D-galactose and leucine and for degradation starch, cellulose, gelatin.

Characteristics	Z1027^T	*S. turgidiscabies* JCM10429^T	*S. reticuliscabiei* DSM41804^T	*S. graminifolius* NBRC108882^T
Spore chain	Straight	Flexuous	Flexuous	Straight
NaCl for growth(%,w/v)	0-4	0-3	0-3	0-4
Carbon source utilization (1.0%, w/v)				
myo-inositol	+	-	+	+
D-glucose	+	-	+	+
D-lactose	+	-	+	+
D-mannitol	+	+	+	-
D-raffinose	+	+	-	+
L-rhamnose	+	+	+	-
Sucrose	+	-	+	+
D-xylose	+	+	-	+
Nitrogen source utilization (0.1%, w/v)				
L-alanine	-	-	-	+
L-asparagine	+	-	+	+
L-histidine	-	+	+	+
L-cysteine	-	-	+	-
Degradation				
Tween 20	+	-	-	-
Tween 80	-	-	-	+
Urease test	+	-	+	-

Table 2. Cellular fatty acid composition of strain Z1027^T and related type species.

Strains: 1, Strain Z1027^T; 2, *S. turgidiscabies* JCM 10429^T; 3, *S. reticuliscabiei* DSM 41804^T;4, *S. graminilatus* NBRC 108882^T. All data were obtained in this study. Tr,Trace (<1%); -, not detected; ECL, equivalent chain length (i.e. the identity of the fatty acids is unknown). Fatty acids amounting to <1% of the total fatty acids in all strains are not shown.

Fatty acid	1	2	*3*	4
Saturated				
$C_{14:0}$	1.56	1.54	Tr	2.03
$C_{15:0}$	-	-	-	4.46
$C_{16:0}$	14.78	13.98	4.34	9.97
$C_{18:0}$	1.06	Tr	-	-
Branched				
iso-$C_{14:0}$	2.39	6.03	4.77	7.76
iso-$C_{15:0}$	5.46	8.83	10.15	25.34
anteiso-$C_{15:0}$	9.79	13.86	8.96	5.79
iso-$C_{16:0}$	18.79	20.44	33.92	17.53
iso-$C_{16:1}$ H	4.90	5.09	8.71	1.78
iso-$C_{17:0}$	Tr	1.35	3.06	2.56
anteiso-$C_{17:0}$	5.08	5.30	5.37	4.32
anteiso-$C_{17:1}$ ω9c	3.82	2.80	3.61	1.45
iso-$C_{18:1}$ H	-	Tr	1.84	Tr
Unsaturated				Tr
$C_{17:1}$ ω8c	1.98	1.74	Tr	
Cyclo				
cyclo-$C_{17:0}$	4.22	1.02	Tr	-
Sum In Feature* 3	17.94	11.15	4.35	8.44
Sum In Feature* 9	2.82	2.97	7.69	1.03

*Sum In Feature are groups of two or three fatty acids that are treated together for the purpose of evaluation in the MIDI system and include both peaks with discrete ECLs as well as those where the ECLs are not reported separately (del Carmen Montero-Calasanz *et al.*, 2013).

Summed Feature 3: $C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c; Summed Feature 9: $C_{16:0}$ 10-methyl or iso-$C_{17:1}$ω9c.