Burkholderia sprentiae sp. nov., isolated from Lebeckia ambigua root nodules

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Seven Gram-stain-negative, rod-shaped bacteria were isolated from Lebeckia ambigua root nodules and authenticated on this host. Based on the 16S rRNA gene phylogeny, they were shown to belong to the genus Burkholderia, with the representative strain WSM5005T being most closely related to Burkholderia tuberum (98.08 % sequence similarity). Additionally, these strains formed a distinct group in phylogenetic trees based on the housekeeping genes gyrB and recA. Chemotaxonomic data including fatty acid profiles and analysis of respiratory quinones supported the assignment of the strains to the genus Burkholderia. Results of DNA–DNA hybridizations, and physiological and biochemical tests allowed genotypic and phenotypic differentiation of our strains from the closest species of the genus Burkholderia with a validly published name. Therefore, these strains represent a novel species for which the name Burkholderia sprentiae sp. nov. (type strain WSM5005T = LMG 27175T = HAMBI 3357T) is proposed.

The south-west of Western Australia historically receives on average 250–500 mm annual rainfall. However, a decline in precipitation of 10% between 1976 and 1999, followed by an additional 15% in the subsequent seven years has been recorded (George et al., 2008), with the driest winter for 100 years recorded in 2010. Leguminous pasture species are important in Western Australian agriculture; nonetheless, due to these changes in rainfall patterns the reproduction from annual species is challenged. Perennial species might be more able to adapt to climate change, though few commercial perennial forage pasture species are important in Western Australian agriculture; nonetheless, due to these changes in rainfall patterns the reproduction from annual species is challenged. Perennial species might be more able to adapt to climate change, though few commercial perennial forage pasture systems exposed to a drying climate (Howieson et al., 2008), with the introduction of further dry years becoming recognized as N2-fixing occupants of legume root nodules (Gyaneshwar et al., 2011; Mishra et al., 2012). Currently, seven species of the genus Burkholderia, including Burkholderia diazotrophica (Sheu et al., 2013), Burkholderia mimosarum (Chen et al., 2006), Burkholderia nodosa (Chen et al., 2007), Burkholderia phymatum (Vandamme et al., 2002), Burkholderia sabiae (Chen et al., 2008), Burkholderia symbiotica (Sheu et al., 2012) and Burkholderia tuberum (Vandamme et al., 2002) have been confirmed to harbour nod and nif genes and to nodulate and fix nitrogen in symbiosis with legumes.

As part of a programme to develop robust perennial pasture legume symbioses for southern Australian agricultural systems exposed to a drying climate (Howieson et al.,
2013), root nodules of *Lebeckia ambiguha* plants growing in the Western Cape of South Africa were collected during four expeditions between 2002 and 2007 and stored as previously described (Yates *et al.*, 2004). Seven strains of root nodule bacteria were isolated from surface-sterilized *L. ambiguha* root nodules (Table S1, available in IJSEM Online) according to the methods of Yates *et al.* (2007). Strains WSM3617 and WSM3618 were isolated from two nodules from two plants collected on sampling site 10 in 2004; WSM4184–WSM4186 and WSM5005 came from two different nodules from two plants collected at site 11 in 2007; WSM4205 was isolated from one nodule collected at site 5 in 2007 (Table S1; Howieson *et al.*, 2013). Strain WSM50055 has been deposited in the BCCCM/LMG bacteria collection, Belgium (http://bcccm.belspo.be/) and in the HAMBI Culture Collection, University of Helsinki, Finland (http://www.helsinki.fi/hambi/). Additionally, strain WSM3617 has been deposited in the BCCCM/LMG bacteria collection as LMG 27178 and in the HAMBI Culture Collection as HAMBI 3358.

The strains were routinely subcultured on half-strength LA medium (Yates *et al.*, 2007) at 28 °C unless otherwise indicated. For PCR, genomic DNA of all isolates was prepared using the GE® method as described by Pitcher *et al.* (1989). (GTG)5-PCR analysis was performed as described by Gevers *et al.* (2001). The fingerprints were analysed using the BioNumerics 5.1 software package (Applied Maths). The similarity among the digitized profiles was calculated using the Pearson correlation coefficient (expressed for convenience as a percentage similarity value) and an UPGMA dendrogram was derived on LMG medium 14 (http://bccm.belspo.be/db/media_search_form.php) after 48 h. Strains were Gram stained. Cell morphology and motility were observed by phase-contrast microscopy. Oxidase activity was detected by immersion of cells in 1 % N,N,N’,N’-tetramethyl-p-phenylenediamine solution and catalase activity was determined by flooding a colony with 10 % H2O2 and checking for the presence of bubbles. Other biochemical tests were performed by inoculating API 20NE and API 20E strips (bioMérieux) according to the manufacturer’s instructions and incubating for 48 h at 28 °C. Growth was tested at 28 °C on LMG medium 14 and nutrient broth (NB; BD Difco) with 0 to 10 % NaCl and with pH 2–9, measured using an Orion 420A pH meter and adjusted with 35 % HCl or 5 M NaOH. Growth on half-strength LA medium (Yates *et al.*, 2007) was tested at 4, 10, 15, 21, 28, 30, 37 and 40 °C. The results of the phenotypic and biochemical tests are given in the species description and in Table 1. Most notably, the strains investigated in this study were positive for β-galactosidase, arginine dihydrolase, tryptophan deaminase, acetoin production, and assimilation of D-glucose, L-arabinose and phenylacetic acid; nitrate was not reduced and negative reactions were observed for assimilation of maltose, capric acid and adipic acid. The strains investigated in this study could be distinguished from closely related species of the genus *Burkholderia* (Table 1).
performed on nutrient agar medium (Oxoid) using the antibiotic Sensi-disc dispenser system (Oxoid) with bio-
discs (Oxoid) containing ampicillin (10 μg), chlorampheni-
col (30 μg), gentamicin (10 μg), kanamycin (30 μg),
penicillin G (10 μg), streptomycin (10 μg) and tetracycline
(30 μg). The strains were grown on half-strength LA
medium for 48 h prior to testing. The plates were incubated
at 28 °C and read between 2 and 5 days. All strains analysed
in this study were resistant to ampicillin and penicillin, and
sensitive to chloramphenicol and tetracycline.

Whole-cell fatty acid methyl esters were extracted accord-
ing to the MIDI protocol (http://www.microbialid.com/
PDF/TechNote_101.pdf). All characteristics such as tem-
perture, medium and physiological age (overlap area of
the second and third quadrant from a quadrant streak)
were as in the MIDI protocol. The profiles were generated
using an Agilent Technologies 6890N gas chromatograph,
and identified and clustered using the Microbial
Identification System software and MIDI TSBA database
version 5.0. Fatty acid profiles are listed in Table 2. The most abundant fatty acids for our strains were C_{18:1}ω7c
(25.2–38.9 %), C_{16:0} (17.0–29.3 %) and summed feature 3
(10.1–18.9 %). Moderate amounts of C_{17:0} cyclo (12.9–
7.0 %), summed feature 2 (7.6–10.0 %), C_{16:0} 3-OH (6.2–
7.8 %), C_{14:0} (4.1–5.4 %), C_{19:0} cyclo ω8c (2.8–4.5 %),
C_{16:1} 2-OH (2.7–4.4 %), C_{16:0} 2-OH (1.8–2.4 %) and
C_{18:1} 2-OH (1.0–1.7 %) were present and trace amounts
(<1 %) of C_{18:0} were detected. The presence of C_{16:0} 3-
OH supports the placement of these strains in the genus
Burkholderia (Yabuuchi et al., 1992; Garrity et al., 2005).
Additionally, there were noticeable differences between the fatty acid profiles of the L. ambigua strains and other type
strains of species of the genus Burkholderia (Table 2). Cell
biomass for respiratory lipoquinone analysis was obtained from
late-exponential phase culture grown in half-strength
Burkholderia sprentiae sp. nov.

LA broth. Lipoquinones were extracted from lyophilized biomass by a modified one-phase Bligh/Dyer extraction method and analysed using high performance liquid chromatography/electrospray/tandem mass spectrometry as described by Ardley et al. (2012). For strain WSM5005T, ubiquinone Q-8 was the major respiratory lipoquinone (approx. 98 %), with ubiquinone Q-7 (approx. 1 %) and ubiquinone Q-10 (approx. 1 %) also present. Q-8 as major respiratory lipoquinone is in agreement with other species of the genus Burkholderia (Valverde et al., 2006; Aizawa et al., 2010a, b, 2011; Sheu et al., 2012, 2013).

For DNA–DNA hybridization and for the determination of the DNA G+C content, high-molecular mass DNA was prepared as described by Pitcher et al. (1989). DNA–DNA hybridizations were performed using a microplate method and biotinylated probe DNA (Ezaki et al., 1989). The hybridization temperature was 45 ± 1 °C. Reciprocal reactions (A × B and B × A) were performed for each DNA pair and their variation was within the limits of this method (Goris et al., 1998). A summary of the hybridization values is given in Table S2. A representative from each recA gene sequence cluster was chosen and the values presented are the mean of at least three replicates. The DNA–DNA relatedness between WSM5005T and WSM3617 was about 85 %; values towards B. tuberum LMG 21444T were between 56 and 59 %. The G+C content of DNA was determined by HPLC according to the method of Mesbah et al. (1989) using a Waters Breeze HPLC system and XBridge Shield RP18 column thermostabilized at 37 °C. The solvent was 0.02 M NH₄H₂PO₄ (pH 4.0) with 1.5 % (v/v) acetonitrile. Non-methylated lambda phage (Sigma) and Escherichia coli DNA were used as calibration reference and control, respectively. The DNA G+C content of our strains, was 61.6 mol% (Table 1), which is within the range reported for species of the genus Burkholderia (59–69.9 mol%) (Yabuuchi et al., 1992; Gillis et al., 1995; Garrity et al., 2005).

The nodulation and nitrogen fixation capacity of all seven strains was previously studied (Howieson et al., 2013) on

Fig. 2. Maximum-likelihood tree based on the gyrB gene sequences (481 bp) of our strains and phylogenetically related species. Bootstrap values after 1000 replicates are expressed as percentages, values less than 50 % are not shown. Cupriavidus taiwanensis LMG 19424T is included as an outgroup. Bar, 0.02 substitutions per site.
their original host (L. ambigua) as well as on Lebeckia sepiaria, using the axenic sand-culture system described by Yates et al. (2007). These results confirmed that all seven novel strains could form effective N₂-fixing symbioses with L. ambigua and L. sepiaria.

The genotypic and phenotypic data presented in this study demonstrate that the strains isolated from South African L. ambigua root nodules represent a novel species in the genus Burkholderia, that can be distinguished phenotypically (Tables 1 and 2) as well as genotypically (Figs 1–3 and Table S2) from its nearest phylogenetic neighbours. Genetically, B. tuberum is the closest neighbour, which nodulates Cyclopia, another South African papilionoid legume (Elliott et al., 2007). Although especially the analysis of the recA genes (Fig. 3) revealed a bifurcation in this novel species, this subdivision was not further substantiated by the results of chemotaxonomic or phenotypic analyses. Therefore, we propose to classify the strains as representatives of Burkholderia sprentiae sp. nov. with WSM5005ᵀ as the type strain.

**Description of Burkholderia sprentiae sp. nov.**

*Burkholderia sprentiae* (spren’ti.əe. N.L. fem. gen. n. sprentiae of Sprent, named after Janet Sprent, a scientist who has devoted her life to legume research).

Cells are rod-shaped and motile (approx. 0.9 × 3.0 μm). Gram-stain-negative, catalase and oxidase-positive. Colonies are white, smooth, round, 0.5–4.0 mm in diameter and convex with entire margins on half-strength LA medium after 24 h of incubation. Growth is visible on half-strength LA medium between 10 and 40 °C. Growth is visible in NB medium with 0–10 % NaCl and pH 4.5–9 at 28 °C. Positive reactions for β-galactosidase, arginine dihydrolase, assimilation of trisodium citrate, acetoin production, assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, potassium gluconate, malate and phenylacetic acid. Weakly positive reaction for tryptophan deaminase. Does not reduce nitrate to nitrite and has negative reactions for lysine and ornithine decarboxylases, H₂S and indole production, urease, gelatinase, β-glucosidase, protease, fermentation of D-glucose, D-mannitol, inositol, D-sorbitol, and D-saccharose.
L-rhamnose, sucrose, melibiose, amygdalin and L-arabinose, and assimilation of maltose, capric and adipic acid. Strain WSM5005<sup>T</sup> is resistant to ampicillin and penicillin, shows intermediate sensitivity to gentamicin and is sensitive to chloramphenicol, kanamycin, streptomycin and tetracycline. The whole-cell fatty acid profile is given in Table 2.

**Table 1.** Phenotypic characteristics distinguishing *Burkholderia sprentiae* sp. nov. from other species of the genus *Burkholderia*

<table>
<thead>
<tr>
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<th>10</th>
<th>11</th>
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<td>Rhizo</td>
<td>Soil</td>
<td>Rn</td>
<td>Rn</td>
<td>Rn</td>
<td>Soil</td>
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<td>Rn</td>
<td>Rn</td>
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<td>Arginine dihydrolase</td>
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<td>–</td>
<td>–</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>V</td>
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<td>Acetoin production</td>
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<td>ND</td>
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<td>V</td>
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<td>Trisodium citrate</td>
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<td>–</td>
<td>ND</td>
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<td>Fermentation/oxidation of:</td>
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<tr>
<td>D-Glucose</td>
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<td>–</td>
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<td>–</td>
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<tr>
<td>D-Manitol</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Melibiose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>ND</td>
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<tr>
<td>D-Sorbitol</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>ND</td>
<td>V</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>L-Arabinose</td>
<td>–</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>L-Rhamnose</td>
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<td>ND</td>
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<td>–</td>
<td>ND</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>61.6</td>
<td>62.0</td>
<td>63.1</td>
<td>63–65</td>
<td>62.1</td>
<td>64.8</td>
<td>62.8</td>
<td>63.9</td>
<td>62.8</td>
<td>64.5</td>
<td>64.2–65.7</td>
</tr>
</tbody>
</table>

L-rhamnose, sucrose, melibiose, amygdalin and L-arabinose, and assimilation of maltose, capric and adipic acid. Strain WSM5005<sup>T</sup> is resistant to ampicillin and penicillin, shows intermediate sensitivity to gentamicin and is sensitive to chloramphenicol, kanamycin, streptomycin and tetracycline. The whole-cell fatty acid profile is given in Table 2.

**Table 2.** Fatty acid composition of members of the genus *Burkholderia*

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;14:0&lt;/sub&gt;</td>
<td>4.7</td>
<td>4.7</td>
<td>4.2</td>
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<td>5.6</td>
<td>4.2</td>
<td>4.8</td>
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<td>4.6</td>
</tr>
<tr>
<td>C&lt;sub&gt;16:0&lt;/sub&gt;</td>
<td>19.9</td>
<td>13.6</td>
<td>16.3</td>
<td>19.0</td>
<td>20.9</td>
<td>15.2</td>
<td>20.1</td>
<td>14.3</td>
<td>17.6</td>
<td>18.3</td>
</tr>
<tr>
<td>C&lt;sub&gt;16:1&lt;/sub&gt;Δ9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.1</td>
<td>2.4</td>
<td>2.9</td>
<td>TR</td>
<td>1.4</td>
<td>3.1</td>
<td>2.2</td>
<td>3.5</td>
<td>2.2</td>
<td>1.6</td>
</tr>
<tr>
<td>C&lt;sub&gt;16:1&lt;/sub&gt;Δ6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.7</td>
<td>6.0</td>
<td>5.7</td>
<td>5.5</td>
<td>5.8</td>
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<td>6.1</td>
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<td>6.5</td>
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<td>2.4</td>
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<td>2.0</td>
<td>5.1</td>
<td>5.3</td>
<td>3.1</td>
<td>1.8</td>
<td>TR</td>
</tr>
<tr>
<td>C&lt;sub&gt;17:0&lt;/sub&gt;cyclo</td>
<td>10.6</td>
<td>8.4</td>
<td>11.8</td>
<td>3.6</td>
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<tr>
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<td>ND</td>
<td>TR</td>
<td>TR</td>
<td>1.3</td>
<td>ND</td>
<td>ND</td>
<td>TR</td>
<td>ND</td>
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<tr>
<td>C&lt;sub&gt;18:1&lt;/sub&gt;Δ9&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>1.1</td>
<td>1.3</td>
<td>ND</td>
<td>TR</td>
<td>1.8</td>
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<tr>
<td>C&lt;sub&gt;19:0&lt;/sub&gt;cyclo Δ11&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>3.7</td>
<td>6.6</td>
<td>2.2</td>
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<td>ND</td>
<td>7.1</td>
<td>7.1</td>
<td>3.8</td>
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<tr>
<td>Summed feature 2&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>7.4</td>
<td>7.1</td>
<td>7.0</td>
<td>8.3</td>
<td>11.9</td>
<td>7.9</td>
<td>8.2</td>
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<tr>
<td>Summed feature 3&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>14.5</td>
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</tbody>
</table>

*Summed features are groups of two or three fatty acids that cannot be separated by GLC using the MIDI system. Summed feature 2 comprises C<sub>14:0</sub>Δ3<sup>OH</sup>, C<sub>16:1</sub>Δ15<sup>c</sup> iso I, an unidentified fatty acid with an equivalent chain-length value of 10.928 or C<sub>12:0</sub> ALDE. Summed feature 3 comprises C<sub>16:1</sub>Δ9<sup>c</sup>c and/or C<sub>15:0</sub> iso 2-OH.
The type strain, WSM5005\textsuperscript{T} (=LMG 27175\textsuperscript{T}=HAMI 3357\textsuperscript{T}), was isolated from root nodules of *Lebeckia ambigua* from the Western Cape of South Africa in 2007. The DNA G+C content of the type strain is 61.6 mol%.

**Acknowledgements**

The authors would like to thank Regina Carr (School of Biological Sciences and Biotechnology, Murdoch University) for skilled technical assistance. Special thanks to Frances Briggs and David Berryman from the SABC (Western Australian State Agricultural Biotechnology Centre, Murdoch University) for using their facilities and skilled assistance. We also thank the Australian Centre for International Agricultural Research (ACIAR) for funding the germplasm collection activities.

**References**


