Sphingobacterium griseoflavum sp. nov., isolated from the insect Teleogryllus occipitalis living in deserted cropland

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A Gram-stain-negative, aerobic, rod-shaped, non-motile bacterial strain, designated SCU-B140T, was isolated from the insect Teleogryllus occipitalis. Phylogenetic analysis on the basis of 16S rRNA gene sequence showed that strain SCU-B140T belonged to the genus Sphingobacterium. Sphingobacterium bambusae KCTC 22814T (97.87 %) was identified as the most closely related phylogenetic neighbour of strain SCU-B140T. The novel strain was able to grow at salt concentrations of 0–4 % (w/v), at temperatures of 10–40 °C, and at a pH of 6.0–9.0. The major cellular fatty acids were iso-C15 : 0, summed feature 3 (iso-C15 : 02-OH and/or C16 : 1ω7c), C16 : 0, C16 : 0 3-OH, C18 : 0 and C14 : 0. The major polar lipids consisted of phosphatidylethanolamine, three unknown aminophospholipids, an unknown glycolipid and three unknown polar lipids. MK-7 was the major isoprenoid quinone. The DNA G + C content was 41.2 mol%. The DNA–DNA relatedness value between SCU-B140T and S. bambusae KCTC 22814T was found to be 30.15 %. According to these results, strain SCU-B140T represents a novel species of the genus Sphingobacterium, for which the name Sphingobacterium griseoflavum sp. nov. is proposed. The type strain is SCU-B140T (=KCTC 42158T=CGMCC 1.12966T).

The genus Sphingobacterium comprises Gram-stain-negative, non-spore-forming, straight rod-shaped bacteria containing high quantities of sphingophospholipids in their cells. This genus was first described by Yabuuchi et al. (1983) with the description of Sphingobacterium spiritivorum, S. multivorum and S. mizutaii. The descriptions of the genus and S. mizutaii were amended by Wauters et al. (2012). Members of the genus Sphingobacterium have been isolated from clinical materials, raw milk, lichen, fresh leaves, water, soil, compost, activated sludge and soybean plants. Currently, the genus Sphingobacterium includes 32 species with validly published names: S. multivorum, S. mizutaii and the type species, S. spiritivorum (Yabuuchi et al., 1983); S. faecium and S. thalphophilum (Takeuchi &Yokota, 1992); S. daejeonense (Kim et al., 2006); S. composti (Ten et al., 2006); S. canadense (Mehnaz et al., 2007); S. siyangense (Liu et al., 2008); S. kitahiroshimense (Matuyama et al., 2008); S. anhuiense (Wei et al., 2008); S. bambusae (Duan et al., 2009); S. shyense (He et al., 2010); S. kyonggiense (Choi & Lee, 2012); S. alimentarium and S. lactis (Schmidt et al., 2012); S. detergens (Marquès et al., 2012); S. nematocida (Liu et al., 2012); S. wenxiniaie (Zhang et al., 2012); S. caeni (Sun et al., 2013); S. changzhhouense (Liu et al., 2013); S. cladoniae (Lee et al., 2013); S. hotanense (Xiao et al., 2013); S. psychroaquaticum (Albert et al., 2013); S. thermophilum (Yabe et al., 2013); S. arenai (Jiang et al., 2014); S. ginsenosidimutans (Son et al., 2013); S. pakistanense (Ahmed et al., 2014); S. yanglingense (Peng et al., 2014);
We isolated a novel strain, SCU-B140\textsuperscript{T}, from the insect *Teleogryllus occipitalis*, captured from a deserted cropland in Chengdu, China (30° 33’N 103° 58’E; altitude 495 m). The insect was crushed and then put on tryptose soya agar (TSA) medium [1.5 % (w/v) tryptone, 0.5 % (w/v) soya peptone, 0.5 % (w/v) NaCl, 1.5 % (w/v) agar, pH adjusted to pH 7.2 by HCl/NaOH] at 30 °C for 1 week. Single colonies on the plates were purified by transfer onto fresh plates and subsequent reincubation. Strain SCU-B140\textsuperscript{T} was thus obtained, preserved in a glycerol suspension (20 %, v/v) and maintained at −80 °C.

Genomic DNA of SCU-B140\textsuperscript{T} was extracted as described by Li *et al.* (2007). PCR amplification of the 16S rRNA gene was performed with the primers 27F (5’-GAGTTTTGATCCTGGTCTAG-3’) and 1492R (5’-ACGGCTACCTGTATCGACTT-3’). Sequencing reactions were performed by Shanghai Sangon (Shanghai, China) using the dideoxy chain-termination method with an ABI 3730XL (Applied Biosystems). We obtained an almost-complete 16S rRNA gene sequence (1494 bp). We used NCBI’s BLAST search (www.blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul *et al.*, 1990) and the EzTaxon-e server (www.eztaxon-e.ezbiocloud.net; Kim *et al.*, 2012) to identify phylogenetic neighbours and calculate pairwise sequence similarities. *S. bambusae* KCTC 22814\textsuperscript{T}, *S. lactis* DSM 22361\textsuperscript{T} and *S. paludis* CGMCC 1.12801\textsuperscript{T} exhibited the greatest similarity to SCU-B140\textsuperscript{T} (97.87, 95.7 and 95.25 % identity, respectively). We used MEGA 5.2 (Tamura *et al.*, 2011) to reconstruct phylogenetic trees based on maximum-likelihood (Felsenstein, 1981), neighbour-joining (Saitou & Nei, 1987) and minimum-evolution (Rzhetsky & Nei, 1992) models with bootstrap values under 1000 replications (Felsenstein, 1985). Calculation of evolutionary distances utilized Kimura’s two-parameter model (Kimura, 1980, 1983). The maximum-likelihood tree demonstrated that strain SCU-B140\textsuperscript{T} belongs to the genus *Sphingobacterium* and forms a cluster with *S. bambusae* KCTC 22814\textsuperscript{T} with a high bootstrap value (98 %) (Fig. 1). Although strain *S. lactis* DSM 22361\textsuperscript{T} showed 95.76 % similarity to SCU-B140\textsuperscript{T}, these strains were distributed in different clades in the maximum-likelihood trees. The neighbour-joining and minimum-evolution trees showed essentially the same topology. In conclusion, *S. bambusae* KCTC 22814\textsuperscript{T} and *S. paludis* CGMCC 1.12801\textsuperscript{T} were chosen as the reference strains.

DNA–DNA hybridization is a commonly used method for defining separate bacterial species (Wayne *et al.*, 1987). According to Wayne *et al.* (1987), a relatedness value below 70 % indicates that the two samples represent different species. DNA–DNA hybridization was performed between SCU-B140\textsuperscript{T} and *S. bambusae* KCTC 22814\textsuperscript{T} with six replications using the microplate method as described by Ezaki *et al.* (1989). The mean DNA–DNA relatedness values were 30.15 ± 0.4 % (SCU-B140\textsuperscript{T} × *S. bambusae* KCTC, 22814\textsuperscript{T}) and 30.81 ± 1.0 % (*S. bambusae* KCTC 22814\textsuperscript{T} × SCU-B140\textsuperscript{T}). As these are well below the cut-off of 70 %, we are confident that these two strains indeed belong to two different species. The G+C content of the DNA for SCU-B140\textsuperscript{T} was determined by HPLC (Mesbah *et al.*, 1989) and was found to be 41.2 mol%, which is within the range for other members of the genus *Sphingobacterium*.

We then performed phenotypic and chemotaxonomic analyses of strain SCU-B140\textsuperscript{T}. For this part of the study, cells were cultured in tryptose soya broth (TSB) medium [1.5 % (w/v) tryptone, 0.5 % (w/v) soya peptone, 0.5 % (w/v) NaCl, pH adjusted to 7.2 by HCl/NaOH]. We observed cell morphology and size under a scanning electron microscope (JSM-7500F, JEOL) using cells in the exponential growth phase (Fig. S1 available in the online Supplementary Material). Gram staining was performed as described by Smibert & Krieg (1994). Motility was examined by stab-culture in semi-solid medium as described by Gerhardt *et al.* (1981). Salt tolerance tests were performed in basal medium [1.5 % (w/v) tryptone, 0.5 % (w/v) soya peptone, pH adjusted to 7.2 by HCl/NaOH] using different concentrations of NaCl (0, 0.1, 0.5, 1, 2, 4, 6, 8, 10 and 15 %, w/v). The optimal pH and temperature for growth of SCU-B140\textsuperscript{T} were determined by incubating the strain in TSB medium at different pH levels (pH 2.0, 4.0, 6.0, 7.0, 7.2, 7.5, 8.0, 9.0, 10.0 and 12.0; glycine/HCl buffer was used for pH 2.0, Na2HPO4/citric acid buffer was used for pH 4.0–8.0, glycine/NaOH buffer was used for pH 9.0–10.0 and Na2HPO4/NaOH buffer was used for pH 12.0) and temperatures (5, 10, 15, 25, 28, 30, 37, 40, 45 and 50 °C). Bacterial concentration was measured at 600 nm using an UV–vis spectrophotometer. Tests for the ability to reduce nitrate and to hydrolyse gelatin, starch, Tween 20, Tween 80, cellulose, and urea were carried out according to methods previously described by Cappuccino & Sherman (2008). Catalase ability was tested with 3 % H2O2, and oxidase ability was determined using tetramethyl-p-phenylenediamine dihydrochloride according to methods described by Barrow & Feltham (2004). Other enzyme activities were assayed using the API ZYM and API 20NE systems. Other biochemical tests were determined using a Biolog GN2 microplate according to the manufacturer’s instructions. Although strain SCU-B140\textsuperscript{T} shared many phenotypic features with the closely related taxa, *S. bambusae* KCTC 22814\textsuperscript{T} and *S. paludis* CGMCC 1.12801\textsuperscript{T}, there were some differences between them. Biolog GN2 results showed positive results for glycerol, D-galactose, L-rhamnose, L-arabinose, maltose and raffinose for strain SCU-B140\textsuperscript{T}, but negative results for *S. bambusae* KCTC 22814\textsuperscript{T}. For *S. bambusae* KCTC 22814\textsuperscript{T} and *S. paludis* CGMCC 1.12801\textsuperscript{T}, acid was produced from melibiose and L-rhamnose, but the opposite result was observed for SCU-B140\textsuperscript{T}. Phenotypic characteristics are summarized in the species description and a comparison of strain SCU-B140\textsuperscript{T} and related type strains is given in Table 1.
When determining the composition of fatty acids, SCU-B140\textsuperscript{T}, S. bambusae KCTC 22814\textsuperscript{T} and S. paludis CGMCC 1.12801\textsuperscript{T} were all assayed in order to examine differences between the novel strain and the most closely related species. The isolates were cultured under aerobic conditions on TSA medium at 30°C until the exponential growth phase according to the cell growth curve. Fatty acid methyl esters were prepared and identified with a MIDI Sherlock Microbial Identification System (Sherlock license CD version 6.1). Whole-cell fatty acid analysis revealed that the predominant fatty acids in SCU-B140\textsuperscript{T} are iso-C\textsubscript{15} : 0, summed feature 3 (iso-C\textsubscript{15} : 0 2-OH and/or C\textsubscript{16} : 1\textit{\textit{v}}\textsubscript{7}c), C\textsubscript{16} : 0, C\textsubscript{16} : 0 3-OH, C\textsubscript{18} : 0 and C\textsubscript{14} : 0. These results were in line with other members of the genus \textit{Sphingobacterium}; however, there were several differences in the proportions of some fatty acids (Table 2).

The polar lipids were extracted from 1 g freeze-dried cells using methanol/chloroform/saline extraction (2 : 1 : 0.8 ratio by vol.), as described by Kates et al. (1972). We separated and identified the polar lipids using two-dimensional chromatography on a silica gel TLC plate (10 x 10 cm), as previously described by Raj et al. (2013). For the presence of lipids, the following spraying reagents were used: molybdatophosphoric acid, ninhydrin, molybdenum blue and \alpha-naphthol. For SCU-B140\textsuperscript{T}, the polar lipids were extracted from 1 g freeze-dried cells using methanol/chloroform/saline extraction (2 : 1 : 0.8 ratio by vol.), as described by Kates et al. (1972). We separated and identified the polar lipids using two-dimensional chromatography on a silica gel TLC plate (10 x 10 cm), as previously described by Raj et al. (2013). For the presence of lipids, the following spraying reagents were used: molybdatophosphoric acid, ninhydrin, molybdenum blue and \alpha-naphthol. For SCU-B140\textsuperscript{T}, the polar lipids were extracted from 1 g freeze-dried cells using methanol/chloroform/saline extraction (2 : 1 : 0.8 ratio by vol.), as described by Kates et al. (1972). We separated and identified the polar lipids using two-dimensional chromatography on a silica gel TLC plate (10 x 10 cm), as previously described by Raj et al. (2013). For the presence of lipids, the following spraying reagents were used: molybdatophosphoric acid, ninhydrin, molybdenum blue and \alpha-naphthol.
Table 1. Differential phenotypic and chemotaxonomic characteristics of SCU-B140T and related strains

Strains: 1, SCU-B140T; 2, S. bambusae KCTC 22814T; 3, S. paludis CGMCC 1.12801T. All data were from this study except where indicated. All strains were positive for oxidase and catalase activities, and assimilation of D-glucose. All strains were negative for Gram-staining, motility, sporulation and assimilation of D-mannitol. +, Positive; −, negative; w, weakly positive.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<th>3</th>
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</thead>
<tbody>
<tr>
<td>Colour of colony</td>
<td>Greyish yellow</td>
<td>Yellow</td>
<td>Yellow</td>
</tr>
<tr>
<td>Biolog GN2 results</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>L-Rhamnose</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Melibiose</td>
<td>+</td>
<td>w*</td>
<td>+</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Raffinose</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Acid production from:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>+</td>
<td>w*</td>
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<tr>
<td>Melibiose</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Rhamnose</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>41.2</td>
<td>41.0†</td>
<td>43.8‡</td>
</tr>
</tbody>
</table>

* Some differences were observed between the literature and this study.
† Data from Duan et al. (2009).
‡ Data from Feng et al. (2014).

Table 2. Cellular fatty acid compositions comparison of strain SCU-B140T and related members of the genus Sphingobacterium

Strains: 1, SCU-B140T; 2, S. bambusae KCTC 22814T; 3, S. paludis CGMCC 1.12801T. All data were from this study and represent percentages of total fatty acids. Summed feature 3 contained C16:0 15c/15t, C18:1ω7c, and C18:2ω6c. TR, Trace amount (<0.1%).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
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</thead>
<tbody>
<tr>
<td>iso-C15:0</td>
<td>43.3</td>
<td>34.6</td>
<td>40.1</td>
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<tr>
<td>iso-C15:0</td>
<td>1.6</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>iso-C17:0</td>
<td>TR</td>
<td>6.8</td>
<td>9.6</td>
</tr>
<tr>
<td>C16:0 3-OH</td>
<td>6.6</td>
<td>10.9</td>
<td>2.9</td>
</tr>
<tr>
<td>C14:0</td>
<td>2.0</td>
<td>1.6</td>
<td>1.4</td>
</tr>
<tr>
<td>C16:0</td>
<td>7.1</td>
<td>3.7</td>
<td>3.9</td>
</tr>
<tr>
<td>C18:0</td>
<td>4.6</td>
<td>5.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Summed feature 3</td>
<td>36.3</td>
<td>34.2</td>
<td>36.4</td>
</tr>
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</table>

by TLC, and analysed by HPLC (Hiraishi & Hoshino, 1984) using the quinones of the reference type strains as standards. MK-7 was found to be the major quinone, in agreement with other members of the genus Sphingobacterium.

In conclusion, the characteristics of the novel species are consistent with the description of the genus Sphingobacterium according to morphological, biochemical and chemotaxonomic properties; however, there are several differences between SCU-B140T and other members of the genus Sphingobacterium. Phylogenetic and chemotaxonomic analyses demonstrate that strain SCU-B140T represents a novel species within the genus Sphingobacterium. Based on these results, we propose the name Sphingobacterium griseoflavum sp. nov. for this species.

Description of Sphingobacterium griseoflavum sp. nov.

Sphingobacterium griseoflavum (gri.se.o fla’vum. L. adj. griseus grey; L. neut. adj. flavum yellow; N.L. neut. adj. griseoflavum greyish yellow).

Cells are Gram-stain-negative, aerobic, rod-shaped (0.4–0.6 μm × 1.1–2.0 μm) and non-motile. Colonies grown on TSA medium are smooth, round and greyish-yellow in colour. Growth occurs at 10–40 °C (optimum 30 °C) and at pH 6.0–9.0 (optimum pH 7.2). The salt tolerant range for growth is 0–4 % (w/v) NaCl (optimum 1 %). Catalase and oxidase reactions are positive. Nitrate reductase, indole production, and hydrolysis of Tweens 20 and 80 are present. Cells are negative for urease, methyl red, Voges-Proskauer, H2S production test, and hydrolysis of gelatin and starch. Assimilates dextrin, Tween 40, Tween 80, N-acetyl-D-galactosamine, L-arabinose, cellobiose, D-fructose, D-galactose, gentiobiose, α-D-glucose, α-lactose, lactulose, maltose, D-mannose, melibiose, methyl β-D-glucoside, raffinose, L-rhamnose, sucrose, trehalose, turanose, acetic acid, glycyl-L-glutamic acid, L-serine and L-threonine, but not α-cyclodextrin, adonitol, L-erythritol, L-fucose, D-mannitol, D-sorbitol, xylitol, cis-aconitic acid, citric acid, D-galactonic acid lactone, D-glucosaminic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, β-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketoglutaric acid, DL-lactic acid, malonic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, succinic acid, glucuronamide, D-alanine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, DL-carnitine, γ-aminobutyric acid, urocanic acid, inosine, thymidine, phenylglyamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, α-DL-glyceraldehyde phosphate, glucose 1-phosphate or glucose 6-phosphate. Acids are produced from D-glucose, D-fructose, D-galactose, D-mannose, D-arabinose, L-arabinose, maltose, raffinose, cellobiose, D-ribose, D-sorbitol, trehalose, glyccogen, inositol, inulin and starch, but not from L-sorbose, L-rhamnose, dulcitol,
D-xyllose, L-xylitol, D-fucose, L-fucose, D-arabitol, L-arabitol, turanose or melibiose. The major cellular fatty acids are iso-C_{15:0}, summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:0} 3-OH), C_{16:0} 3-OH, C_{18:0} and C_{14:0}.

The major polar lipids consist of phosphatidylethanolamine, three unknown aminophospholipids, an unknown glycolipid and three unknown polar lipids. The major isoprenoid quinone is MK-7.

The type strain is SCU-B140\textsuperscript{T} (=KCTC 42158\textsuperscript{T}=CGMCC 1.12966\textsuperscript{T}), isolated from the insect *Teleogryllus occipitalis*, which was found living in deserted cropland in Chengdu, China. The DNA G+C content of the type strain is 41.2\%.

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**References**


