International Journal of Systematic and Evolutionary Microbiology (2006), **56**, 1847–1851 DOI 10.1099/ijs.0.64325-0

Burkholderia mimosarum sp. nov., isolated from root nodules of *Mimosa* spp. from Taiwan and South America

Wen-Ming Chen,[1] Euan K. James,[2] Tom Coenye,[3] Jui-Hsing Chou,[4] Edmundo Barrios,[5] Sergio M. de Faria,[6] Geoffrey N. Elliott,[2] Shih-Yi Sheu,[7] Janet I. Sprent[2] and Peter Vandamme[3]

Correspondence
Wen-Ming Chen
p62365@ms28.hinet.net

[1]Department of Seafood Science, National Kaohsiung Marine University, No. 142, Hai-Chuan Road, Nan-Tzu, Kaohsiung City 811, Taiwan

[2]School of Life Sciences, University of Dundee, Dundee DD1 5EH, UK

[3]Laboratorium voor Microbiologie, Universiteit Gent, K. L. Ledeganckstraat 35, B-9000 Gent, Belgium

[4]Department of Soil Environmental Science, College of Agriculture and Natural Resources, National Chung Hsing University, Taichung, Taiwan

[5]Tropical Soil Biology and Fertility Institute of Centro Internacional de Agricultura Tropical (TSBF-CIAT), A.A. 6713, Cali, Colombia

[6]EMBRAPA-Agrobiologia, km 47, Seropedica, 23851-970 Rio de Janeiro, Brazil

[7]Department of Marine Biotechnology, National Kaohsiung Marine University, Kaohsiung, Taiwan

Fourteen strains were isolated from nitrogen-fixing nodules on the roots of plants of the genus *Mimosa* growing in Taiwan, Brazil and Venezuela. On the basis of 16S rRNA gene sequence similarities, all of the strains were previously shown to be closely related to each other and to belong to the genus *Burkholderia*. A polyphasic approach, including DNA–DNA reassociation, whole-cell protein analysis, fatty acid methyl ester analysis and extensive biochemical characterization, was used to clarify the taxonomic position of these strains: all 14 strains were classified as representing a novel species, for which the name *Burkholderia mimosarum* sp. nov. is proposed. The type strain, PAS44^T (=LMG 23256^T =BCRC 17516^T), was isolated from *Mimosa pigra* nodules in Taiwan.

It is now generally accepted that legumes are not nodulated exclusively by members of the *Rhizobiaceae* in the *Alphaproteobacteria*, but may also be nodulated by members of the *Betaproteobacteria* (so-called 'legume-nodulating *β*-proteobacteria' or '*β*-rhizobia') (Moulin *et al.*, 2001). These include *Cupriavidus taiwanensis* (Chen *et al.*, 2001, 2003a, b; Vandamme & Coenye, 2004) and various *Burkholderia* strains (Moulin *et al.*, 2001; Vandamme *et al.*, 2002), two of which were classified as the novel species *Burkholderia tuberum* and *Burkholderia phymatum* (Vandamme *et al.*, 2002). Until recently, the best evidence for nodulation of legumes by *β*-rhizobia had come from work with strains of *C. taiwanensis*. This species has been isolated from nodules of *Mimosa pudica*, *Mimosa diplotricha* and *Mimosa pigra* (synonym *Mimosa pellita*) in Taiwan (Chen *et al.*, 2001, 2003a, 2005b) and from *M. pudica* in northern and southern India (Verma *et al.*, 2004), and the type strain, LMG 19424^T, has been shown to nodulate *M. pudica* and *M. diplotricha* effectively (Chen *et al.*, 2003b). More recently, there has been a greater focus on *β*-rhizobia in the genus *Burkholderia*, as these are being isolated from *Mimosa* and related species with much greater frequency than is *C. taiwanensis*, particularly in South America and Central America (Barrett & Parker, 2005, 2006; Chen *et al.*, 2005a), but also in Taiwan from the invasive legume *M. pigra* (Chen *et al.*, 2005b). However, with the exception of *Burkholderia caribensis* TJ182, *B. phymatum* STM815^T and *B. tuberum* STM678^T (Vandamme *et al.*, 2002), the taxonomic positions of *Burkholderia* legume symbionts have not yet been described. The aim of the present study was to clarify the taxonomic affiliation of a group of strains – isolated

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain PAS44^T is AY752958.

Tables showing the DNA–DNA binding values, G+C contents and fatty acid compositions of strain PAS44^T and related taxa, together with an extended neighbour-joining phylogenetic tree, are available as supplementary material in IJSEM Online.

Table 1. Sources of the strains analysed

Strain	Host plant	Geographical origin
Reported by Chen *et al.* (2005b)		
PAS44^T (=LMG 23256^T=BCRC 17516^T)	*M. pigra*	Taiwan
PTK1 (=LMG 23330)	*M. pigra*	Taiwan
PTK31	*M. pigra*	Taiwan
PTU38	*M. pigra*	Taiwan
PTU17	*M. pigra*	Taiwan
PTU10	*M. pigra*	Taiwan
Reported by Chen *et al.* (2005a)		
MAP3-1	*M. pigra*	Venezuela
MAP3-2	*M. pigra*	Venezuela
MAP3-3	*M. pigra*	Venezuela
MAP3-4	*M. pigra*	Venezuela
MAP3-5 (=LMG 23331)	*M. pigra*	Venezuela
MAP3-6	*M. pigra*	Venezuela
Br3454	*M. scabrella*	Brazil
Br3467	*M. pigra*	Brazil

from *Mimosa* nodules in Taiwan and South America – that have previously been shown via 16S rRNA gene sequence analyses to be very closely related (Chen *et al.*, 2005a, b).

The strains used in these studies are listed in Table 1. All 14 strains were isolated from root nodules of *M. pigra*, except for Br3454, which was isolated from *Mimosa scabrella* (de Faria *et al.*, 1988). Details of the geographical origins of the strains have been described previously (Chen *et al.*, 2005a, b). All were grown on yeast extract-mannitol agar plates (Vincent, 1970) and incubated at 28 °C unless otherwise indicated. The *Burkholderia* reference strains have been described previously (Vandamme *et al.*, 2002).

The 16S rRNA gene sequences of nine of the strains have been reported by Chen *et al.* (2005a, b) (see Fig. 1). These sequences were compared with published 16S rRNA gene sequences of other *Burkholderia* species, as described previously (Chen *et al.*, 2001, 2005a). The phylogenetic analysis showed that these nine strains form a single cluster (99·5–100·0 % 16S rRNA gene sequence similarity) and belong to the genus *Burkholderia* within the *Betaproteobacteria*. Comparison of the 16S rRNA gene sequences of the nine strains with those of their closest neighbours, *Burkholderia sacchari*, *Burkholderia unamae* and *Burkholderia tropica*, revealed 97·1–97·9, 96·5–97·5 and 96·0–96·5 % similarity, respectively (Fig. 1). Sequence similarities with respect to other *Burkholderia* species were below 96 %.

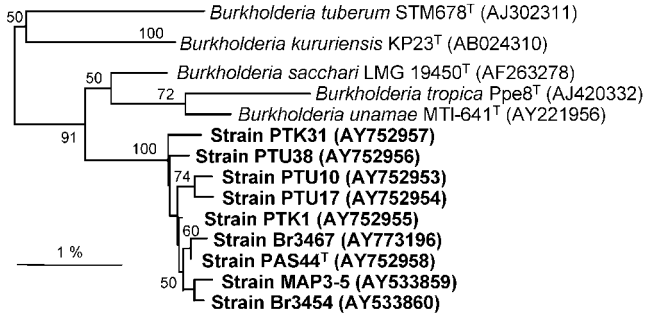

Fig. 1. Neighbour-joining phylogenetic tree of the novel strains (*Burkholderia mimosarum* sp. nov.) and related bacteria, based on 16S rRNA gene sequence comparisons. Accession numbers are given in parentheses. Bar, 1 % sequence dissimilarity. The full tree from which Fig. 1 was taken is available as Supplementary Fig. S1 in IJSEM Online.

DNA samples were prepared from strains PAS44^T, PTK1, MAP3-5, PTU17, PTK31, PTU38, PTU10, Br3467 and Br3454 as described by Pitcher *et al.* (1989). For determination of the DNA G+C contents, DNA was degraded enzymically into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained 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 contents of the strains examined were in the range 63·8–64·8 mol%; the DNA–DNA binding values among these nine strains varied between 79 and 100 % (see Supplementary Table S1 in IJSEM Online). The mean binding values with respect to the closest phylogenetic neighbours, *B. unamae*, *B. tropica* and *B. sacchari*, were found to be 32, 46 and 53 %, respectively (Fig. 1), and values of 56 % or less were calculated in relation to the type strains of other *Burkholderia* species (Supplementary Table S1).

Differentiation of the novel strains from their closest phylogenetic neighbours was examined by using several approaches. For the analysis of protein electrophoretic patterns, strains were grown on nutrient agar (CM3; Oxoid) 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. The preparation of whole-cell proteins and the performance of SDS-PAGE were carried out as described by Pot *et al.* (1994). Densitometric analysis, normalization and interpolation of the protein profiles and numerical analysis using the Pearson product-moment correlation coefficient were performed using the GelCompar 4.2 software package (Applied Maths). Whole-cell protein extracts from the *Mimosa* isolates were prepared and compared with others present in our database. All of the novel strains formed a single cluster with similarities of more than 72 %; this compares with similarities of less than 68 % with other *Burkholderia* species (Fig. 2). For fatty acid methyl ester analyses, 10 µl cell culture was harvested after incubation at 28 °C for 24 h. Fatty acid methyl esters were then prepared, separated and identified using the Microbial Identification System (Microbial ID) as described previously (Vandamme *et al.*, 2002). The fatty acid profiles of strains PAS44^T, PTK1 and MAP3-5 were determined and then compared with those of other *Burkholderia* species (see Supplementary Table S2 available in IJSEM Online). The fatty acid profiles of these three *Mimosa* strains and other reference strains were similar and showed a predominance of the following fatty acids: 16 : 0, 18 : 1ω7*c*, summed feature 2 (comprising 14 : 0 3-OH, 16 : 1 iso I, an unidentified fatty acid with an equivalent chain-length value of 10·928 or 12 : 0 ALDE, or any combination of these fatty acids) and summed feature 3 (comprising 16 : 1ω7*c* or 15 iso 2-OH or both). The fatty acid profile of strain PAS44^T consisted of the following: 14 : 0 (3·6 ± 0·1 %), 16 : 0 (19·1 ± 0·4 %), 16 : 0 2-OH (1·6 ± 0·1 %), 16 : 0 3-OH (4·2 ± 0·1 %), 16 : 1 2-OH (0·9 ± 0·1 %), 17 : 0 cyclo (3·3 ± 0·3 %), 18 : 1ω7*c* (45·6 ± 1·0 %), 18 : 1 2-OH (1·0 ± 0·1 %), 19 : 0 cyclo ω8*c* (1·8 ± 0·2 %) and summed features 2 (5·2 ± 0·1 %) and 3 (12·6 ± 0·6 %). Finally, amplified rDNA restriction analysis of all 14 *Mimosa* strains has been described previously by Chen *et al.* (2005b). The *Burkholderia* strains isolated from Taiwan contained two amplified rDNA restriction analysis types and formed a single cluster together with the Venezuelan strains (MAP3-1, MAP3-2, MAP3-3, MAP3-4, MAP3-5) and the Brazilian strains (Br3454 and Br3467) with at least 91 % banding similarity. This compared with a figure of less than 85 % for banding similarity with other nodulating *Burkholderia* strains (Chen *et al.*, 2005b).

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. Early exponential phase cultures were used as inocula for the test plates (150 µl per well). Plates were incubated at 28 °C and examined after 24 and 48 h to allow the development of a purple colour indicative of substrate oxidation. When the API 20NE microtest gallery was used, the following characteristics were present for all strains: oxidase activity, catalase activity and the assimilation of glucose, arabinose, mannitol, *N*-acetylglucosamine and malate. The following characteristics were uniformly absent: indole production, glucose fermentation, arginine dihydrolase activity, aesculin hydrolysis, gelatin hydrolysis, β-galactosidase activity and the assimilation of maltose, caprate, adipate and citrate. The following characteristics were strain-dependent: nitrate reduction, urease activity and the assimilation of mannose, phenylacetate and gluconate.

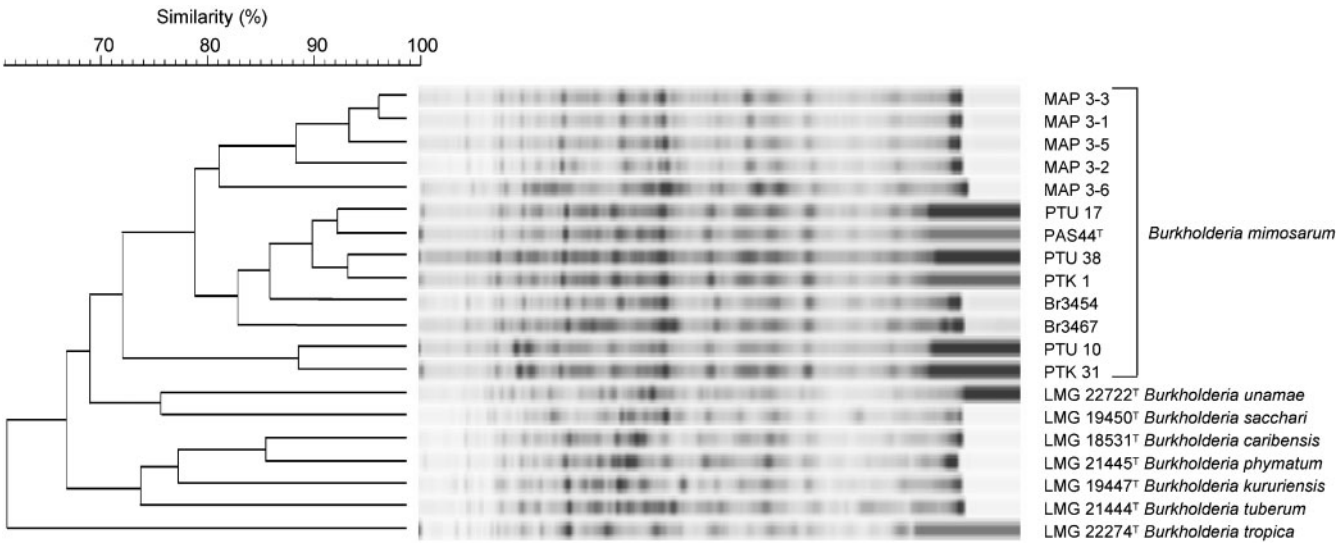

Fig. 2. Dendrogram showing whole-cell protein profiles and the result of a numerical comparison of the protein profiles of the *Mimosa* isolates and type strains of *Burkholderia* species.

When the API ZYM microtest gallery was used, the following characteristics were present in all strains: alkaline phosphatase, C8 lipase, leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities. The following characteristics were uniformly absent: 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 activities. C4 esterase activity was strain-dependent.

When the Biolog GN2 microtitre test system was used, the following substrates were oxidized: dextrin, glycogen, Tweens 40 and 80, *N*-acetyl-D-glucosamine, arabinose, arabitol, i-erythritol, D-fructose, L-fucose, D-galactose, α-D-glucose, *myo*-inositol, D-mannitol, D-mannose, D-sorbitol, methyl pyruvate, monomethyl succinate, acetic acid, *cis*-aconitic acid, citrate, formic acid, D-galacturonic acid, D-glucosaminic acid, α- and β-hydroxybutyric acids, *p*-hydroxyphenylacetic acid, α-ketoglutaric acid, DL-lactate, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, D- and L-alanine, L-aspartic acid, L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-phenylalanine, L-proline, L-pyroglutamic acid, L-serine, L-threonine and γ-aminobutyric acid. None of the strains oxidized α-cyclodextrin, *N*-acetyl-D-galactosamine, adonitol, cellobiose, gentiobiose, α-D-lactose, maltose, D-melibiose, methyl β-D-glucoside, D-raffinose, L-rhamnose, sucrose, D-trehalose, turanose, xylitol, D-glucuronic acid, γ-hydroxybutyric acid, itaconic acid, α-ketovaleric acid, glucuronamide, inosine, uridine, thymidine, 2,3-butanediol, DL-α-glycerol phosphate, glucose 1-phosphate or glucose 6-phosphate. Oxidation of the following substrates was strain-dependent: lactulose, D-psicose, D-galactonic acid lactone, D-gluconic acid, α-ketobutyric acid, malonic acid, succinamic acid, alaninamide, L-alanyl glycine, L-asparagine, glycyl L-aspartic acid, glycyl L-glutamic acid, L-ornithine, D-serine, DL-carnitine, urocanic acid, phenylethylamine, putrescine, 2-aminoethanol and glycerol.

A comparison of the biochemical characteristics of strain PAS44^T with those of the type strains of 20 closely related *Burkholderia* species is shown in Table 2. Strain PAS44^T can be differentiated from *B. sacchari* by the oxidation of adonitol, raffinose and sucrose, and from *B. unamae* and *B. tropica* by the oxidation of adonitol, arabitol, cellobiose, rhamnose and trehalose. Strain PAS44^T is the only strain within the *Burkholderia* cluster tested in this work that is negative for the oxidation of adonitol, rhamnose, sucrose and trehalose.

In conclusion, the present study demonstrated that 14 isolates from root nodules of *M. pigra* and *M. scabrella* from Taiwan, Brazil and Venezuela represent a single species that is readily distinguished from its nearest phylogenetic neighbours by means of their whole-cell protein (Fig. 2) and amplified rDNA restriction analysis profiles (Chen *et al.*, 2005b), the results of DNA–DNA reassociation experiments (Supplementary Table S1) and the biochemical characterization (Table 2). Therefore the 14 novel strains can be classified as representing a novel species, for which we propose the name *Burkholderia mimosarum* sp. nov., with strain PAS44^T as the type strain. Isolates PAS44^T, PTK1,

Table 2. Comparison of the oxidation of carbon sources by strain PAS44^T and type strains of *Burkholderia* species

Species/strain: 1, *B. sacchari*; 2, *B. kururiensis*; 3, *B. phenazinium*; 4, *B. glathei*; 5, *B. cepacia*; 6, *B. pyrrocinia*; 7, *B. vietnamiensis*; 8, *B. glumae*; 9, *B. plantarii*; 10, *B. gladioli*; 11, *B. caryophylli*; 12, *B. andropogonis*; 13, *B. mallei*; 14, *B. pseudomallei*; 15, *B. graminis*; 16, *B. caribensis*; 17, *B. unamae*; 18, *B. tropica*; 19, *B. phymatum*; 20, *B. tuberum*; 21, strain PAS44^T. The data in columns 1–18 are from Brämer *et al.* (2001), Reis *et al.* (2004) and Caballero-Mellado *et al.* (2004).

Carbon source	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Adonitol	+	+	+	+	+	+	−	+	−	+	+	+	−	−	+	+	+	+	+	+	−
Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+
Arabitol	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	−	+	+	+
Cellobiose	−	−	−	−	+	+	+	−	−	−	−	−	−	−	+	−	+	+	−	−	−
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+
Fucose	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+
Lactose	−	−	−	+	−	−	−	−	−	−	−	+	−	−	+	+	−	+	−	−	−
Maltose	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Melibiose	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−
Raffinose	+	−	−	−	−	−	+	+	−	−	+	−	−	−	+	−	−	−	−	−	−
Rhamnose	−	+	+	+	−	−	−	−	+	−	+	−	−	−	+	+	+	+	+	+	−
Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+
Sucrose	+	−	+	−	+	+	+	−	−	−	+	−	+	+	+	−	−	−	+	−	−
Trehalose	−	−	−	−	+	+	+	+	+	+	+	−	+	−	+	+	+	+	−	−	−
Xylitol	−	+	+	+	+	+	−	−	−	−	+	−	−	−	+	+	−	−	+	+	−

PTU38, PTU17, PTU10, PTK31, MAP3-5 and Br3454 effectively nodulated *Mimosa* species, and the presence of *nif* and *nod* genes in the genomes of strains PAS44^T, MAP3-5 and Br3454 has been demonstrated (Chen *et al.*, 2005a, b). Moreover, green fluorescent protein-expressing transconjugant derivatives of PAS44^T and MAP3-5 produced N_2-fixing nodules on their original host, *M. pigra* (Chen *et al.*, 2005a, b). These results strongly confirm that these *Burkholderia* strains can form effective symbioses with legumes of *Mimosa* species.

Description of *Burkholderia mimosarum* sp. nov.

Burkholderia mimosarum [mi.mo.sa′rum. N.L. gen. pl. n. *mimosarum* of mimosas (of *Mimosa* spp.), from which all the strains, including the type strain, were isolated].

Cells are Gram-negative, non-spore-forming and rod-shaped. After 24 h growth on yeast extract-mannitol agar at 28 °C, the mean cell size is about 0·5–0·7 μm (width) × 0·8–2·0 μm (length). Growth is observed at 28, 30 and 37 °C. Catalase- and oxidase-positive. Assimilates glucose, arabinose, mannitol, *N*-acetylglucosamine and malate. Indole is not produced, gelatin and aesculin are not hydrolysed and glucose is not fermented. Does not assimilate maltose, caprate, adipate or citrate. Additional characteristics are listed above. The DNA G+C content is about 63·8–64·8 mol%. Strains have been isolated from root nodules of *Mimosa pigra* and *Mimosa scabrella*.

The type strain, PAS44^T (=LMG 23256^T=BCRC 17516^T), was isolated from *M. pigra* nodules at Anso in south-east Taiwan. The phenotypic characteristics of the type strain are the same as those described for the species. Its DNA G+C content is 64·8 mol%.

Acknowledgements

W.-M. C. was supported by grants from the National Science Council, Taipei, Taiwan, Republic of China (NSC 94-2320-B-022-001 and 94-2313-B-022-001). E. K. J., G. N. E. and J. I. S. were supported by the Natural Environment Research Council (NERC). We thank J. Euzéby for his advice on the nomenclature of the novel species.

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