Pseudoclavibacter endophyticus sp. nov., isolated from roots of Glycyrrhiza uralensis

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A Gram-stain-positive, aerobic, rod-shaped, non-motile actinomycete strain, designated EGI 60007T, was isolated from healthy roots of Glycyrrhiza uralensis F. collected from Yili County, Xinjiang Province, north-west China. A polyphasic approach was applied to study the taxonomic position of the new isolate. The 16S rRNA gene sequence of strain EGI 60007T had highest similarities with members of the genus Pseudoclavibacter, including Pseudoclavibacter chungangensis CAU 59T (96.98% 16S rRNA gene sequence similarity), Pseudoclavibacter helvolus DSM 20419T (96.43%) and Pseudoclavibacter terrae THG-MD12T (96.14%). The neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showed that strain EGI 60007T clustered with members of the genus Pseudoclavibacter, and formed a distinct clade with P. chungangensis CAU 59T. The polar lipids detected for strain EGI 60007T were phosphatidylglycerol, diphosphatidylglycerol, one unidentified glycolipid and one unidentified lipid. The DNA G+C content was determined to be 63.3 mol%. The chemotaxonomic features of strain EGI 60007T showed typical characteristics of the genus Pseudoclavibacter, with MK-9 as the respiratory quinone, 2,4-diaminobutyric acid as the diamino acid in the peptidoglycan, and anteiso-C15 : 0, anteiso-C17 : 0 and iso-C16 : 0 as the major fatty acids. The sugars of whole-cell hydrolysates were mainly mannose, rhamnose, ribose and glucose, and a minor amount of xylose. Based on the results of the phylogenetic analysis supported by morphological, physiological, chemotaxonomic and other differentiating phenotypic characteristics, strain EGI 60007T is considered to represent a novel species of the genus Pseudoclavibacter, for which the name Pseudoclavibacter endophyticus sp. nov. is proposed. The type strain is EGI 60007T ( = CGMCC 1.15081T = KCTC 39112T = DSM 29943T).

The genus Pseudoclavibacter (family Microbacteriaceae, class Actinobacteria) was first described by Manaia et al. (2004) with the reclassification of ‘Brevibacterium helvolum’ DSM 20419 as Pseudoclavibacter helvolus, the type species for the genus. At the time of writing, the genus comprises five species with validly published names (http://www.bacterio.net/pseudoclavibacter.html; Parte 2014): Pseudoclavibacter helvolus (Manaia et al., 2004), Pseudoclavibacter soli (Kim & Jung, 2009), Pseudoclavibacter chungangensis (Cho et al., 2010), Pseudoclavibacter caeni (Srinivasan et al., 2012) and Pseudoclavibacter terrae (Du et al., 2015). Members of the genus Pseudoclavibacter are Gram-stain-positive, rod-shaped, catalase-positive and have chemotaxonomic features comprising of a B2c-type peptidoglycan, MK-9 as the predominant respiratory quinone, anteiso-C15 : 0, anteiso-C17 : 0 and iso-C16 : 0 as major fatty acids, and phosphatidylglycerol (PG), diphosphatidylglycerol (DPG) and glycolipid as major polar lipids (Manaia et al., 2004; Cho et al., 2010). During an investigation of the diversity of cultivable endophytic bacteria from

†These authors contributed equally to this work.

Abbreviations: DPG, diphosphatidylglycerol; PG, phosphatidylglycerol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain EGI 60007T is KP721484.

A supplementary table and three supplementary figure are available with the online Supplementary Material.
Glycyrrhiza uralensis F., a novel actinobacterial strain, EGI 60007T, belonging to the genus Pseudoclavibacter was isolated.

Healthy roots samples of the plant Glycyrrhiza uralensis F. were collected from Yili County, Xinjiang Province, northwest China (43.39° N 83.84° E). The roots were washed thoroughly with running tap water to remove adhering soil particles, followed by surface-sterilization as described by Qin et al. (2009). The surface-sterilized root samples were then sliced into small pieces, and plated on Reasoner's 2A agar (R2A) medium (Difco). Plates were incubated at 30 °C. Cells were grown on R2A medium for 4 days at 30 °C and main-
tained on R2A agar slants at 4 °C. The strain grew well on GYEA, TSA and LB media. Strain EGI 60007T was found to grow in 3 % (v/v) H2O2, and oxidase activity was determined using 1 % (w/v) tetramethyl-ß-phenylenedia-
mine (Cappuccino & Sherman, 2002). Other enzyme activities and biochemical characteristics were determined using API ZYM, API50CH/B and API 20 E kits (bioMérieux) according to the manufacturer’s instructions. Carbon source utilization and antibiotic susceptibility tests were tested using GEN III microplates from the Microlog system (Biolog) according to the manufacturer’s instructions.

Strain EGI 60007T was Gram-stain-positive. Transmission electron microscopy showed that strain EGI 60007T was rod-shaped and measured 0.4–0.6 μm in width and 1.1–2.3 μm in length (Fig. 1). Colonies of strain EGI 60007T were opaque, white and circular on R2A medium after cul-
tivation for 7 days at 30 °C. The strain grew well on GYE,
TSA and LB media. Strain EGI 60007T was found to grow at pH 7.0–9.0 (optimum pH 8.0), at 10–60 °C (optimum 25–35 °C) and in the presence of 0–11 % (w/v) NaCl (opti-
mum 4 % NaCl). The strain was catalase-positive, but ox-
dase-negative. Comparative physiological properties of strain EGI 60007T and the most closely related type strains Pseudoclavibacter chungangensis CAU 59T and Pseudoclavi-
bacter helvolus DSM 20419T are shown in Table 1.

The biomass for fatty acid analysis of strain EGI 60007T, P. chungangensis CAU 59T and P. helvolus DSM 20419T were harvested from cells grown on TSA at 30 °C until late exponential phase. Fatty acid methyl esters were prepared according to the method described by Sasser (1990) and the manufacturer’s instructions, and analysed by GC (7890A GC System; Agilent Technologies) using the Microbial Identification software package (Sherlock version 6.1; MIDI database TSBA6). Polar lipids were extracted (Minnikin et al., 1979) and identified by two-
dimensional TLC (Collins & Jones, 1980). Menaquinones were extracted and analysed by HPLC as described by Col-

lins et al. (1977) and Kroppenstedt (1982). Purified cell-
wall preparation and hydrolysis were done according to method of Schleifer & Kandler (1972). Amino acids in cell-wall hydrolysates were analysed by HPLC as described by Tang et al. (2009). The sugars of whole-cell hydrolysates were determined according to the procedures described by Lechevalier & Lechevalier (1970). The G + C content of the genomic DNA was determined as described by Mesbah et al. (1989) using reversed-phase HPLC.
Table 1. Comparison of phenotypic and biochemical characteristics of strain EGI 60007<sup>T</sup> and type strains of closely related species of the genus *Pseudoclavibacter*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony pigmentation on GYEA</td>
<td>White</td>
<td>Yellow</td>
<td>Light yellow</td>
</tr>
<tr>
<td>Cell size (length x width, μm)</td>
<td>1.1–2.3 × 0.4–0.6</td>
<td>1.3–1.7 × 0.1–0.2*</td>
<td>0.9–1.1 × 0.4–0.5†</td>
</tr>
<tr>
<td>Temperature range for growth (°C)</td>
<td>10–60</td>
<td>4–50*</td>
<td>10–35†</td>
</tr>
<tr>
<td>NaCl range (w/v, %)</td>
<td>0–11</td>
<td>0–15*</td>
<td>1–8†</td>
</tr>
<tr>
<td>pH range for growth</td>
<td>7–9</td>
<td>3–11*</td>
<td>6–10†</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>63.3</td>
<td>66.2*</td>
<td>67†</td>
</tr>
<tr>
<td>Polar lipids</td>
<td>DPG, PG, GL, L</td>
<td>DPG, PG, GL</td>
<td>DPG, PG, GL</td>
</tr>
<tr>
<td>Sugars of whole-cell hydrolysates</td>
<td>Man, Rha, Rib, Glu and a minor amount of Xyl</td>
<td>Man, Rha, Rib, Glu and a minor amount of Xyl</td>
<td>Man and minor quantities of Rha, Rib, Glu, Xyl and Fuc</td>
</tr>
<tr>
<td>Enzyme activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidase</td>
<td>–</td>
<td>–</td>
<td>W†</td>
</tr>
<tr>
<td>Gelatinase</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>–</td>
<td>W</td>
<td>+</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Acid production from:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Inositol</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Melibiose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Arabinose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Glycerol</td>
<td>–</td>
<td>W</td>
<td>+</td>
</tr>
<tr>
<td>d-Xylose</td>
<td>–</td>
<td>W</td>
<td>–</td>
</tr>
<tr>
<td>d-Fructose</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>d-Mannose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Aesculin</td>
<td>+</td>
<td>W</td>
<td>+</td>
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<tr>
<td>Growth on sole carbon source (Biolog GEN III)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-d-Glucosamine</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>d-Mannitol</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>myo-Inositol</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Glycerol</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>d-Glucose 6-phosphate</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>d-Fructose 6-phosphate</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>l-Aspartic acid</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>l-Glutamic acid</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pectin</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>d-Gluconic acid</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>p-Hydroxyphenylacetic acid</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Methyl pyruvate</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>l-Lactic acid</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Citric acid</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>α-Ketoglutaric acid</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>d-Malic acid</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>l-Malic acid</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>α-Hydroxybutyric acid</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>β-Hydroxy-DL-butyric acid</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>α-Ketobutyric acid</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Acetoacetic acid</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>
The fatty acid methyl ester profile (≥1 %) of strain EGI 60007T was found to contain anteiso-C15:0 (37.67 %), anteiso-C17:0 (34.08 %), iso-C16:0 (19.61 %), iso-C15:0 (3.37 %), C16:0 (2.13 %) and iso-C17:0 (1.93 %). The complete fatty acid profiles of strain EGI 60007T and closely related type strains are given in Table S1 (available in the online Supplementary Material). Two-dimensional TLC analysis of polar lipids extracted from strain EGI 60007T.

Table 1. cont.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Formic acid</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Strains: 1, EGI 60007T; 2, *P. chungangensis* CAU59T; 3, *P. helvolus* DSM 20419T. All data are from this study unless otherwise indicated. All strains were negative for urease activity and positive for indole production, catalase activity and growth on D-fructose as a sole carbon source. +, Positive; −, negative; w, weakly positive; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; GL, unidentified glycolipid; L, unidentified lipid. Man, mannose; Rha, rhamnose; Rib, ribose; Glu, glucose; Xyl, xylose; Fuc, fucose. *Data from Cho et al. (2010). †Data from Manaia et al. (2004).

The fatty acid methyl ester profile (≥1 %) of strain EGI 60007T was found to contain anteiso-C15:0 (37.67 %), anteiso-C17:0 (34.08 %), iso-C16:0 (19.61 %), iso-C15:0 (3.37 %), C16:0 (2.13 %) and iso-C17:0 (1.93 %). The complete fatty acid profiles of strain EGI 60007T and closely related type strains are given in Table S1 (available in the online Supplementary Material). Two-dimensional TLC analysis of polar lipids extracted from strain EGI 60007T.

**Fig. 2.** Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of strain EGI 60007T and close relatives. Bootstrap values (expressed as percentages of 1000 replications) >50 % are shown at the branch points. Asterisks denote nodes that were also recovered using the maximum-parsimony and maximum-likelihood methods. The sequence of *Micrococcus luteus* NCTC 2665T (GenBank accession no. CP001628) was used as the outgroup. Bar, 0.005 substitutions per nucleotide position.
(Fig. S1) showed that the isolate contained PG, DPG and one unidentified glycolipid similar to other species of the genus Pseudoclavibacter. Strain EGI 60007T contained an additional unidentified lipid, which was absent in the type strains *P. chungangensis* CAU 59T and *P. helvulus* DSM 20419T. The respiratory quinone was MK-9, which is in accordance with members of the genus *Pseudoclavibacter* (Manaia et al., 2004; Cho et al., 2010). The diamino acid in the peptidoglycan of strain EGI 60007T was 2,4-diaminobutyric acid, while alanine, glycine and glutamate were also detected. Although the peptidoglycan contained 2,4-diaminobutyric acid, the ratio of the amino acids were different from the other species of the genus (molar ratio, 1.5:2.5:2.7:2.6). The sugars of whole-cell hydrolysates were mainly mannose, rhamnose, ribose and glucose, and a minor amount of xylose. The DNA G+C content of strain EGI 60007T was 63.3 mol%.

Extraction of genomic DNAs and PCR amplification of 16S rRNA genes were performed as described by Li et al. (2007). The sequences obtained were compared with available 16S rRNA gene sequences of species with validly published names from the EzTaxon-e server (http://www.ezbiocloud.net/eztaxon; Kim et al., 2012). Multiple alignments with sequences of the most closely related taxa and calculations of sequence similarity values were carried out using the CLUSTAL X 2.1 program (Larkin et al., 2007). Phylogenetic analyses were performed by three tree-making algorithms: neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971). Trees were reconstructed by using the MEGA version 6.0 software package (Tamura et al., 2013). Kimura’s two-parameter model was used to calculate evolutionary distance matrices of the phylogenetic trees (Kimura, 1980). Bootstrap analysis was performed with 1000 replications (Felsenstein, 1985). The phylogenetic trees were rooted using *Micrococcus luteus* NCTC 2665T as an outgroup.

Analysis of the almost-complete 16S rRNA gene sequence of strain EGI 60007T (1490 bp; GenBank accession no. KP721484) using the EzTaxon server database revealed highest similarities to strains belonging to the genus *Pseudoclavibacter*, including *P. chungangensis* CAU 59T (96.98 % 16S rRNA gene sequence similarity), *P. helvulus* DSM 20419T (96.43 %) and *P. terrae* THG-MD12T (96.14 %); lower 16S rRNA gene sequence similarities were observed with other genera. The neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showed strain EGI 60007T formed a distinct clade with *P. chungangensis* CAU 59T (Fig. 2). The stability of the neighbour-joining tree was further supported by maximum-likelihood and maximum-parsimony trees (Figs S2 and S3).

On the basis of the phylogenetic analysis and the differences in the phenotypic characteristics (Tables 1 and S1), strain EGI 60007T is considered to represent a novel species of the genus *Pseudoclavibacter*, for which the name *Pseudoclavibacter endophyticus* sp. nov. is proposed.

**Description of Pseudoclavibacter endophyticus sp. nov.**

*Pseudoclavibacter endophyticus* (en.do.phy’ti.cus. Gr. pref. endo- within; Gr. n. phytон plant; L. fem. suff. -icus adjectival suffix used with the sense of belonging to; N.L. masc. endophyticus within plant, endophytic).

Cells are Gram-stain-positive, aerobic and rod-shaped, measuring 0.4–0.6 μm in width and 1.1–2.3 μm in length. Forms white, opaque and circular colonies on R2A after incubation for 7 days at 30 °C. Grows well on LB, TSA and GYE medium. Growth occurs at 10–60 °C and pH 7.0–9.0, with optimum growth at 25–35 °C and pH 8.0. Growth occurs in the presence of up to 11% (w/v) NaCl (optimum 4%). Positive for catalase activity, but negative for oxidase and urease activities. Produces indole, but does not hydrolyse gelatin. In the API ZYM system, positive for esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase, chymotrypsin and β-glucosidase activities, but negative for lipase (C14), alkaline phosphatase, acid phosphatase, trypsin, z-galactosidase, β-galactosidase, β-glucuronidase, z-glucosidase, β-fucosidase z-mannosidase and N-acetyl-β-glucosaminidase activities. In the API 50CH/B system, only aesculin is fermented. In the Biolog GEN III system, d-fructose, d-mannitol, l-aspartic acid, l-glutamic acid, p-hydroxyphenylacetic acid, β-hydroxy-DL-butyrionic acid, acetoacetic acid, acetic acid and formic acid are utilized. Positive for tolerance of 1, 4 and 8% NaCl, pH 6, 1% sodium lactate, trehaloseomyacin, lincomycin, tetrazolium violet, nalidixic acid, potassium tellurite, aztreonam, sodium butyrate and sodium bromate, but negative in the remaining tests. The respiratory quinone is MK-9. The major fatty acids (>5 %) are anteiso-C15:0, anteiso-C17:0 and iso-C16:0. The polar lipids consist of DPG, PG, an unidentified glycolipid and an unidentified lipid. The diamino acid in the peptidoglycan is 2,4-diaminobutyric acid, while alanine, glycine and glutamate are primary amino acids in the cell-wall hydrolysates. The sugars of whole-cell hydrolysates are mainly mannose, rhamnose, ribose and glucose, and a minor amount of xylose.

The type strain EGI 60007T (= CGMCC 1.15081T = KCTC 39112T = DSM 29943T) was isolated from healthy roots of *Glycyrrhiza uralensis* F. collected from Yili County, Xinjiang Province, north-west China. The G+C content of the genomic DNA of the type strain is 63.3 mol%.

**Acknowledgements**

We are grateful to Dr Rüdiger Pukall (DSMZ, Germany) and Miss Sang-Mi Lee (KCTC, Korea) for providing the reference type strains. This research was supported by the National Natural Science Foundation of China (no. 31200008), projects of China Tobacco Yunnan Industrial Co. Ltd (nos 2014YL01 and 2015CP01), the Hundred Talents Program of Chinese Academy of Sciences, the High-level Talents Program of Xinjiang Autonomous Region and the West Light Foundation of Chinese Academy of Sciences. W.-J. L. was also...
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References


