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Burkholderia sabiae sp. nov., isolated from root nodules of *Mimosa caesalpiniifolia*

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Two rhizobial strains, Br3407^T and Br3405, were isolated from nitrogen-fixing nodules on the roots of *Mimosa caesalpiniifolia*, a legume tree native to Brazil. On the basis of 16S rRNA gene sequence similarities, both strains were shown previously to belong to the genus *Burkholderia*. A polyphasic approach, including DNA–DNA hybridizations, pulsed-field gel electrophoresis of whole-genome DNA profiles, whole-cell protein analyses, fatty acid methyl ester analysis and extensive biochemical characterization, was used to clarify the taxonomic position of these strains further; the strains are here classified within a novel species, for which the name *Burkholderia sabiae* sp. nov. is proposed. The type strain is strain Br3407^T (=LMG 24235^T =BCRC 17587^T).

Plants in the legume genus *Mimosa* (in the subfamily Mimosoideae) are largely nodulated by members of the *Betaproteobacteria*, such as *Cupriavidus taiwanensis* and *Burkholderia* strains (Chen *et al.*, 2001, 2003a, b, 2005a, b; Vandamme & Coenye, 2004; Verma *et al.*, 2004; Barrett & Parker, 2005, 2006; Elliott *et al.*, 2007). Symbiont diversity within the genus *Cupriavidus* appears to be quite low based upon 16S rRNA gene sequences, and only one nodulating species of *Cupriavidus*, *C. taiwanensis* (Chen *et al.*, 2001), has been described so far. However, in contrast to *Cupriavidus*, the diversity of *Burkholderia* symbionts associated with legumes is very high (Chen *et al.*, 2005a, b; Barrett & Parker, 2005, 2006), and two novel *Mimosa*-nodulating species, *Burkholderia mimosarum* (Chen *et al.*, 2006) and *Burkholderia nodosa* (Chen *et al.*, 2007), have recently been added to the genus. Together with *Burkholderia phymatum* and *Burkholderia tuberum* (Moulin *et al.*, 2001; Vandamme *et al.*, 2002), this brings the number of legume-nodulating *Burkholderia* species to four, but 16S rRNA gene sequences in the literature and databases suggest that several other species await description. For example, amongst the group of South American *Mimosa* symbionts described by Chen *et al.* (2005a) that gave rise to both *B. mimosarum* and *B. nodosa* (Chen *et al.*, 2006, 2007), there are other clades containing strains that cluster very closely together. In this paper, we describe a novel *Mimosa*-nodulating species based upon two strains, Br3405 and Br3407^T, both isolated from *Mimosa caesalpiniifolia*, a legume tree native to Brazil.

Both Br3405 and Br3407^T were grown on yeast extract-mannitol agar plates (Vincent, 1970) and incubated at 28 °C unless otherwise indicated. *Burkholderia* reference strains have been described previously (Achouak *et al.*, 1999; Vandamme *et al.*, 2002; Goris *et al.*, 2002; Yang *et al.*, 2006).

The 16S rRNA gene sequences of strains Br3407^T and Br3405 have been reported by Chen *et al.* (2005a). 16S rRNA gene sequences of related taxa were obtained from the Ribosomal Database Project and GenBank databases.

Abbreviation: PFGE, pulsed-field gel electrophoresis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains Br3407^T and Br3405 are respectively AY773186 and AY773185.

An extended neighbour-joining tree, detailed DNA–DNA hybridization results and fatty acid compositions of strain Br3407^T and related strains are available as supplementary material with the online version of this paper.

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Multiple alignments were performed by using the CLUSTAL_X program (Thompson *et al.*, 1997) and gaps were edited in the BioEdit program, version 5.0.9 (Hall, 1999). Evolutionary distances were calculated using Kimura's two-parameter model (Kimura, 1983). Phylogenetic trees were constructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch & Margoliash, 1967) methods, using the MEGA program, version 3.1 (Kumar *et al.*, 2004), and with bootstrap values based on 1000 replications (Felsenstein, 1981). The 16S rRNA gene sequence similarity between strains Br3407^T and Br3405 was 99.7 %. In the phylogenetic tree based on the neighbour-joining algorithm, strains Br3407^T and Br3405 formed a well-supported clade with type strains of *B. phymatum*, *Burkholderia caribensis*, *Burkholderia terrae* and *Burkholderia hospita* (Fig. 1 and Supplementary Fig. S1, available in IJSEM Online). The 16S rRNA gene sequence of strain Br3407^T has 98.5, 98.5, 98.2 and 97.8 % similarity, respectively, to these four type strains, and similarity of 97.8–97.0 % to the type strains of *Burkholderia terricola*, *B. fungorum*, *B. phenoliruptrix*, *B. phytofirmans*, *B. xenovorans*, *B. graminis*, *B. caryophylli* and *B. caledonica*. The similarity to other *Burkholderia* species is less than 97.0 %.

DNA samples were prepared from strains Br3407^T and Br3405 as described by Pitcher *et al.* (1989). For determination of the DNA base composition, DNA was degraded enzymically into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture was separated by HPLC using a Waters Symmetry Shield C8 column at 37 °C. The solvent was 0.02 M $NH_4H_2PO_4$ (pH 4.0) with 1.5 % acetonitrile. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference. DNA–DNA hybridizations were performed with photobiotin-labelled probes as described by Ezaki *et al.* (1989). The hybridization temperature was 50 °C and the reaction was carried out in 30 % formamide. Each value obtained was the mean of two hybridization experiments. The DNA G+C content of strains Br3407^T and Br3405 was 64.0±0.5 mol%. The DNA–DNA binding value between strains Br3407^T and Br3405 was 97–100 % (Supplementary Table S1). The high degree of DNA–DNA relatedness and the high levels of 16S rRNA gene similarity between the two isolates indicate that they represent a single species. Their separate species status was demonstrated by the DNA–DNA hybridization values of 15–43 % between strain Br3407^T and its closest phylogenetic neighbours, the type strains of *B. phymatum*, *B. terrae*, *B. caribensis* and *B. hospita* (Supplementary Table S1).

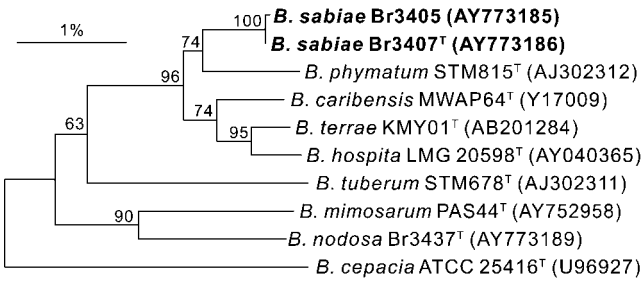

Fig. 1. Phylogenetic tree of strains Br3407^T and Br3405 (*Burkholderia sabiae* sp. nov.) and related *Burkholderia* type strains based on 16S rRNA gene sequence comparisons. Distances were calculated and clustering with the neighbour-joining method was performed by using the software package BioEdit. Numbers at nodes are percentage bootstrap values based on 1000 resampled datasets; only values >50 % are given. Bar, 1 % sequence dissimilarity. The sequence of *Burkholderia cepacia* ATCC 25416^T was used as an outgroup. A tree including a wider selection of reference sequences is available as Supplementary Fig. S1 in IJSEM Online.

Differentiation of the proposed novel taxon from its closest phylogenetic neighbours was examined by several approaches. For the analysis of protein electrophoretic patterns, strains were grown on nutrient agar (Oxoid CM3) supplemented with 0.04 % (w/v) KH_2PO_4 and 0.24 % (w/v) $Na_2HPO_4 . 12H_2O$ (pH 6.8) and incubated for 48 h at 28 °C. 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 strains Br3407^T and Br3405 and compared with others present in our database. Strains Br3407^T and Br3405 formed a single cluster with similarities of >98 %, in comparison with similarities of less than 79 % to other *Burkholderia* species (Fig. 2). For pulsed-field gel electrophoresis (PFGE) genome organization analysis as described by Chen *et al.* (2003b), intact genomic DNA in agarose plugs was electrophoresed on an 0.8 % agarose gel in TAE for 41 h with a pulse time of 500 s at 100 V (CHEF-III system; Bio-Rad). Strains Br3407^T and Br3405 have similar PFGE profiles and contained three replicons (5.0–5.2, 2.4–2.6 and 0.5–0.6 Mb). The PFGE profiles of the two *Mimosa* isolates clearly differentiate them from their closest neighbour, the type strain of *B. phymatum*, which contained four replicons of 3.5, 2.8, 2.0 and 0.5 Mb (Chen *et al.*, 2003b) (Fig. 3).

For fatty acid methyl ester analysis, cells were harvested after an incubation period of 48 h at 28 °C; fatty acid methyl esters were then prepared, separated and identified using the Microbial Identification System (Microbial ID) as described previously (Vandamme *et al.*, 2002). Fatty acid profiles of strains Br3407^T and Br3405 were determined and compared with those of other *Burkholderia* species. Fatty acid profiles of strains Br3407^T and Br3405 and other reference strains were similar, and were dominated by 16:0, 18:1ω7*c*, summed feature 2 (any combination of 14:0 3-OH, 16:1 iso I, an unidentified fatty acid with an equivalent chain-length of 10.928 or 12:0 ALDE) and summed feature 3 (16:1ω7*c* and/or 15:0 iso 2-OH), which supported the affiliation of strains Br3407^T and Br3405 to the genus

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Fig. 2. Dendrogram based on numerical analysis of the whole-cell protein profiles of *Mimosa* isolates (Br3407^T and Br3405) and type strains of closely related *Burkholderia* species.

Burkholderia (Supplementary Table S2). In general, all these organisms had very similar whole-cell fatty acid profiles, which were not useful for species discrimination. The fatty acid profile of strain Br3407^T consisted of 14:0 (3.7±0.1 %), 16:0 (17.9±0.4 %), 18:0 (1.4±0.2 %), 17:0 cyclo (3.7±0.3 %), 16:0 3-OH (4.1±0.1 %), 18:1ω7c (43.1±1.0 %), 19:0 cyclo ω8c (3.3±0.2 %) and summed features 2 (5.0±0.1 %) and 3 (16.0±0.5 %).

Cell morphology was examined by phase-contrast microscopy (DM 2000; Leica) in the lag, exponential and stationary phases of growth. A Gram-stain set (BD Difco) and the Ryu non-staining KOH method (Powers, 1995) were used to ascertain the Gram reaction. The motility of cells was tested by using the hanging drop method. Poly-β-hydroxybutyrate granule accumulation was observed under light microscopy after staining of cells with Sudan black. Catalase activity was determined by bubble production in a 3 % (v/v) H_2O_2 solution. Oxidase activity was determined on filter paper moistened with a 1 % (w/v) aqueous solution of *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine.

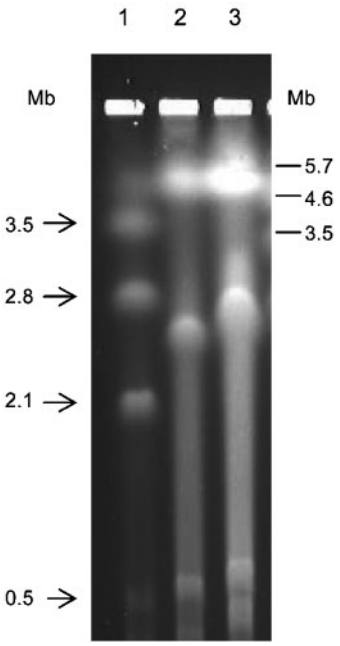

Fig. 3. PFGE of undigested whole-genome DNA profiles. Lanes: 1, *B. phymatum* STM815^T (sizes of replicons are indicated on the left); 2, strain Br3405; 3, strain Br3407^T. Molecular sizes of *Schizosaccharomyces pombe* marker DNA (Bio-Rad) are indicated on the right.

For biochemical characterization, the API 20NE and API ZYM microtest systems were used according to the recommendations of the manufacturer (bioMérieux). For carbon substrate assimilation tests, Biolog GN2 microtitre test plates were used. *B. phymatum* LMG 21445^T, *B. caribensis* LMG 18531^T, *B. hospita* LMG 20598^T and *B. terrae* LMG 23368^T were included as reference strains.

When using the API 20NE microtest gallery, the following characteristics were present in all strains: nitrate reduction, activity of oxidase, catalase, urease and β-galactosidase and assimilation of glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, gluconate, caprate, citrate, malate and phenylacetate. The following characteristics were uniformly absent: indole production, glucose fermentation, aesculin hydrolysis, gelatin hydrolysis and assimilation of adipate and maltose.

When using the API ZYM microtest gallery, activities of alkaline phosphatase, C4 esterase, C8 lipase, leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase were present in all strains. Activities of C14 lipase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase were uniformly absent.

Detailed results of the biochemical characterization and antibiotic sensitivity tests are given in Table 1 and in the species description. Strain Br3407^T can be distinguished from representatives of its close phylogenetic relatives by using a combination of phenotypic attributes, especially nitrate reduction, activity of urease, arginine dihydrolase, esterase C4 and esterase lipase C8 and oxidation of various substrates (Table 1). Strain Br3407^T had been shown previously to form N_2-fixing nodules on four *Mimosa* species, but not on its original host (Chen *et al.*, 2005a; Table 1). Therefore, it was inoculated on to *M. caesalpiniifolia* seedlings according to Elliott *et al.* (2007) and, after 6 months growth, it was shown to produce very active

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Table 1. Comparison of phenotypic characters of strain Br3407^T and the type strains of related *Burkholderia* species

Strains: 1, strain Br3407^T (*B. sabiae* sp. nov.); 2, *B. phymatum* LMG 21445^T; 3, *B. caribensis* LMG 18531^T; 4, *B. hospita* LMG 20598^T; 5, *B. terrae* LMG 23368^T. +, Positive; −, negative; w, weak reaction. Data for reference strains were obtained in this study with the exception of sources of isolation and DNA G+C contents, which were taken from Vandamme *et al.* (2002), Achouak *et al.* (1999), Goris *et al.* (2002) and Yang *et al.* (2006). All the strains have the following features: positive for the oxidation of Tweens 40 and 80, *N*-acetyl-D-glucosamine, adonitol, D-arabitol, D-fructose, L-fucose, D-galactose, α-D-glucose, *myo*-inositol, D-mannitol, D-mannose, L-rhamnose, D-sorbitol, pyruvic acid methyl ester, succinic acid monomethyl ester, *cis*-aconitic acid, formic acid, D-galactonic acid lactone, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, β-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, DL-lactic acid, quinic acid, succinic acid, bromosuccinic acid, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, L-leucine, L-phenylalanine, L-proline, L-pyroglutamic acid, γ-aminobutyric acid and 2-aminoethanol. Negative for the oxidation of α-cyclodextrin, i-erythritol, gentiobiose, maltose, methyl β-D-glucoside, raffinose, γ-hydroxybutyric acid, itaconic acid, α-ketovaleric acid, uridine, thymidine and putrescine.

Characteristic	1	2	3	4	5
Isolation source	Root nodules	Root nodules	Soil	Soil	Soil
Nitrate reduction	+	+	+	+	−
Urease	+	−	+	+	+
Arginine dihydrolase	−	−	+	−	+
Esterase C4	+	−	+	+	−
Esterase lipase C8	+	−	−	−	+
Oxidation of (Biolog):					
Dextrin	w	−	−	−	−
Glycogen	w	−	−	−	−
N-Acetyl-D-galactosamine	−	−	w	−	+
L-Arabinose	+	+	+	−	+
Lactulose	+	−	+	−	+
D-Psicose	−	+	w	−	+
Trehalose	+	−	+	−	+
α-Hydroxybutyric acid	+	w	+	−	+
Propionic acid	+	w	w	−	+
Succinamic acid	+	w	−	−	+
Glycyl L-glutamic acid	+	−	−	−	+
L-Ornithine	−	w	−	+	+
D-Serine	+	w	−	w	+
Inosine	w	−	+	−	+
Glycerol	w	−	+	+	+
DL-α-Glycerol phosphate	−	−	+	−	+
Nodulation on *Mimosa pudica*	+	+	−	−	−
DNA G+C content (mol%)	64.5	62.1	63.1	62.0	62.0

nodules giving high acetylene reduction activities (3.776 ± 1.542 nmol C_2H_4 h^{-1} per plant).

In conclusion, the present study demonstrated that the two isolates from root nodules of *M. caesalpiniifolia* from Brazil represent a single species that is readily distinguished from its nearest phylogenetic neighbours by whole-cell protein profiles (Fig. 2), whole-genome PFGE patterns (Fig. 3), DNA–DNA reassociation experiments (Supplementary Table S1) and biochemical characterization (Table 1). Moreover, strain Br3407^T effectively nodulated its original host, *M. caesalpiniifolia*, and other *Mimosa* species (Table 1), and the presence of *nif* and *nod* genes in the genome of strain Br3407^T has been demonstrated (Chen *et al.*, 2005a). We propose the name *Burkholderia sabiae* sp. nov. Two other strains with 16S rRNA gene sequences that are closely related to those of strains Br3405 and Br3407^T, strain Br3446 isolated from *Mimosa laticifera* (Chen *et al.*, 2005a) and Br3452 isolated from *M. caesalpiniifolia* (Menna *et al.*, 2006), were not included in this study as Br3452 could not be obtained and the protein profile of Br3446 was very different from those of Br3405 and Br3407^T (not shown); the latter strain therefore cannot be considered to be a third strain of *B. sabiae*.

Description of *Burkholderia sabiae* sp. nov.

Burkholderia sabiae (sa′bi.ae. N.L. gen. n. *sabiae* from sabiá, the Portuguese name of *Mimosa caesalpiniifolia*, the tree from which the type strain was isolated).

Cells are Gram-negative, non-spore-forming rods. Poly-β-hydroxybutyrate granules are accumulated. After 24 h growth on yeast extract-mannitol agar at 28 °C, the mean cell size is about 0.4–0.6 × 0.6–1.5 μm. Growth is observed at 28, 30 and 37 °C. Catalase- and oxidase-positive. Assimilation of glucose, arabinose, mannose, mannitol,

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N-acetylglucosamine, gluconate, caprate, citrate, malate and phenylacetate is observed. No indole production, gelatin hydrolysis, aesculin hydrolysis, glucose fermentation or assimilation of adipate or maltose is observed. The following carbon sources are oxidized (positive in the Biolog GN2 system): dextrin, glycogen, Tweens 40 and 80, *N*-acetyl-D-glucosamine, adonitol, arabinose, arabitol, cellobiose, D-fructose, L-fucose, D-galactose, lactulose, α-D-glucose, *myo*-inositol, D-mannitol, D-mannose, L-rhamnose, D-sorbitol, trehalose, methyl pyruvate, monomethyl succinate, acetic acid, *cis*-aconitic acid, citrate, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α-ketobutyric acid, α-ketoglutaric acid, DL-lactate, malonic acid, propionic acid, quinic acid, D-saccharic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, alaninamide, D- and L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, L-leucine, L-phenylalanine, L-proline, L-pyroglutamic acid, L- and D-serine, L-threonine, DL-carnitine, γ-aminobutyric acid, inosine, phenylethylamine, 2-aminoethanol, glycerol and glucose 6-phosphate. The following substrates are not utilized as carbon sources (negative in the Biolog GN2 system): α-cyclodextrin, *N*-acetyl-D-galactosamine, i-erythritol, gentiobiose, α-D-lactose, maltose, melibiose, methyl β-D-glucoside, D-psicose, raffinose, sucrose, turanose, xylitol, γ-hydroxybutyric acid, itaconic acid, α-ketovaleric acid, sebacic acid, L-ornithine, urocanic acid, uridine, thymidine, putrescine, 2,3-butanediol, DL-α-glycerol phosphate and glucose 1-phosphate. Sensitive to amikacin (30 μg), cefotaxime (30 μg), chloramphenicol (30 μg), kanamycin (30 μg), nalidixic acid (30 μg), novobiocin (30 μg), piperacillin (100 μg), rifampicin (5 μg), streptomycin (10 μg) and tetracycline (30 μg). Known strains were isolated from root nodules of *Mimosa caesalpiniifolia*.

The type strain is strain Br3407^T (=LMG 24235^T=BCRC 17587^T). Phenotypic characteristics of the type strain are the same as described for the species. Its DNA G+C content is 64.5 mol% and the genome size is approximately 8 Mb.

Acknowledgements

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