**Burkholderia dipogonis** sp. nov., isolated from root nodules of *Dipogon lignosus* in New Zealand and Western Australia

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Seven strains, ICMP 19430ᵀ, ICMP 19429, ICMP 19431, WSM4637, WSM4638, WSM4639 and WSM4640, were isolated from nitrogen-fixing nodules on roots of the invasive South African legume *Dipogon lignosus* (subfamily Papilionoideae, tribe Phaseoleae) in New Zealand and Western Australia, and their taxonomic positions were investigated by using a polyphasic approach. All seven strains grew at 10–37 °C (optimum, 25–30 °C), at pH 4.0–9.0 (optimum, pH 6.0–7.0) and with 0–2 % (w/v) NaCl (optimum growth in the absence of NaCl). On the basis of 16S rRNA gene sequence analysis, the strains showed 99.0–99.5 % sequence similarity to the closest type strain, *Burkholderia phytofirmans* PsJNT, and 98.4–99.7 % sequence similarity to *Burkholderia caledonica* LMG 19076ᵀ. The predominant fatty acids were C₁₈ : ₁ω7c (21.0 % of the total fatty acids in strain ICMP 19430ᵀ), C₁₆ : ₀ (19.1 %), C₁₇ : ₀ cyclo (18.9 %), summed feature 3 (C₁₆ : ₁ω7c and/or C₁₆ : ₁ω6c; 10.7 %) and C₁₉ : ₀ cyclo ω8c (7.5 %). The polar lipid profile consisted of a mixture of phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and several uncharacterized aminophospholipids and phospholipids. The major isoprenoid quinone was Q-8 and the DNA G + C content of strain ICMP 19430ᵀ was 63.2 mol%. The DNA–DNA relatedness of the novel strains with respect to the closest neighbouring members of the genus *Burkholderia* was 55 % or less. On the basis of 16S rRNA and recA gene sequence similarities and chemotaxonomic and phenotypic data, these strains represent a novel symbiotic species in the genus *Burkholderia*, for which the name *Burkholderia dipogonis* sp. nov. is proposed, with the type strain ICMP 19430ᵀ (=LMG 28415ᵀ=HAMBI 3637ᵀ).

The genus *Burkholderia*, belonging to the family *Burkholderiaceae* of the Betaproteobacteria, was proposed by Yabuuchi et al. (1992). At the time of writing, it comprises 87 species with validly published names (http://www.bacterio.net/burkholderia.html). Members of the genus *Burkholderia* are characterized as Gram-stain-negative, aerobic, non-spore-forming, non-fermentative, straight rod-shaped, catalase-positive bacteria, and most species are motile by using a single polar flagellum or a tuft of polar flagella. They possess high metabolic versatility and
Recently, 10 nodule isolates from the invasive South African legume *Dipogon lignosus* (tribe Phaseoleae) growing in New Zealand were sampled and surveyed for their symbiotic diversity. Sequences of their 16S rRNA, recA, nifH, nodA and nodC genes showed that seven of these isolates belonged to the genus *Burkholderia* (Liu et al., 2014). Three isolates were deposited in the International Collection of Microorganisms from Plants (ICMP), Landcare Research, Auckland, New Zealand, as ICMP 19430T, ICMP 19429 and ICMP 19431. ICMP 19429, ICMP 19430T and ICMP 19431 nodulated and fixed nitrogen with their original host *D. lignosus* and with *Phaseolus vulgaris* (also in tribe Phaseoleae), but were unable to nodulate *Mimosa pudica* (Liu et al., 2014). In addition, ICMP 19430T was shown to produce N2-fixing nodules on the South African native legumes *Cyclopia subternata, Hypocaidxus sophoroides, Podalyria calyptrata* and *Virgilia oroboides* (Liu et al., 2014).

Four isolates were obtained from four nitrogen-fixing nodules of *D. lignosus* growing in sandy soil in the Dugalup Brook vegetation reserve in the coastal town of Dunsborough, south-western Australia (33°36'55"S 115°06'13"E), and were deposited in the Western Australian Soil Microbiology (WSM) culture collection at the Centre for Rhizobium Studies, Murdoch University, Western Australia, as WSM4637, WSM4638, WSM4639 and WSM4640. All isolates were tested and confirmed as able to form N2-fixing nodules on *D. lignosus*, using the axenic sand-culture system described previously (Howieson et al., 1995). The ERIC PCR (Versalovic et al., 1991) banding patterns obtained for WSM4637, WSM4638, WSM4639 and WSM4640 indicated that they either were very closely related to each other or were clones (data not shown). Preliminary 16S rRNA gene sequence data showed that these four isolates belonged to the genus *Burkholderia* and were most closely related to the New Zealand *D. lignosus* isolates. Subsequent analysis by DNA–DNA hybridization (Table S1, available in the online Supplementary Material) and whole-cell protein profiles (Fig. S1) indicated that these four isolates, along with the three isolates from *D. lignosus* growing in New Zealand, were not clones but constituted seven separate strains.

As the study by Liu et al. (2014) suggested that ICMP 19429, ICMP 19430T and ICMP 19431 formed a novel taxonomic group, these strains, together with the four Western Australian *D. lignosus* strains, were subjected to a polyphasic taxonomic approach. All strains were grown on yeast extract-mannitol (YEM) agar plates (Vincent, 1970) and incubated at 25 °C, unless indicated otherwise. Subculturing was performed on YEM agar at 25 °C for 2 days. The strains were stored at −80 °C in YEM broth with 20 % (v/v) glycerol or by lyophilization. *Burkholderia phytofirmans* LMG 22146T, *B. caledonica* LMG 19076T, *B. phenoliruptrix* LMG 22037T, *B. fungorum* LMG 16225T, *B. xenovorans* LMG 21463T and *B. rhynchosiaea* LMG 27174T were obtained from the BCCM/LMG Bacteria Collection, Belgium, and *Burkholderia ginsengisoli* NBRC 100965T was obtained from the NITE Biological Research Center, Japan, and used as references for phenotypic and genotypic tests.

Bacterial cells grown on YEM agar at 25 °C for 2 days were observed by phase-contrast microscopy (DM2000; Leica). Flagellar motility was tested using the hanging-drop method, and the Spot Test flagella stain (BD Difco) was used for flagellum staining (Beveridge et al., 2007). The Gram Stain Set S (BD Difco) kit and the Ryu non-staining KOH method (Powers, 1995) were used for testing the Gram reaction. The presence of a capsule was assessed by using the Hiss staining method (Beveridge et al., 2007). Poly-β-hydroxybutyrate granule accumulation was examined under light microscopy after staining the cells with Sudan black (Schlegel et al., 1970) and visualized by UV illumination after growing bacteria on plates containing Nile red at 25 °C for 2 days (Spiekermann et al., 1999). Colony morphology was observed on YEM agar using a stereoscopic microscope (SMZ 800; Nikon).

The pH range for growth was determined for all investigated strains by measuring the OD600 of cultures in YEM broth. The medium was adjusted prior to sterilization to pH 4.0–9.0 (at intervals of 0.5 pH units) using the following biological buffers (Breznak & Costilow, 2007): citrate/Na2HPO4 (pH 4.0–5.5), phosphate (pH 6.0–7.5) and Tris/HCl (pH 8.0–9.0). The requirement for NaCl was determined using YEM broth containing 0, 0.5 and 1.0–8.0 % (w/v) NaCl. Growth at 4–50 °C was observed in YEM broth. Cellular growth under the different conditions mentioned above was determined by measuring the OD600 of the cultures. Anaerobic growth was determined after incubating the strains on YEM agar in the Oxoid AnaeroGen system (Miller et al., 1995).

All investigated strains were examined for a broad range of phenotypic properties. Activities of catalase, oxidase, DNase, urease and lipase (corn oil) and hydrolysis of starch, casein and Tween 20, 40, 60 and 80 were determined using standard methods (Tindall et al., 2007). Hydrolysis of alginase [1 % (w/v) sodium alginate] was examined on YEM agar. Chitin hydrolysis was assessed by chitinase-detection...
agar (Wen et al., 2002) and visualized by the formation of clear zones around the colonies. Hydrolysis of CM-cellulose was tested as described by Bowman (2000) using YEM agar as the basal medium. Additional biochemical tests were performed using the API 20NE and API ZYM kits (bioMérieux) and carbon-source utilization was evaluated using the GN2 microplate (Biolog). All commercial phenotypic tests were performed according to the manufacturers’ recommendations.

Antibiotic sensitivities of the novel strains and the reference strains were analysed by the diffusion method after spreading cell suspensions (0.5 McFarland) on YEM agar. The following antibiotic discs (Oxoid) were used: ampicillin (10 μg), chloramphenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), novobiocin (30 μg), rifampicin (5 μg), penicillin G (10 U), streptomycin (10 μg), sulfamethoxazole plus trimethoprim (23.75/1.25 μg) and tetracycline (30 μg). The effect of antibiotics on cell growth was assessed after 2 days at 25 °C. Strains were considered susceptible or resistant as described by Nokhal & Schlegel (1983). Detailed results of the biochemical characterization and antibiotic sensitivity tests are given in the species description and in Tables 1 and S2. Our strains can be distinguished from type strains of closely related species of the genus Burkholderia by using a combination of phenotypic attributes, especially the activity of urease, assimilation of phenylacetate and utilization of putrescine, 2-β-glucose 1-phosphate and d-alanine as sole carbon sources (Table S2).

The 16S rRNA and recA gene sequences of strains ICMP 19430T, ICMP 19429 and ICMP 19431 were obtained previously by Liu et al. (2014). For strains WSM4637, WSM4638, WSM4639 and WSM4640, 16S rRNA gene sequences were obtained as reported by Ardley et al. (2012) and recA gene sequences were obtained as reported by Liu et al. (2014). The 16S rRNA gene sequences were compared to those available in EzTaxon-e (Kim et al., 2009) and their phylogenetically closest neighbours within the genus Burkholderia by using a combination of phenotypic attributes, especially the activity of urease, assimilation of phenylacetate and utilization of putrescine, 2-β-glucose 1-phosphate and d-alanine as sole carbon sources (Table S2).

The 16S rRNA and recA gene sequences of strains ICMP 19430T, ICMP 19429 and ICMP 19431 were obtained previously by Liu et al. (2014). For strains WSM4637, WSM4638, WSM4639 and WSM4640, 16S rRNA gene sequences were obtained as reported by Ardley et al. (2012) and recA gene sequences were obtained as reported by Liu et al. (2014). The 16S rRNA gene sequences were compared to those available in EzTaxon-e (Kim et al., 2012), the Ribosomal Database Project (Cole et al., 2009) and the GenBank database (http://blast.ncbi.nlm.nih.gov/ Blast.cgi). Sequence analyses were performed using the software packages BioEdit (Hall, 1999) and MEGA 5 (Tamura et al., 2011), after multiple alignments of the data by CLUSTAL_X (Thompson et al., 1997). Distances were calculated using Kimura’s two-parameter model (Kimura, 1983) and clustering was performed with the neighbour-joining method (Saitou & Nei, 1987). Maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) trees were generated using the treeing algorithms contained in the PHYLIP software package (Felsenstein, 1993). In each case, bootstrap values were calculated based on 1000 replications.

The phylogenetic tree based on 16S rRNA gene sequence comparison (Figs 1 and S2) showed that the strains formed a separate phylogenetic branch within the genus Burkholderia. The overall topologies of the phylogenetic trees obtained with the neighbour-joining, maximum-likelihood and maximum-parsimony methods were similar (not shown). The 16S rRNA gene sequences of the strains showed high similarity to each other (more than 99.5 %) and were closely related to those of Burkholderia phytofirmans PsJN (99.0–99.5 % similarity), Burkholderia caledonica W50D (98.1–99.1 %), Burkholderia phenoliruptrix AC1100 (98.1–98.5 %), Burkholderia ginsengisoli KMY03 (97.2–97.5 %), Burkholderia fungorum P763-2 (98.1–99.1 %), Burkholderia xenovorans LB400 (98.0–98.8 %) and Burkholderia rhynchosiae WSM3937 (98.0–98.6 %). Lower sequence similarities (<97.0 %) were found with the type strains of all other species included in Fig. 1.

According to pairwise recA gene sequence comparisons, the similarity of the investigated strains with each other ranged from 99.9 to 100 %. Strain ICMP 19430T showed the highest similarity (97.8 %) to Burkholderia phytofirmans PsJN, and the levels of the recA gene sequence similarity to members of other species of the genus Burkholderia with validly published names were below 94.5 %. Phylogenetic analyses of the partial recA sequences were performed using MEGA6. Neighbour-joining, maximum-likelihood and maximum-parsimony trees were generated and bootstrap values were calculated based on 1000 replications. The overall topologies of the phylogenetic trees were similar, and showed that all seven strains formed a separate monophyletic cluster within the genus Burkholderia (Fig. S3).

Whole-genome DNA–DNA hybridization experiments were performed at 50 °C with photobiotin-labelled probes as described by Ezaki et al. (1989). DNA–DNA hybridization experiments were performed with all investigated strains, and their phylogenetically closest neighbours within the genus Burkholderia. The phylogenetic analysis performed at 50 °C using photobiotin-labelled probes for the genus Burkholderia showed that all seven strains formed a separate monophyletic cluster within the genus Burkholderia (Fig. S3).

Preparation of whole-cell proteins and SDS-PAGE were performed as described by Pot et al. (1994). Densitometric analysis, normalization and interpolation of the protein profiles and numerical analysis using Pearson's product-moment correlation coefficient were performed using the GelCompar 4.2 software package (Applied Maths). Whole-cell protein extracts were prepared from all investigated strains and compared with those of type strains of closely related species. Our strains formed a single cluster with similarities ≥93 %, in comparison with similarities of less than 86 % to other members of the genus Burkholderia (Fig. S1).
The fatty acid profiles of strain ICMP 19430<sup>T</sup>, *B. phytofirmans* LMG 22146<sup>T</sup>, *B. caledonica* LMG 19076<sup>T</sup>, *B. phenoliruptrix* LMG 22037<sup>T</sup>, *B. fungorum* LMG 16225<sup>T</sup>, *B. xenovorans* LMG 21463<sup>T</sup>, *B. rhynchosiae* LMG 27174<sup>T</sup> and *B. ginsengisoli* NBRC 100965<sup>T</sup> were determined using cells grown on YEM agar at 25 °C for 2 days. The physiological age of the different bacterial cultures at the time of harvest was standardized by selecting a sector from a

### Table 1. Phenotypic characteristics that distinguish the novel strains from the type strains of other species of the genus *Burkholderia*

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<td>DNA G+C content (mol%)</td>
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<sup>a</sup>Data taken from: <i>a</i>, Sessitsch et al. (2005); <i>b</i>, Coenye et al. (2001); <i>c</i>, Coenye et al. (2004); <i>d</i>, Goris et al. (2004); <i>e</i>, De Meyer et al. (2013a); <i>f</i>, Kim et al. (2006).
quadrant streak on YEM agar plates according to the MIDI protocol (Sasser, 1990). In this study, the different members of the genus *Burkholderia* exhibited very similar growth rates on YEM agar. Fatty acid methyl esters were prepared and separated according to the standard protocol of MIDI (Sherlock Microbiological Identification System, version 6.0), analysed by GC (Hewlett Packard 5890 Series II) and identified by using the RTSBA6.00 database of the Microbial Identification System (Sasser, 1990). The overall fatty acid profile of strain ICMP 19430T was similar to those of the reference strains, although there were differences in the proportions of certain components (Table 2). The major fatty acids (>5 %) of strain ICMP 19430T were C<sub>18</sub>:1ω7c (21.0 %), C<sub>16</sub>:0 (19.1 %), C<sub>17</sub>:0 cyclo (18.9 %), summed feature 3 (C<sub>16</sub>:1ω7c and/or C<sub>16</sub>:ω6c; 10.7 %) and C<sub>19</sub>:0 cyclo ω8c (7.5 %).

Isoprenoid quinones were extracted and purified according to the method of Collins (1994) and analysed by HPLC, which revealed Q-8 as the main respiratory quinone for strain ICMP 19430T. The DNA G+C content of strain ICMP 19430T, as determined by HPLC (Mesbah *et al.*, 1989), was 63.2 mol%, which is within the range reported previously for members of the genus *Burkholderia* (59–69.5 mol%; Yabuuchi *et al.*, 1992; Gillis *et al.*, 1995; Garry *et al.*, 2001). Polar lipids were extracted and analysed by two-dimensional TLC according to Embley & Wait (1994). Molybdophosphoric acid was used for the detection of the total polar lipids, ninhydrin for aminolipids, Dragendorff reagent for choline-containing lipids and α-naphthol reagent for glycolipids. Strain ICMP 19430T exhibited a complex polar lipid profile consisting of phosphatidylethanolamine, phosphatidylycerol, diphasphatidylglycerol, two uncharacterized aminophospholipids (APL1 and APL2) and several uncharacterized phospholipids (Fig. S4). Moreover, the polar lipid profile of strain ICMP 19430T was very similar to those of its closest relatives, *B. phytofirmans* LMG 16225T and *B. ginsengisoli* NBRC 100965T, with phosphatidylethanolamine, phosphatidylglycerol, two uncharacterized aminophospholipids (APL1 and APL2) and several uncharacterized phospholipids (Fig. S4). Moreover, the polar lipid profile of strain ICMP 19430T was very similar to those of its closest relatives, *B. phytofirmans* LMG 16225T, *B. ginsengisoli* NBRC 100965T, *B. xenovorans* LMG 21463T, *B. rhynchosiae* LMG 27174T and *B. ginsengisoli* NBRC 100965T, with phosphatidylethanolamine, phosphatidylglycerol, diphasphatidylglycerol and APL1 as major polar lipids. However, there were differences in the uncharacterized phospholipids.

Based on the phenotypic and genotypic data obtained in this study, the strains investigated constitute a novel species within the genus *Burkholderia*, for which the name *Burkholderia dipogonis* sp. nov. is proposed.

**Fig. 1.** Neighbour-joining phylogenetic tree of the novel strains (*Burkholderia dipogonis* sp. nov.) and related bacteria, based on 16S rRNA gene sequence comparisons. Numbers at nodes are bootstrap percentages >70 % based on the neighbour-joining algorithm. Filled circles indicate branches of the tree that were also recovered using the maximum-likelihood and maximum-parsimony algorithms; open circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. *Cupriavidus taiwanensis* LMG 19424T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position. The full tree from which this tree was taken is available as Fig. S2.
**Table 2.** Cellular fatty acid compositions of *B. dipogonis* sp. nov. ICMP 19430T and type strains of related species 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>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>4.3</td>
<td>3.7</td>
<td>3.6</td>
<td>4.0</td>
<td>3.7</td>
<td>3.4</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>19.1</td>
<td>20.9</td>
<td>16.7</td>
<td>18.4</td>
<td>20.1</td>
<td>22.9</td>
<td>13.0</td>
<td>18.9</td>
</tr>
<tr>
<td>C16:0 3-OH</td>
<td>3.6</td>
<td>4.0</td>
<td>3.5</td>
<td>3.8</td>
<td>3.9</td>
<td>3.6</td>
<td>3.5</td>
<td>3.8</td>
</tr>
<tr>
<td>C16:1 2-OH</td>
<td>1.2</td>
<td>–</td>
<td>–</td>
<td>2.3</td>
<td>–</td>
<td>1.2</td>
<td>–</td>
<td>1.8</td>
</tr>
<tr>
<td>C17:0 cyclo</td>
<td>18.9</td>
<td>–</td>
<td>4.6</td>
<td>9.8</td>
<td>2.3</td>
<td>1.1</td>
<td>1.8</td>
<td>7.9</td>
</tr>
<tr>
<td>C18:0</td>
<td>1.6</td>
<td>1.4</td>
<td>1.1</td>
<td>1.4</td>
<td>2.2</td>
<td>2.9</td>
<td>1.6</td>
<td>3.3</td>
</tr>
<tr>
<td>C19:0 cyclo o8c</td>
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<td>30.4</td>
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<tr>
<td>C18:1ω7c</td>
<td>21.0</td>
<td>37.3</td>
<td>36.6</td>
<td>23.3</td>
<td>35.2</td>
<td>30.4</td>
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<tr>
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<td>4.9</td>
<td>3.8</td>
<td>5.3</td>
<td>4.9</td>
<td>5.3</td>
<td>5.0</td>
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<tr>
<td>Summed feature 3*</td>
<td>10.7</td>
<td>23.6</td>
<td>23.3</td>
<td>16.9</td>
<td>16.6</td>
<td>21.2</td>
<td>20.8</td>
<td>19.5</td>
</tr>
</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 comprised C16:0 3-OH and/or iso-C16:1 i. Summed feature 3 comprised C16:1ω7c and/or C16:1ω6c.

**Description of Burkholderia dipogonis sp. nov.**

*Burkholderia dipogonis* (di.po.go’nis. N.L. gen. n. *dipogonis* of *Dipogon*, a botanical generic name, referring to the isolation of the first strains from *Dipogon lignosus*).

Cells are Gram-stain-negative, motile, aerobic, non-spore-forming rods surrounded by a thick capsule. Poly-β-hydroxybutyrate accumulation is observed. Catalase- and oxidase-positive. After 24 h of growth on YEM agar at 25 °C, cells are 0.6–0.8 μm in diameter and 1.8–2.8 μm long. Colonies on YEM agar are pale yellow, circular, smooth and convex with entire edges. Colonies are approximately 1.2–1.8 mm in diameter on YEM agar after 48 h of incubation at 25 °C. Growth occurs at 10–37 °C (optimum, 25–30 °C), at pH 4.0–9.0 (optimum, pH 6.0–7.0) and with 0–2% (w/v) NaCl (optimum growth in the absence of NaCl). Positive for hydrolysis of Tween 40 and 60, weakly positive for hydrolysis of CM-cellulose and negative for hydrolysis of DNA, starch, chitin, casein, corn oil, alginate and Tween 20 and 80. Positive for activities of urease, alkaline phosphatase, C4 esterase, C8 esterase lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase and assimilation of glucose, arabinose, mannose, mannitol, N-acetylglucoamine, gluconate, caprate, adipate and malate; negative for nitrate reduction, indole production, glucose fermentation, hydrolysis of ascelin and gelatin, activities of arginine dihydروdrolase, C14 lipase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase and assimilation of maltose. Additional chemotaxonomic information can be found in Tables 1 and S2. All known strains are sensitive to chloramphenicol, rifampicin, gentamicin, kanamycin, penicillin G, ampicillin, novobiocin, tetracycline, streptomycin, sulfamethoxazole plus trimethoprim and nalidixic acid. The major fatty acids are C18:1ω7c, C16:0, C17:0 cyclo, summed feature 3 (C16:1ω7c and/or C16:1ω6c) and C19:0 cyclo ω8c. The major respiratory quinone is Q-8. The polar lipid profile consists of a mixture of phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, two uncharacterized aminophospholipids and several uncharacterized phospholipids.

The type strain is ICMP 19430T (=LMG 28415T=HAMI 3637T), which was isolated from root nodules of *Dipogon lignosus* in New Zealand. The DNA G+C content of the type strain is 63.2 mol%.

**References**


Chen, W. M., de Faria, S. M., Chou, J. H., James, E. K., Elliott, G. N., Sprent, J. I., Bontemps, C., Young, J. P. W. & Vandamme, P.,...


Burkholderia dipogonis sp. nov.  


