Lentzea pudingi sp. nov., isolated from a weathered limestone sample in a karst area

Chengliang Cao,¹,² Bo Yuan,¹ Sheng Qin,¹ Jihong Jiang,¹ Faxiang Tao² and Bin Lian³,*

Abstract

A novel Gram-stain-positive, aerobic bacterium, designated strain DHS C021T, was isolated from a limestone sample collected from the Puding Karst Ecosystem Research Station of Guizhou Province, southwest China. This strain developed branched vegetative mycelia, and its aerial mycelia fragmented into rod-shaped spores. The cell-wall peptidoglycan contained meso-diaminopimelic acid and the whole-cell sugars comprised galactose, ribose and mannose. The respiratory quinone was identified as menaquinone MK-9(H₄). The major cellular fatty acids were iso-C₁₄:₀ and iso-C₁₆:₀. The phospholipids detected were diphosphatidylglycerol, phosphatidylethanolamine, hydroxyl-phosphatidylethanolamine, phosphatidylinositol, phosphotidylinositolmannosides and one unidentified phospholipid. The genomic DNA G+C content was 69.8 mol% and 16S rRNA gene sequence analysis showed that the strain belonged to the genus Lentzea and shared highest sequence similarity with Lentzea albida CGMCC 4.1727T (98.8 %) and Lentzea waywayandensis CGMCC 4.1646T (98.5 %). However, it could be distinguished from these reference strains based on the low levels of DNA–DNA relatedness (54.5 ± 2.7 and 41.7±3.2 %, respectively). On the basis of morphological, chemotaxonomic and phylogenetic characteristics, and DNA–DNA hybridization data, strain DHS C021T represents a novel species of the genus Lentzea, for which the name Lentzea pudingi sp. nov. is proposed. The type strain is DHS C021T (=CGMCC 4.7319T=KCTC 39694T).

The genus Lentzea, with Lentzea albidocapillata as the type strain, was proposed by Yassin [1]. It belongs to the family Pseudonocardiaceae which currently has 18 genera. Lentzea was transferred to a later synonym of Saccharothrix [2]. However, it was subsequently reintroduced to the genus Lentzea, because it could be differentiated from the genera Saccharothrix and Lechevalieria on the basis of extensive chemotaxonomic and molecular systematic data [3]. At the time of writing, the taxon encompasses nine validly named species (www.bacterio.net/lentzea.html), some of which were isolated from human pathological tissue [1], equine placenta [4], limestone rocks [5] and most from different soils [3, 6, 7]. These Lentzea species are Gram-stain-positive, aerobic and usually form branched vegetative mycelia and aerial hyphae that fragment into rod-shaped spores. Their whole-cell hydrolysates are rich in meso-diaminopimelic acid, contain galactose, mannose and ribose, comprise mixtures of saturated or unsaturated fatty acids of the iso and anteiso types, contain major amounts of phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylinositol and phosphatidylglycerol and phosphatidylinositol, and have MK-9(H₄) as the predominant isoprenologue [8, 9]. We have been investigating bacterial communities on limestone rocks using culture-independent and culture-dependent methods. In this paper, a novel species of the genus Lentzea, isolated from a limestone sample, was characterized taxonomically using a polyphasic approach.

Limestone detritus, used for the isolation of strain DHS C021T, was collected from the Puding Karst Ecosystem Research Station of Guizhou Province, southwest China (26°09′–26°31′N, 105°27′–105°58′E). Five grams of limestone detritus was ground into particles and suspended into 10 ml sterile water under shaking conditions (28°C, 180 r.p.m., 1 h), and 200 µl serially diluted suspension was spread onto culture medium consisting (l–): 1.0 g sodium humate, 0.5 g Na₂HPO₄, 1.5 g KCl, 0.05 g MgSO₄·7H₂O, 0.01 g FeSO₄·7H₂O and 0.02 g CaCO₃. The pH was adjusted at 7.2 ±0.1. The sterilized medium was supplemented with the following antibiotics (l–): 20 mg nalidixic acid, 40 mg cycloheximide and 40 mg nystatin. After incubation for 21 days...
at 28 °C, pure colonies were transferred onto International Streptomyces Project ISP 2 agar medium (yeast extract–malt extract agar) [10]. Strain DHS C021T was obtained and maintained on ISP 2 agar slants at 4 °C and as suspensions of hyphal fragments in glycerol (20%, v/v) at −70 °C.

The morphology of the strain, grown on ISP 2 agar medium at 28 °C for 14 days, was observed using a light microscope (DM750, Leica) and a scanning electron microscope (S-3400N, Hitachi). Cultures for observation of characteristics grew on various media at 28 °C for 14 days, including ISP 2, oatmeal agar medium (ISP 3), inorganic salts–starch agar medium (ISP 4), glycerol–asparaginase agar medium (ISP 5) [10], Czapek solution agar [11], nutrient agar (Difco) and potato dextrose agar medium (PDA) [12]. The colours of substrate, aerial mycelia and soluble pigments were examined with reference to the ISCC-NBS centroid chart [13]. Gram-staining was performed according to the standard procedure [14]. Growth temperature (4, 10, 15, 20, 28, 37, 40, 45 and 55 °C) and NaCl tolerance (0–10%, w/v, at intervals of 1%, 28 °C) were tested on ISP 2 agar medium. Growth at pH 4.0–11.0 (at intervals of 1.0 pH unit) in trypti-case soy broth (TSB; Difco), adjusted with different buffer systems [15], was tested on a rotary shaker at 28 °C, 180 r.p.m. for 14 days. Media and procedures used for determination of carbon and nitrogen source utilization, enzyme activity and other physiological properties were as described by Kurup and Schmitt [16], Gordon et al. [17], and Smibert and Krieg [18].

Biomass for chemotaxonomic studies was prepared by growing strain DHS C021T in ISP 2 medium incubated on a rotary shaker at 28 °C, 180 r.p.m. for 5 days. Cells were harvested at 4 °C by centrifugation (Avanti J-26xpi, Beckman Coulter), and washed twice with sterile distilled water before freeze-drying (Christ Beta 180 LD plus, Martin Christ Gefriertrocknungsanlagen). Amino acids and sugars in the whole-cell hydrolysates were determined according to the procedures described by Hasegawa et al. [19]. Phospholipids were extracted [20] and identified by using two-dimensional thin-layer chromatography [21]. Menaquinones were extracted [22] and analysed by high-performance liquid chromatography (1260 Infinity, Agilent Technologies) [23]. Extraction and analysis of cell fatty acids were performed according to the standard procedures of the MIDI system (Sherlock version 6.1, ACTIN6 database, www.midi-inc.com; 7890A GC System, Agilent Technologies) [24, 25]. The G+C content of the genome was determined by using the thermal denaturation method (Evolution 300 UV-VIS, Thermo Scientific) [26, 27].

Genomic DNA extraction, PCR amplification and sequencing of the 16S rRNA gene were performed using the methods described previously [28, 29]. Based on the 16S rRNA gene sequence data, multiple alignments and calculations of sequence similarity were carried out by using the EzTaxon-e server (www.ezbiocloud.net/) [30]. Phylogenetic trees were reconstructed with neighbour-joining [31], maximum-likelihood [32] and maximum-parsimony algorithms by using MEGA software version 7.0.14 [33]. The complete deletion option was chosen for elimination of gaps and missing data from the dataset, and bootstrap analysis with 1000 replicates was conducted for stability evaluation of the tree topology [34]. DNA–DNA hybridization was prepared according to the methods described by Marmur [35], and determined following the thermal renaturation method [36].

Morphological observation revealed that strain DHS C021T showed the typical characteristics of the genus Lentzea. Strain DHS C021T was aerobic and Gram-stain-positive. Colonies of strain DHS C021T grown on ISP 2 medium agar for 14 days produced sparse substrate mycelia and branched aerial hyphae that developed into rod-shaped elements (Fig. 1). It showed better growth on all tested media than the two type strains. All of the strains had no diffusible pigments on any tested media. The colours of the aerial hyphae of strain DHS C021T varied from light grey to yellowish white according to the media used, whereas substrate mycelia were from slight yellow to yellowish dark grey (Fig. S1, available in the online Supplementary Material). In particular, the substrate mycelia of strain DHS C021T was a bit too dark on all tested media, compared to the two type strains. The temperature range for growth of strain DHS C021T was between 15 and 37 °C (optimum temperature 28 °C). Above 45 °C, strain DHS C021T, Lentzea albida CGMCC 4.1727T and Lentzea waywayandensis CGMCC 4.1646T showed no growth, and only L. albida CGMCC 4.1727T had imperceptible growth at 10 °C. The strain grew at pH 6–10 (optimum pH 7–8), and was able to tolerate up to 5% NaCl. Tween 80, casein and starch were hydrolysed or decomposed by the three strains. Details of other physiological and biochemical properties are given in Table 1 and the species description.

Whole-cell hydrolysates of strain DHS C021T contained meso-DAP, but no characteristic amino acids. The whole-cell hydrolysates contained galactose, ribose and a trace of

![Fig. 1. Scanning electron microscopy of strain DHS C021T grown on ISP 2 agar medium for 14 days at 28 °C. Bar, 10 µm.](image-url)
mannose. The predominant menaquinones detected were MK-9(H_{4}) (95.3 %) and MK-8(H_{4}) (4.7 %). The polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, hydroxyl-phosphatidyethanolamine, phosphatidylglycerol, phosphatidylglycerolmannosides and one unidentified phospholipid. In conclusion, strain DHS C021\textsuperscript{T} formed a clade with \textit{L. albida} CGMCC 4.1727\textsuperscript{T}, distinct from other closely related species of the genus \textit{Lentzea} (Fig. 2). The distinction was supported by all tree-making methods used in the study (Figs 2, S3 and S4). Based on the sequence similarities of the 16S rRNA genes and phylogenetic analysis, strains \textit{L. albida} CGMCC 4.1727\textsuperscript{T} and \textit{L. waywayandensis} CGMCC 4.1646\textsuperscript{T} were selected as reference strains for polyphasic taxonomy and DNA–DNA hybridization studies. The mean DNA–DNA relatedness between strain DHS C021\textsuperscript{T} and \textit{L. albida} CGMCC 4.1727\textsuperscript{T} and \textit{L. waywayandensis} CGMCC 4.1646\textsuperscript{T} were 54.5±2.7 and 41.7±3.2 %, respectively, which were lower than the threshold value (70 %) recommended for distinguishing novel species [37].

As described above, strain DHS C021\textsuperscript{T} exhibited typical characteristics of the genus \textit{Lentzea}, including well-differentiated aerial hyphae that fragment into rod-shaped elements, a diagnostic cell-wall amino acid (\textit{meso}-diaminopimelic acid) and sugars (galactose, mannose and ribose), and MK-9(H_{4}) as the predominant menaquinone. However, strain DHS C021\textsuperscript{T} could be distinguished from its closest phylogenetic neighbours, \textit{L. albida} CGMCC 4.1727\textsuperscript{T} and \textit{L. waywayandensis} CGMCC 4.1646\textsuperscript{T}, by several properties. Substrate mycelia of strain DHS C021\textsuperscript{T} varied from slight yellow to yellowish brown or dark grey–reddish brown in the tested media, which is very different to its close phylogenetic neighbours (Fig. S1). The new strain also had a different fatty acid profile (Table S1). Phosphatidylethanolamine was the diagnostic phospholipid for \textit{L. waywayandensis} CGMCC 4.1646\textsuperscript{T} [38]. However, strain DHS C021\textsuperscript{T} had more phospholipids, including diphosphatidylglycerol, phosphatidylethanolamine, hydroxyl-phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositolmannosides and one unidentified phospholipid. In conclusion, strain DHS C021\textsuperscript{T} can be clearly differentiated from its closest phylogenetic neighbours by phenotypic and phylogenetic data, and strain DHS C021\textsuperscript{T}, therefore, is considered to represent a novel species of the genus \textit{Lentzea}, for which the name \textit{Lentzea pudingi} sp. nov. is proposed.

**DESCRIPTION OF LENTZEA PUDINGI SP. NOV.**

\textit{Lentzea pudingi} (pu.ding'i. N.L. masc. gen. \textit{pudingi} of Puding Karst Ecosystem Research Station of Guizhou Province, southwest China, referring to the source of the isolate).

Aerobic, Gram-stain-positive, non-motile actinomycete. Branched vegetative mycelium develops well; a mature aerial mycelium develops into rod-shaped spore chains. Grows well on ISP 2, ISP 3, ISP 4, ISP 5, Czapek solution agar, nutrient agar and PDA. Diffusible pigment is not observed on these tested media. The colours of the aerial mycelium and substrate mycelium on ISP 2 agar medium are light grey and slightly yellow, respectively. Growth occurs at 15–37 °C (optimum at 28 °C) at pH 6–10 (optimum pH 7–8) and in the presence of up to 5 % NaCl (w/v) (optimum 0 %). D-Arabino, lactose, raffinose, D-rhamnose and xylitol can be utilized as sole carbon sources for

### Table 1. Differential characteristics of strain DHS C021\textsuperscript{T} and type strains of related species of genus \textit{Lentzea}

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Strain 1</th>
<th>Strain 2</th>
<th>Strain 3</th>
</tr>
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<tbody>
<tr>
<td>Isolation source</td>
<td>Limestone</td>
<td>Soil</td>
<td>Soil</td>
</tr>
<tr>
<td>Nitrate reductase</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Degradation of:</td>
<td></td>
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<td></td>
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<tr>
<td>Urea</td>
<td>w</td>
<td>w</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Xanthine</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Conditions for growth</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>6–10</td>
<td>5–10</td>
<td>5–10</td>
</tr>
<tr>
<td>Temperatures °C</td>
<td>15–37</td>
<td>10–37</td>
<td>15–37</td>
</tr>
<tr>
<td>NaCl tolerance, %</td>
<td>0–5</td>
<td>0–3</td>
<td>0–2</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-Mannose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Raffinose</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>d-Rhamnose</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>d-Ribose</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Xylitol</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Acid production from:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-Arabinose</td>
<td>w</td>
<td>–</td>
<td>w</td>
</tr>
<tr>
<td>d-Mannose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Raffinose</td>
<td>w</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>d-Rhamnose</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Major fatty acids (&gt;10 %)</td>
<td>\textit{Iso-C_{14}:0}</td>
<td>\textit{Iso-C_{15}:0}</td>
<td>\textit{Iso-C_{14}:0}</td>
</tr>
<tr>
<td>DNA G+C content (mol %)</td>
<td>69.8</td>
<td>ND</td>
<td>71*</td>
</tr>
</tbody>
</table>

*Data from Labeda et al. [38].
growth, but not D-galactose, D-mannose, D-ribose, D-sorbitol. L-Histidine, L-glutamic acid and L-valine can be used as sole nitrogen sources for growth, but not L-lysine. Catalase is positive. Nitrate can be reduced. Hydrolyses casein, starch and Tween 80. Urea is weakly degraded. It can peptonize milk and fails to produce H₂S. Whole-cell hydrolysates contain meso-DAP as the diagnostic cell-wall amino acid, and galactose, mannose, ribose as diagnostic sugars. The predominant menaquinones detected is MK-9 (H₄). The polar lipids are diphosphatidylglycerol,
phosphatidylethanolamine, hydroxyl-phosphatidylethanolamine, phosphatidylinositol, phosphotidylinositolmannosides and one unidentified phospholipid. The major fatty acids are iso-C₁₄:₀ and iso-C₁₆:₀.

The type strain, DHS C021^T (=CGMCC 4.7319^T=KCTC 39694^T), was isolated from a limestone sample collected in the Puding Karst Ecosystem Research Station of Guizhou Province, southwest China. The G+C content of the DNA of the type strain is 69.8 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References


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