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ORIGINAL PAPER

Lentzea guizhouensis sp. nov., a novel lithophilous actinobacterium isolated from limestone from the Karst area, Guizhou, China

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Abstract A novel filamentous actinobacterium, designated strain DHS C013^T, was isolated from limestone collected in Guizhou Province, South-west China. Morphological and chemotaxonomic characteristics of the strain support its assignment to the genus *Lentzea*. Phylogenetic analyses showed that strain DHS C013^T is closely related to *Lentzea jiangxiensis* FXJ1.034^T (98.7 % 16S rRNA gene similarity) and *Lentzea flaviverrucosa* 4.0578^T (98.0 % 16S rRNA gene similarity), but it can be distinguished from these strains based on low levels of DNA:DNA relatedness (~44 and ~37 %, respectively). Physiological and biochemical tests also allowed phenotypic differentiation of the novel strain from these closely related species. On the basis of the evidence presented here, strain DHS C013^T is concluded to represent a novel species of the genus *Lentzea*, for which the name *Lentzea guizhouensis* sp. nov. is proposed. The type strain is DHS C013^T (=KCTC 29677^T = CGMCC 4.7203^T).

Keywords *Lentzea guizhouensis* sp. nov. · Lithophilous · Karst area · Polyphasic taxonomy

Introduction

The genus *Lentzea* was established by Yassin et al. (1995) as a member of the family *Pseudonocardiaceae* (Labeda et al. 2011). However, the type species of the genus was transferred into the genus *Saccharothrix* as *Saccharothrix albidocapillata* (Lee et al. 2000). Then the genus *Lentzea* was revived by Labeda et al. (2001), who transferred *Saccharothrix waywayandensis* NRRL B-16159^T, *Saccharothrix aerocolonigenes* NRRL B-16137^T and *Asiosporangium albidum* IFO 16102^T into the genus *Lentzea*, on the basis of extensive molecular phylogenetic and

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chemotaxonomic analyses. At the time of writing, the genus *Lentzea* contains eight validly named species (http://www.bacterio.net/lentzea.html), including *Lentzea albidocapillata* (Yassin et al. 1995), *Lentzea flaviverrucosa* (Xie et al. 2002), *Lentzea kentuckyensis* (Labeda et al. 2007), *Lentzea jiangxiensis* (Li et al. 2012), *Lentzea californiensis*, *Lentzea waywayandensis*, *Lentzea albida* and *Lentzea violacea* (Labeda et al. 2001). All members of the genus are Gram-positive, typically aerobic, non-motile actinomycetes with branched aerial mycelia which fragment into rod-shaped elements. These organisms contain *meso*-diaminopimelic acid, galactose, mannose and ribose in their cell walls. Their phospholipid patterns consist of significant amounts of phosphatidylethanolamine along with diphosphatidylglycerol, phosphatidylglycerol, and phosphatidylinositol. The principal menaquinone is MK-9(H_4). The G+C content of the genomic DNA ranges from 68.6 to 79.6 mol %. Fatty acid profiles contain straight-chain saturated or unsaturated, and branched-chain saturated fatty acids of the iso and anteiso types, in addition to tuberculostearic acid (Labeda et al. 2011; Labeda 2012).

During our study of the microbial diversity associated with limestone and their effects on bioweathering processes, several lithophilous actinobacteria (i.e. bacteria which can live in stony places) were isolated from limestone samples collected in Karst area, Guizhou Province, South-west China. The present study was devoted to establish the taxonomic status of a *Lentzea*-like strain, designated DHS C013^T, based on polyphasic taxonomic methods.

Materials and methods

Strains and cultural conditions

Strain DHS C013^T was isolated from limestone collected from the Puding Karst Ecosystem Research Station of the Chinese Academy of Sciences in Guizhou Province, China (26°09′−26°31′N, 105°27′−105°58′E). The average annual precipitation and temperature of this region are 1390 mm and 15.1 °C, respectively. These limestone samples were dried at room temperature, ground into powder and then suspended in sterile distilled water and serially diluted. After incubation at 28 °C for 21 days, the organism was isolated on modified ATCC-172 medium at 1/10 concentration, which contained glucose 1.0 g, soluble starch 2.0 g, yeast extract 0.5 g, N-Z Amine Type A (Sigma co.626) 0.5 g, $CaCO_3$ 0.1 g, agar 15 g and distilled water 1L. The isolate DHS C013^T and the type strains of *L. jiangxiensis* FXJ1.034^T (98.7 %) and *L. flaviverrucosa* 4.0578^T were maintained on yeast extract-malt extract agar (ISP 2 medium) (Shirling and Gottlieb 1966) at 4 °C and as suspensions of hyphal fragments in glycerol (20 %, v/v) at -20 and -80 °C. Strain DHS C013^T has been deposited in the China General Microbiological Culture Collection Center (CGMCC) as strain CGMCC 4.7203^T and in the Korean Collection for Type Cultures (KCTC) as strain KCTC 29677^T.

Phenotypic properties

Colonial morphologies and cultural characteristics were observed after incubation at 28 °C for 2 weeks on the following media: ISP 2, oatmeal agar medium (ISP 3) inorganic salts-starch agar medium (ISP 4), glycerol-asparagine agar medium (ISP 5) (Shirling and Gottlieb 1966), Czapek solution agar, nutrient agar (Difco) and potato dextrose agar medium (PDA). The colours of substrate and aerial mycelia and soluble pigments were tested with reference to the ISCC–NBS centroid chart (Kelly 1964). The morphological properties of strain DHS C013^T were examined by optical microscopy (OM, SA3300-PL) and scanning electron microscopy (SEM, Hitachi S-3400 N), using cultures grown on ISP 2 medium at 28 °C for 14 days. The growth at 4, 10, 15, 20, 28, 37, 45 and 55 °C and in 0–20 % (w/v) NaCl (at intervals of 1 %, 28 °C) were examined on ISP 2 basal medium. Growth at pH 4.0–11.0 (at intervals of 1 pH unit) was examined as described by Xu et al. (2005). Media and procedures used for determination of physiological features, carbon and nitrogen source utilisation were as described by Kurup and Schmitt (1973), Gordon et al. (1974) and Williams et al. (1989).

Chemotaxonomy

Mycelia biomass for chemotaxonomic and molecular analyses was obtained from cultures grown in tryptic soy broth (TSB) at 180 rpm at 28 °C for 4 days. Mycelia were harvested by centrifugation, washed with distilled water and freeze-dried. The isomer of diaminopimelic acid in the cell wall peptidoglycan was determined by the method of Hasegawa et al.

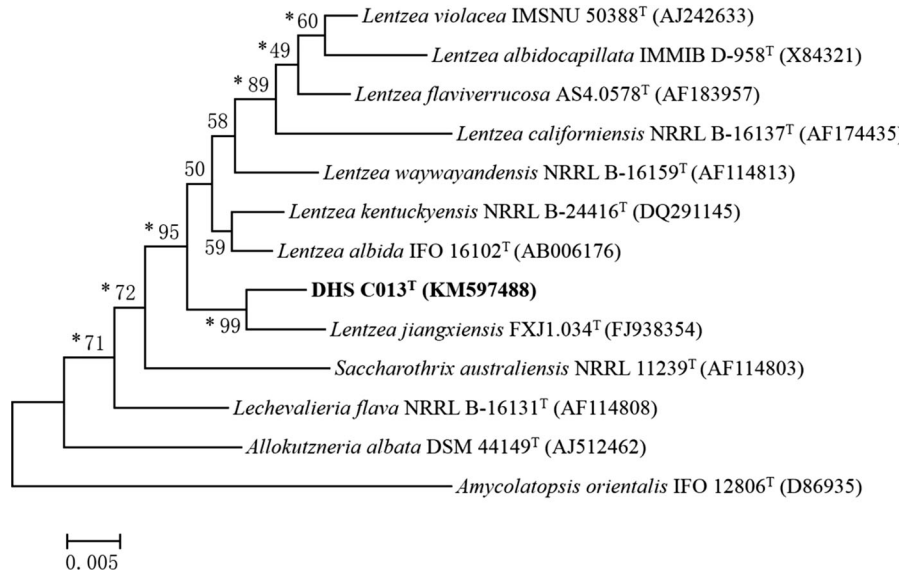

Fig. 1 Neighbour-joining tree based on 16S rRNA gene sequences showing the relationship between strain DHS C013^T and species of the genus *Lentzea*. The sequence of *Amycolatopsis orientalis* IFO 12806^T (D86935) was used as outgroup. *Numbers* at branch nodes are bootstrap values (1000 resamplings) and *asterisks indicate* branches of the tree that were also found using the maximum-likelihood and maximum-parsimony tree-making algorithms. *Bar* 0.005 sequence variation

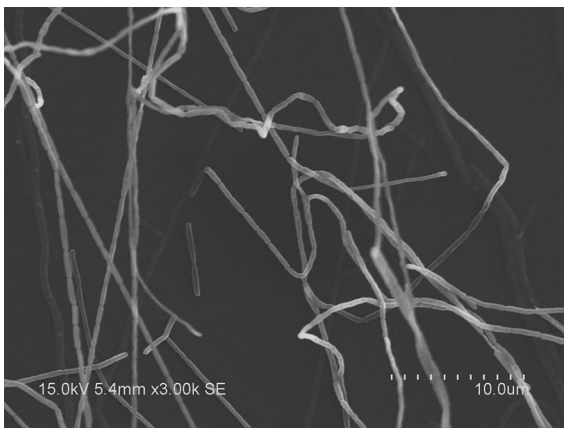

Fig. 2 Scanning electron micrograph of strain DHS C013^T, showing aerial mycelium fragmenting into spore chains after growth on ISP 2 agar medium at 28 °C for 2 weeks. *Bar* 10 μm

(1983). The whole cell sugar compositions were analysed by the methods of Lechevalier and Lechevalier (1970). Phospholipids were analysed by two-dimensional Thin Layer Chromatography and identified referring to the method of Minnikin et al. (1984). The fatty acid profile was determined using the method of Sasser (1990) and characterised by using the Microbial Identification System (MIDI, Sherlock Version 6.1). Menaquinones were extracted and purified according to the procedures described by Collins et al. (1977) and analysed by HPLC (Groth et al. 1997). The G+C content of the genomic DNA was tested using the method of Mesbah et al. (1989).

16S rRNA gene sequence analyses

Extraction of genomic DNA and 16S rRNA gene amplification were carried out according to the procedures described by Qin et al. (2009). The resultant almost complete 16S rRNA gene sequence of the isolate was subjected to BLAST sequence similarity search at the GenBank database. Then the identification of phylogenetic neighbours and the calculation of pairwise 16S rRNA gene sequence identities were achieved using the EzTaxon-e database (Kim et al. 2012). Phylogenetic trees showing the relationship between the isolate and closely related strains were inferred using the neighbour-joining (Saitou and Nei 1987), maximum-parsimony (Kluge and Farris 1969) and maximum-likelihood (Felsenstein 1981) tree-making algorithms from the MEGA software (Version 5.0), with bootstrap values based on 1000 repeats (Felsenstein 1985).

DNA–DNA relatedness was performed on DHS C013^T with its two closely related type strains using the fluorometric micro-well method (Ezaki et al. 1989; He et al. 2005). The hybridization values were expressed as a mean of triplicate measurements.

Table 1 Fatty acid composition (%) of isolate DHS C013^T and the type strains of its close phylogenetic neighbours

Fatty acids	Isolate DHS C013^T	*L. jiangxiensis* FXJ1.034^T	*L. flaviverrucosa* 4.0578^T
Iso-$C_{10:0}$	–	0.2	–
$C_{10:0}$ 3OH	–	–	4.2
Iso-$C_{12:0}$	0.6	1.2	0.7
Anteiso-$C_{12:0}$	0.3	–	0.3
$C_{12:0}$	–	–	4.7
Iso-$C_{13:0}$	0.1	2.9	–
Anteiso-$C_{13:0}$	0.3	0.9	0.3
Iso-$C_{14:0}$	0.9	1.6	0.7
Anteiso-$C_{14:0}$	0.1	–	–
$C_{14:0}$	0.4	1.1	2.0
Iso-$C_{15:0}$	26.7	33.5	7.2
Anteiso-$C_{15:0}$	34.1	26.2	18.1
$C_{15:0}$ 2OH	–	0.6	0.4
$C_{16:1}\omega7c$ alcohol	0.3	0.7	–
Iso-$C_{16:0}$	3.8	5.5	2.0
$C_{16:1}\omega11c$	–	0.4	–
$C_{16:0}$ 2OH	–	–	2.2
$C_{16:0}$	4.4	2.9	19.5
Iso-$C_{17:1}\omega10c$	0.6	2.5	
Iso-$C_{17:0}$	5.6	6.7	2.5
Anteiso-$C_{17:0}$	–	8.4	5.2
Cyclo $C_{17:0}$	–	–	21.0
$C_{18:0}$	–	0.4	0.3
$C_{18:1}\omega7c$	–	–	1.2
Cyclo $C_{19:0}\omega8c$	–	–	1.6
Summed feature 2*	–	–	1.1
Summed feature 3*	0.7	2.5	4.8
Summed feature 4*	0.5	1.6	–
Summed feature 5*	7.8	–	–

Sum in feature 3 consisted of 16:1 ω7*c*/16:1ω6*c*, Sum in feature 4 consisted of 17:1 iso I/anteiso B, Sum in feature 5 consisted of 18:2ω6, 9c/18:0 ante

* Summed features represent groups of two or three fatty acids that cannot be separated by GC with the MIDI system

Results

16S rRNA gene sequencing and DNA:DNA relatedness studies

The almost complete 16S rRNA gene sequence (1497 bp) of strain DHS C013^T was determined and deposited in GenBank with the accession number KM597488. Phylogenetic analyses of 16S rRNA gene sequences showed that strain DHS C013^T is a member of the genus *Lentzea*. Furthermore, the strain shared high levels of 16S rRNA gene sequence similarities with respect to the type strains *L. jiangxiensis* FXJ1.034^T (98.7 %) and *L. flaviverrucosa* 4.0578^T (98.0 %), and for other species of the genus *Lentzea* the similarities ranged from 96.5 % (*L. californiensis*) to 97.9 % (*L. violacea*). As shown in the phylogenetic tree based on the neighbour-joining algorithm (Fig. 1), strain DHS C013^T formed a distinct sub-branch with the type strain *L. jiangxiensis* FXJ1.034^T, supported by a bootstrap value of 99 % and by all of the tree-making algorithms (maximum-likelihood and maximum-

Table 2 Growth and cultural characteristics of isolate DHS C013^T and the type strains of closely related species following incubation at 28 °C for 14 days

Medium	Strains		
	Isolate DHS C013^T	*L. jiangxiensis* FXJ1.034^T	*L. flaviverrucosa* 4.0578^T
Yeast extract-malt extract agar (ISP medium 2)			
Growth	+++	++	++
Aerial mycelium	White	White	White (spars)
Color of substrate mycelium	Slight yellow	Slight yellow	Slight yellow
Oatmeal agar (ISP medium 3)			
Growth	+	+	++
Aerial mycelium	White (spars)	Yellow white (spars)	White
Color of substrate mycelium	Pale yellow	Yellow white	Yellow white
Inorganic salts-starch agar (ISP medium 4)			
Growth	+++	+++	+++
Aerial mycelium	White (spars)	None	White (spars)
Color of substrate mycelium	Pale orange yellow	Yellow white	Pale orange yellow
Glycerol-asparagine agar (ISP medium 5)			
Growth	+++	+++	+++
Aerial mycelium	White	None	Yellow white
Color of substrate mycelium	Yellowish white	Light orange	Pale white
Potato dextrose agar			
Growth	++	+++	++
Aerial mycelium	White	White	White (spars)
Color of substrate mycelium	Dark yellow	Deep yellow	Medium yellow
Czapek-dox medium			
Growth	++	++	+++
Aerial mycelium	White	None	White
Color of substrate mycelium	Yellowish white	Dark yellowish brown	Light orange yellow
Nutrient agar			
Growth	++	++	++
Aerial mycelium	Yellowish white	Yellowish white	White
Color of substrate mycelium	Pale yellow	Light orange yellow	Pale yellow

None of the strains formed diffusible pigments on these media

Key +++ abundant growth, ++ moderate growth, + poor growth

parsimony trees are shown in Supplementary Figs. S1 and S2).

Strain DHS C013^T showed low DNA:DNA relatedness values of 43.6 ± 1.6 and 37.4 ± 2.2 % with *L. jiangxiensis* FXJ1.034^T and *L. flaviverrucosa* 4.0578^T, respectively, well below the 70 % cut-off point recommended for the circumscription of bacterial species (Wayne et al. 1987). The DNA G+C content of strain DHS C013^T was determined to be 69.6 mol %, which is within the range for members of the genus *Lentzea*.

Chemotaxonomic, cultural, morphological and phenotypic characteristics

Isolate DHS C013^T was observed to form slightly yellow substrate mycelium and white aerial mycelium that fragmented into straight spore chains on ISP 2 medium (Fig. 2). The isolate was determined to contain *meso*-diaminopimelic acid in the cell wall and galactose, mannose and ribose as diagnostic sugars. The predominant menaquinone was identified as MK-9(H_4) (89.6 %); minor amounts of MK-9(H_2)

Table 3 Phenotypic properties that distinguish between isolate DHS C013^T and the type strains of closely related species

Characteristic	Strains		
	Isolate DHS C013^T	*L. jiangxiensis* FXJ1.034^T	*L. flaviverrucosa* 4.0578^T
Hydrolysis of:			
Casein	+	+	+
Starch	+	+	+
Tween 80	+	+	+
H_2S production	−	−	−
Milk peptonization	+	+	+
Growth temperatures (°C)	10–37	15–37	10–37
NaCl (w/v) for growth	0–5	0–1	0–1
pH range for growth	6–11	5–11	5–11
Assimilation of sole carbon sources:			
D-Arabinose	+	+	+
D-Galactose	−	+	−
α-Lactose	+	+	+
D-Mannose	−	−	+
D-Raffinose	−	+	+
D-Rhamnose	+	+	+
D-Ribose	+	+	+
D-Sorbitol	−	−	+
Xylitol	+	−	+
Assimilation of sole nitrogen sources:			
L-Histidine	+	+	+
L-Lysine	−	−	+
L-Glutamic acid	−	+	+
L-Valine	+	+	+
DNA G+C content (mol %)	69.6	69.6[a]	64.1[b]
Polar lipids	DPG, PL, PE, PI, L,	DPG, PI, PE, OH-PE, PIM[a]	PE, DPG, PIMs[b]

[a] Data from Li et al. (2012)

[b] Data from Xie et al. (2002)

+ positive, − negative

(10.4 %) were also found to be present. The major polar lipids detected were identified as diphosphatidylglycerol and phosphatidylinositol, with trace amounts of phosphatidylethanolamine and an unidentified phospholipid (Fig. S3). The cellular fatty acid profile was determined to consist of major amounts of *anteiso*-$C_{15:0}$ (34.1 %) and *iso*-$C_{15:0}$ (26.7 %) (Table 1).

In addition, the isolate was found to show good growth on ISP 2, ISP 4 and ISP 5 media. Like its close phylogenetic neighbours, the isolate was observed to form light to dark yellow substrate mycelia and white aerial mycelia on most of agar media, and none of the strains formed diffusible pigments on these media (Table 2). As shown in Table 3, isolate DHS C013^T can be readily distinguished from the two reference type strains using a broad range of phenotypic properties. All of these strains were found to be able to hydrolyse casein and starch; peptonize milk; and to grow on D-arabinose, α-lactose, D-rhamnose, D-ribose, L-histidine, L-valine as sole carbon and nitrogen sources. In contrast, none of them were found to be able to produce H_2S, to grow at 4 or 45 °C, or in the presence of 7 % NaCl (w/v).

Discussion

The present results confirm that isolate DHS C013^T has chemotaxonomic and morphological properties consistent with those of the members of the genus *Lentzea* (Yassin et al. 1995). It is also evident that strain DHS C013^T can be readily distinguished from the type strains *L. jiangxiensis* FXJ1.034^T and *L. flaviverrucosa* 4.0578^T, based on genotypic and phenotypic data. Therefore, it is proposed that strain DHS C013^T should be recognised as representing a new *Lentzea* species, for which the name *Lentzea guizhouensis* sp. nov. is proposed.

Description of *Lentzea guizhouensis* sp. nov.

Lentzea guizhouensis (gui.zhou.en'sis. N.L. fem. adj. *guizhouensis* of or belonging to Guizhou Province, South-west China, the source of the type strain).

Aerobic, Gram-positive actinobacterium which forms branched, slightly to dark yellow substrate mycelia and white aerial mycelia on various agar media. The mature aerial mycelium fragments into short spore chains on all media in Table 2 except for nutrient agar. No soluble pigments are produced on these media. Hydrolyses casein and starch, can peptonize milk, and does not produce H_2S. D-arabinose, α-lactose, D-rhamnose, D-ribose and xylitol can be used as sole carbon source. Growth occurs at 10–37 °C (optimum at 25–28 °C), at pH 6–11 (optimum pH 7) and in the presence of up to 5 % NaCl (w/v) (optimum near 0 %). The diagnostic amino acid of the peptidoglycan is *meso*-diaminopimelic acid. The polar lipids include diphosphatidylglycerol, phosphatidylinositol and phosphatidylethanolamine. The predominant menaquinone is MK-9(H_4). The cellular major fatty acids (>10 %) are *anteiso*-$C_{15:0}$ and *iso*-$C_{15:0}$.The DNA G+C content of the type strain is 69.6 mol %.

The type strain, DHS C013^T (= KCTC 29677^T = CGMCC 4.7203^T), was isolated from limestone in Karst area, Guizhou Province, China. The GenBank accession number of the 16S rRNA gene sequence of the type strain is KM597488.

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