Burkholderia tropica sp. nov., a novel nitrogen-fixing, plant-associated bacterium

V. M. Reis,[1]† P. Estrada-de los Santos,[2]† S. Tenorio-Salgado,[2] J. Vogel,[3] M. Stoffels,[4] S. Guyon,[5] P. Mavingui,[2,5] V. L. D. Baldani,[1] M. Schmid,[4] J. I. Baldani,[1] J. Balandreau,[3,5] A. Hartmann[4] and J. Caballero-Mellado[2]

Correspondence
J. Caballero-Mellado
jesuscab@cifn.unam.mx

[1]Centro Nacional de Pesquisa de Agrobiologia (EMBRAPA-Agrobiologia), km 47, Seropédica, 23851-970, CP 74505, Rio de Janeiro, Brazil

[2]Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Ap. Postal 565-A, Cuernavaca, Morelos, México

[3]SASA Experiment Station, Private Bag X02, Mt Edgecombe, KZN, 4300 South Africa

[4]GSF – National Research Center for Environment and Health, Institute of Soil Ecology, Department of Rhizosphere Biology, Ingolstädter Landstr.1, D-85764 Neuherberg/Munich, Germany

[5]Ecologie Microbienne, UMR CNRS 5557 Université Claude Bernard Lyon I, 43 Bd du 11 Novembre 1918, 69622 Villeurbanne cedex, France

In an ecological survey of nitrogen-fixing bacteria isolated from the rhizosphere and as endophytes of sugarcane, maize and teosinte plants in Brazil, Mexico and South Africa, a new phylogenetically homogeneous group of N_2-fixing bacteria was identified within the genus *Burkholderia*. This polyphasic taxonomic study included microscopic and colony morphology, API 20NE tests and growth on different culture media at different pH and temperatures, as well as carbon source assimilation tests and whole-cell protein pattern analysis. Analysis of 16S rRNA gene sequences showed 99·2–99·9 % similarity within the novel species and 97·2 % similarity to the closest related species, *Burkholderia sacchari*. The novel species was composed of four distinct amplified 16S rDNA restriction analysis groups. The DNA–DNA reassociation values within the novel species were greater than 70 % and less than 42 % for the closest related species, *B. sacchari*. Based on these results and on many phenotypic characteristics, a novel N_2-fixing species is proposed for the genus *Burkholderia*, *Burkholderia tropica* sp. nov., with the type strain Ppe8^T (=ATCC BAA-831^T=LMG 22274^T=DSM 15359^T). *B. tropica* was isolated from plants grown in geographical regions with climates ranging from temperate subhumid to hot humid.

INTRODUCTION

Thirty different *Burkholderia* species have been described so far (Coenye & Vandamme, 2003). For a long time, N_2-fixing ability in bacteria of the genus *Burkholderia* was recognized only in the species *Burkholderia vietnamiensis* (Gillis *et al.*, 1995). This species was isolated from the rhizosphere of young rice plants grown on a Vietnamese soil under laboratory conditions (Trân Van *et al.*, 1994). Concomitant with the creation of the genus *Burkholderia* in the early 1990s, diazotrophic bacteria were obtained in a survey of root-associated diazotrophs in sugarcane grown in Brazil, and the first indication of their affiliation to the genus *Burkholderia* was gained from partial 23S rRNA gene sequences (Hartmann *et al.*, 1995). On the basis of the 23S rRNA gene sequence data, an oligonucleotide probe (PPe8) was developed, which was characteristic of these isolates (Kirchhof *et al.*, 1997). Among diazotrophic isolates obtained from the banana and pineapple rhizosphere in Brazil, several grouped within the genus *Burkholderia* using 23S rRNA gene oligonucleotide probing and phenotypic techniques (Weber *et al.*, 1999). Two isolates from pineapple showed an amplified 16S rDNA restriction analysis (ARDRA) pattern identical to the sugarcane isolate Ppe8^T (Cruz *et al.*, 2001). Recently, the analysis of N_2-fixing bacteria associated with maize and coffee plants grown under

Abbreviation: ARDRA, amplified 16S rDNA restriction analysis.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequences of the *Burkholderia tropica* strains determined in this work are AJ420332, AY128103, AY321306, AY128105 and AY128104.

†V. M. Reis and P. Estrada-de los Santos contributed equally to this study.

Detailed Biolog results, ARDRA profiles and an extended 16S rRNA gene-based phylogenetic tree are available as supplementary material in IJSEM Online.

field conditions revealed the presence of *B. vietnamiensis*, as well as the richness of novel diazotrophic bacterial species belonging to the genus *Burkholderia* (Estrada-de los Santos *et al.*, 2001; Estrada *et al.*, 2002). These N_2-fixing isolates exhibited high diversity in the ARDRA profiles. In addition, two nodulating strains recovered from legume plants were recently assigned to the genus *Burkholderia* according to their 16S rRNA gene sequences (Moulin *et al.*, 2001). These strains have been formally described as *Burkholderia tuberum* and *Burkholderia phymatum* (Vandamme *et al.*, 2002).

In this study, a polyphasic approach was undertaken to determine the taxonomic status of bacterial isolates recovered from sugarcane, maize and teosinte plants grown in different geographical and climatic regions of Brazil, Mexico and South Africa. The analysis revealed that these isolates belong to a novel species within the genus *Burkholderia*, for which the name *Burkholderia tropica* sp. nov. is proposed.

METHODS

Isolation and cultivation of diazotrophic bacterial strains. The sources of the 41 *Burkholderia* isolates analysed are shown in Table 1. N_2-fixing *Burkholderia* isolates were recovered using three different strategies. In Mexico, the diazotrophic isolates were recovered from the rhizosphere, rhizoplane and inner tissues of maize and teosinte plants using the nitrogen-free semi-solid BAz medium and BAc agar plates as described previously (Estrada-de los Santos *et al.*, 2001). In Brazil, diazotrophic bacteria were isolated from sugarcane in nitrogen-free semi-solid LGI-P medium containing cane juice (Reis *et al.*, 1994). Stems and roots of the sugarcane plants were washed and then macerated and aliquots were inoculated into vials containing semi-solid LGI-P medium. After incubation for 4–5 days at 30 °C, a fine subsurface pellicle was formed. The contents of vials showing pellicles were transferred to semi-solid JMV medium with the following composition (g l^{-1}): 5·0 mannitol, 0·6 K_2HPO_4, 1·8 KH_2PO_4, 0·2 $MgSO_4.7H_2O$, 0·1 NaCl, 0·02, $CaCl_2.2H_2O$, 0·05 yeast extract, 1·6 agar, adjusted to pH 5·5–5·7. New growth was streaked out on JMV or LGI-P solid medium supplemented with yeast extract (100 mg l^{-1}). Grown colonies were inoculated into fresh semi-solid JMV medium and finally transferred to LGI-P solid medium for characterization. *Burkholderia* strains recovered in South Africa were isolated from the roots of sugarcane. Roots were washed gently with tap water and then blended and aliquots were plated onto PCAT medium (Burbage & Sasser, 1982). After incubation for 48 h, bacterial colonies were transferred to PCAT agar plates once more and purified on tryptic soy agar plates.

Phenotypic characterization. Strains were grown at 29 °C unless otherwise indicated. The presence of capsules was determined microscopically by the presence of white haloes surrounding the bacteria suspended in black ink dye (Black ink; Hering ref. 12250). To determine other phenotypic features, cells were prepared by growing the isolates for 12 h in BSE medium (Estrada-de los Santos *et al.*, 2001). Cultures were washed twice in 10 mM $MgSO_4$, adjusted to an OD of 0·2 (3×10^6 c.f.u. ml^{-1}) and each culture was streaked onto solid media. The effects of temperature and pH on growth were determined in BSE agar medium. The effects of temperature and pH on growth and nitrogenase activity of some strains (Ppe5, Ppe6, Ppe7 and Ppe8^T) were also evaluated in a nitrogen-free semi-solid JMV medium. The optimal growth temperature was determined indirectly by measuring nitrogenase activity by the acetylene reduction method (Burris, 1972). Growth on MacConkey agar (Difco) plates as well as on BCSA medium (Henry *et al.*, 1997) was determined after 72 h at 29 and 37 °C. Tests such as oxidase, catalase and hydrolysis of gelatin and Tween 80 were performed by the methods of Smibert & Krieg (1981). Strains were analysed with the API 20NE, API 50CH (bioMérieux) and Biolog MicroLog systems. In the case of API 20NE and API 50CH tests, the inoculation was performed according to the recommendations of the manufacturer (bioMérieux). The results for API 20NE were obtained after 24 or 48 h of incubation as recommended by the manufacturer and API 50CH galleries were obtained after 6 days of incubation. When the Biolog system was used, strains were incubated on biological universal growth medium (Biolog) at 30 °C for 24 h. GN2 microplates were inoculated according to the manufacturer's instructions and incubated at 30 °C for 24 h. The quantitative data of carbon source utilization by each strain were transformed to categories using the CategVar module in ADE-4 software (Biolog).

SDS-PAGE of whole-cell proteins and siderophore production. Preparation of whole-cell proteins as well as SDS-PAGE assays were performed as described previously (Estrada-de los Santos *et al.*, 2001). Siderophores were detected using the universal chemical assays on chromeazurol-S agar plates and in chromeazurol-S solution as described previously (Schwyn & Neilands, 1987). Hydroxamate-type siderophores were identified using the test of Czàky (1948).

ARDRA and sequencing. Genomic DNA was isolated from bacterial cells using published protocols (Kirchhof *et al.*, 1997; Ausubel *et al.*, 1987). Primers fD1 and rD1 were used for amplification of the 16S rRNA gene (Weisburg *et al.*, 1991) using PCR conditions described previously (Estrada-de los Santos *et al.*, 2001). The amplified 16S rRNA genes were restricted with *Alu*I, *Dde*I, *Hae*III, *Hha*I, *Hin*fI, *Msp*I and *Rsa*I. The restriction fragments were separated by electrophoresis in 3 % agarose gels and the patterns were compared. Each isolate was assigned to one of the ARDRA genotypes 16, 17 or 19 as described previously (Estrada-de los Santos *et al.*, 2001). For the strain Ppe8^T, an almost full-length bacterial 16S rRNA gene fragment was amplified by PCR as described by Juretschko *et al.* (1998) and sequenced by Sequiserve (Vaterstetten, Germany). For strains MOc-725, MTo-672 and MTo-293, PCR products were cloned first into the pCRII vector (Invitrogen). 16S rRNA genes were restricted into small fragments (0·3–0·8 kb) using *Eco*RI and subcloned into vector pUC18. 16S rRNA gene sequencing was performed by Medigenomix. The sequences of both strands were determined using universal primers for the pUC18 vector.

DNA base composition and DNA–DNA relatedness analysis. The mean mol% G+C content of genomic DNA was measured by the DSMZ. DNA–DNA relatedness was based on relative levels of hybridization to ^{32}P-labelled DNA as described previously (Estrada-de los Santos *et al.*, 2001).

Species-specific PCR primers. Available *Burkholderia* 16S rRNA gene sequences were aligned to identify regions specific for the novel species; a region corresponding to positions 456–475 of *Escherichia coli* (GenBank accession no. V00348) was identified. This region was chosen to define the forward primer 5′-TCCCTGGTCCTAATATG-3′. The reverse primer (5′-CAACCCTCTGTTCCGA-3′) was identified in a 16S rRNA gene region described previously (Pallud *et al.*, 2001). PCR conditions were as follows: initial denaturation for 7 min at 95 °C followed by 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 48 °C and 1 min elongation at 72 °C, followed by a final 15 min elongation at 72 °C.

Table 1. Source and locality of *Burkholderia tropica* sp. nov. strains analysed

B. tropica strains have been deposited in the ATCC and Collection de l'Institut Pasteur (CIP) with the following numbers: strain MOc-725 (ATCC BAA-567; CIP 107590), strain MTo-672 (ATCC BAA-568; CIP 107591) and strain MTo-293 (ATCC BAA-569; CIP 107592). Climate class codes: 1, semi-hot subhumid; 2, semi-hot humid; 3, hot subhumid; 4, hot humid; 5, temperate subhumid. ND, Not determined.

Strain	Source	Soil pH	Country	Locality	Climate class	Reference
ARDRA profile 16						
MCh-1054	Maize roots*	6·76	Mexico	Chilpancingo, Guerrero	1	This study
MCh-1057	Maize rhizoplane	6·76	Mexico	Chilpancingo, Guerrero	1	This study
MCo-7712	Maize roots*	6·69	Mexico	Coatepec, Veracruz	2	This study
MOc-255	Maize rhizosphere	6·40	Mexico	Ocotepec, Morelos	3	Estrada-de los Santos *et al.* (2001)
MOc-332	Maize rhizosphere	6·22	Mexico	Ocotepec, Morelos	3	This study
MOc-725	Maize rhizoplane	6·35	Mexico	Ocotepec, Morelos	3	Estrada-de los Santos *et al.* (2001)
MOc-3412	Maize roots*	6·55	Mexico	Ocotepec, Morelos	3	This study
MTe-73523	Maize roots*	5·04	Mexico	Tepoztlan, Morelos	1	This study
MTe-7363	Maize rhizoplane	5·04	Mexico	Tepoztlan, Morelos	1	This study
MTl-5681	Maize roots*	4·56	Mexico	Tlayacapan, Morelos	1	This study
MTl-582	Maize rhizosphere	4·84	Mexico	Tlayacapan, Morelos	1	This study
TTe-225	Teosinte rhizosphere	7·10	Mexico	Tepoztlan, Morelos	1	This study
ARDRA profile 17						
MCa-9022	Maize rhizosphere	5·15	Mexico	Cacaohatan, Chiapas	4	This study
MCo-7931	Maize rhizosphere	6·89	Mexico	Coatepec, Veracruz	2	This study
MCu-831	Maize rhizoplane	7·07	Mexico	Cuernavaca, Morelos	1	This study
MMi-786	Maize rhizoplane	ND	Mexico	Miacatlán, Morelos	3	Estrada-de los Santos *et al.* (2001)
MSj-805	Maize rhizosphere	6·20	Mexico	San J. Atenco, Puebla	5	This study
MTl-6311	Maize rhizoplane	4·87	Mexico	Tlayacapan, Morelos	1	This study
MTo-432	Maize rhizoplane	ND	Mexico	Totontepec, Oaxaca	2	Estrada-de los Santos *et al.* (2001)
MTo-672	Maize stem*	ND	Mexico	Totontepec, Oaxaca	2	This study
MXo-435	Maize rhizoplane	7·15	Mexico	Xoxocotla, Morelos	3	This study
MXo-437	Maize rhizosphere	7·15	Mexico	Xoxocotla, Morelos		This study
TSj-832	Teosinte rhizosphere	6·29	Mexico	San J. Atenco, Puebla	5	This study
TTe-1910	Teosinte stem*	7·03	Mexico	Tepoztlan, Morelos	1	This study
ARDRA profile 19						
Ppe8^T	Sugarcane stem	ND	Brazil	Pernambuco State	4	This study
BM-16	Maize stem*	ND	Mexico	Totontepec, Oaxaca	2	Estrada *et al.* (2002)
BM-273	Maize roots*	ND	Mexico	Totontepec, Oaxaca	2	Estrada *et al.* (2002)
MCo-761	Maize roots*	6·79	Mexico	Coatepec, Veracruz	2	This study
MCo-8562	Maize rhizoplane	6·62	Mexico	Coatepec, Veracruz	2	This study
MCu-82	Maize rhizosphere	7·07	Mexico	Cuernavaca, Morelos	1	This study
MCu-833	Maize roots*	7·07	Mexico	Cuernavaca, Morelos	1	This study
MCu-842	Maize rhizoplane	7·07	Mexico	Cuernavaca, Morelos	1	This study
MSj-8432	Maize rhizosphere	6·29	Mexico	San J. Atenco, Puebla	5	This study
MTo-16	Maize rhizosphere	ND	Mexico	Totontepec, Oaxaca	2	Estrada-de los Santos *et al.* (2001)
MTo-293	Maize stem*	ND	Mexico	Totontepec, Oaxaca	2	Estrada-de los Santos *et al.* (2001)
ARDRA profile 19a						
LM1-376.8	Sugarcane roots	4·30	South Africa	Tongaat, KwaZulu-Natal	1	This study
LM2-376.3	Sugarcane roots	4·98	South Africa	Tongaat, KwaZulu-Natal	1	This study
RASC	Activated sludge	–	USA	Oregon State	–	Suwa *et al.* (1996)
Not profiled						
Ppe5	Sugarcane stem	ND	Brazil	Pernambuco State	4	This study
Ppe6	Sugarcane stem	ND	Brazil	Pernambuco State	4	This study
Ppe7	Sugarcane stem	ND	Brazil	Pernambuco State	4	This study

*Surface-sterilized.

RESULTS AND DISCUSSION

Growth and phenotypic characteristics of *Burkholderia* isolates

N_2-fixing *Burkholderia* isolates analysed in the present work were recovered from the rhizosphere, rhizoplane, roots and stems of maize, teosinte and sugarcane plants grown in different geographical regions from Brazil, Mexico and South Africa with climates ranging from temperate sub-humid to hot humid (Table 1). In nitrogen-free LGI-P and BAz enriched semi-solid media, the bacterial growth formed a thin yellowish pellicle approximately 2–4 mm below the surface. When the bacterial growth was transferred to LGI-P solid medium, small colonies were formed with a yellow centre and white margins. Colonies growing on BAc medium plates were yellowish, round, smooth and convex, 1–2 mm in diameter, with entire margins after incubation for 4 days as described previously (Estrada-de los Santos *et al.*, 2001). In terms of morphological features, the novel *Burkholderia* isolates were characterized as rod-shaped (0·7–0·8 × 1·5–1·6 μm) Gram-negative bacteria. Cells appeared encapsulated, very motile due to the presence of several (one to four) polar flagella (Fig. 1) and possessed peritrichous fimbriae (data not shown). Spores were not observed but poly-β-hydroxybutyrate granules were viewed under transmission electron microscopy (data not shown). The isolates were oxidase, catalase and urease positive and were able to hydrolyse Tween 80, but not gelatin or starch. Nitrate was reduced to nitrite, but nitrite was not further reduced. The novel isolates grew and showed nitrogenase activity under microaerobic conditions in nitrogen-free semi-solid JMV, LGI-P and BAz media (Table 2), except for strain RASC. Growth and nitrogenase activity were

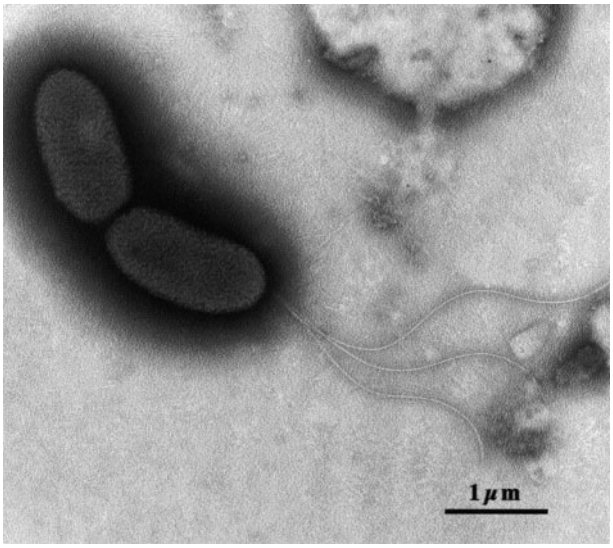

Fig. 1. Transmission electron micrograph of a cell of *B. tropica* sp. nov. strain BM273 showing three polar flagella. Negative staining (aqueous sodium silicotungstate 1 % for 30 s). Photo courtesy of René Rohr (University of Lyon 1). Bar, 1 μm.

Table 2. Comparison of *B. tropica* sp. nov. with other related *Burkholderia* species

Species/strains: 1, *B. tropica* sp. nov. (n=38, including the type strain); 2, *B. sacchari* IPT 101^T; 3, *B. vietnamiensis* (n=30, including the type strain); 4, *B. kururiensis* KP23^T; 5, *B. cepacia* LMG 1222^T. +, Good growth; ±, poor growth; −, no growth; ND, no data available. All species are positive for growth on BSE agar medium at 37 °C and for oxidase [data for *B. sacchari* from Brämer *et al.* (2001) and for *B. kururiensis* from Zhang *et al.* (2000)].

Characteristic	1	2	3	4	5
C_2H_2 reduction activity (N_2 fixation) in N-free semi-solid LGI-P, JMV and BAz media	+	−	+	+	−
Growth on:					
BAc medium	+	±	+	−	+
MacConkey medium at 29 °C	+	−	+	−	+
MacConkey medium at 37 °C	−	−	+	−	+
BCSA medium	−	−	+	−	+
BSE agar medium at 42 °C	−	−	+	+	+
API 50CH test:					
Urease	+*	−	−	−	−
Gelatinase	−	−[a]†	−	−[b]	+
Nitrate reduced to nitrite	+	+[a]	+	+	−
Optimum growth temperature (°C)	30	28–30[a]	30	37[b]	ND
pH range for growth	4·5–7·0	ND	6·5–7·5	6·0–7·8[b]	ND
DNA G+C content (mol%)	63·5	63·7[a]	66·9–68·1	64·8[b]	66·9[c]

**B. tropica* strains gave a negative reaction using the Ferguson medium.

†Data taken from: *a*, Brämer *et al.* (2001); *b*, Zhang *et al.* (2000); *c*, Yabuuchi *et al.* (1992).

stimulated by the addition of yeast extract (100 mg l^{-1}). Optimum temperature for N_2-dependent growth was 30 °C, although they still could fix N_2 at 25 and 36 °C; growth at 40 °C was very poor and no growth was observed below 7 °C or above 42 °C. Similarly, all of the isolates grew on BSE agar medium at 37 °C but not at 42 °C. The novel isolates grew well at pH values between 4·5 and 6·5, but poor growth was observed at pH 7·0–7·5; optimal growth was observed between pH 5·0 and 5·8. While strains of *B. tropica* sp. nov. were found in the rhizosphere and associated with plants growing in soils with a pH in the range 4·5–7·1, isolation from maize plants cultivated on soils with a pH higher than 7·5 was unsuccessful. This result is in accordance with the good growth of the novel isolates on culture media with a pH 4·5–6·5. Bacteria of the genus *Burkholderia* are considered to be characteristic of neutral pH environments (Liesack *et al.*, 1997), but recent evidence suggests a high abundance of *Burkholderia* in acidic soils (Nogales *et al.*, 2001).

Although the expression of N_2-fixing genes in an endosymbiotic *Burkholderia* species has been observed (Minerdi *et al.*, 2001), among the hitherto-described species of the genus *Burkholderia*, N_2-fixing ability has only been found in *B. vietnamiensis* (Gillis *et al.*, 1995) and *Burkholderia kururiensis* (Estrada-de los Santos *et al.*, 2001). In addition, the presence of the *nifH* gene encoding dinitrogenase reductase, a key enzyme in N_2 fixation, has been detected (Moulin *et al.*, 2001) in the recently described nodulating species *B. tuberum* (Vandamme *et al.*, 2002). In the present study, diazotrophic *Burkholderia* isolates recovered from plants grown in Brazil, Mexico and South Africa shared a high similarity in their phenotypic and genomic traits, but exhibited little resemblance to other *Burkholderia* species. Phenotypic characteristics for the differentiation of *B. tropica* sp. nov. from other N_2-fixing *Burkholderia* species as well as from related *Burkholderia* species are shown in Table 2. Differences in the usage of carbon sources by the novel isolates and other related *Burkholderia* species according to the API 50CH test are shown in Table 3. According to the Biolog carbon source utilization tests, a broad spectrum of sugars and alcohols were used (see supplementary data in IJSEM Online).

SDS-PAGE of whole-cell proteins and siderophore production

Whole-cell protein extracts were prepared from 38 strains of *B. tropica* sp. nov. and from several related species. Strains isolated from maize, teosinte and sugarcane plants growing in different regions of Brazil, Mexico and South Africa showed almost identical protein patterns (Fig. 2). These protein patterns were clearly different from other N_2-fixing and from non-N_2-fixing *Burkholderia* species. All of the isolates corresponding to ARDRA genotypes 16, 17 and 19 showed the ability to produce siderophores. Ninety-two per cent of the strains produced hydroxamates as the main type of siderophore.

Table 3. Discriminatory carbon source assimilation of *B. tropica* sp. nov. compared with other related *Burkholderia* species based on the API 50CH system

Species/strains: 1, *B. tropica* sp. nov. ($n=38$); 2, *B. sacchari* IPT 101^T; 3, *B. cepacia* LMG 1222^T; 4, *B. vietnamiensis* TVV75^T; 5, *B. kururiensis* KP23^T; 6, *B. caribensis* MWAP64^T. All of the *B. tropica* strains assimilated the following substrates: *N*-acetylglucosamine, DL-arabinose, D-arabitol, D-fructose, galactose, gluconate, D-glucose, glycerol, inositol, 2-ketogluconate, malate, mannitol, D-mannose, phenylacetate, sorbitol and D-xylose. None of the *B. tropica* strains assimilated erythritol, glycogen, inulin, melezitose, melibiose, methyl α-glucoside, methyl α-D-mannoside, methyl β-xyloside, L-sorbose, starch or D-turanose. *B. tropica* strains: +, >95 % of strains gave a positive reaction; −, >95 % of strains gave a negative reaction; V+, 55–70 % of strains gave a positive reaction.

Substrate	1	2	3	4	5	6
Adonitol	+	+	+	−	+	+
Cellobiose	+	−	+	+	−	+
D-Fucose	+	+	−	+	+	−
L-Fucose	+	+	+	−	+	+
β-Gentiobiose	+	−	+	+	−	−
Rhamnose	+	−	−	−	+	+
Ribose	+	−	+	+	+	+
Lactose	V+	−	−	−	−	−
D-Lyxose	V+	+	+	+	+	−
Salicin	V+	−	+	+	−	−
Trehalose	V+	−	+	+	−	−
Amygdalin	−	−	+	+	−	−
L-Arabitol	−	−	+	−	+	+
Arbutin	−	−	+	−	−	−
Dulcitol	−	−	+	+	−	−
5-Ketogluconate	−	−	+	−	+	−
Maltose	−	−	−	−	−	+
D-Raffinose	−	+	+	+	−	−
Sucrose	−	+	+	+	−	−
D-Tagatose	−	−	+	+	−	−
Xylitol	−	−	+	−	+	+
L-Xylose	−	−	−	−	+	−

ARDRA and phylogenetic 16S rRNA gene analysis

A group of N_2-fixing *Burkholderia* isolates, designated ARDRA genotypes 16, 17 and 19, was described previously (Estrada-de los Santos *et al.*, 2001). These genotypes showed identical ARDRA profiles with enzymes *Alu*I, *Dde*I, *Hae*III, *Hha*I, *Msp*I and *Rsa*I, but could be distinguished by the enzyme *Hin*fI (see Supplementary Fig. A in IJSEM Online). Strain Ppe8^T showed an ARDRA profile identical to genotype 19. Strains LM1-376.8, LM2-376.3 and RASC showed the same profiles as genotype 19 except with *Hae*III, and therefore they were designated ARDRA genotype 19a (Table 1). When the 16S rRNA gene was restricted with each of the seven different enzymes, the sum of the fragments was approximately 1·5 kb, except in the case of strains

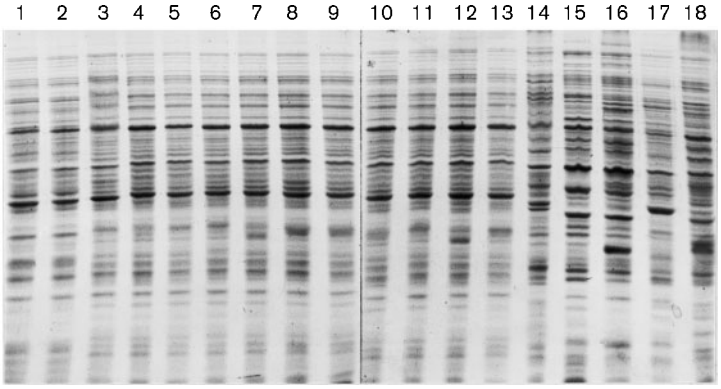

Fig. 2. SDS-PAGE of selected *B. tropica* sp. nov. strains and *Burkholderia* reference strains. Lanes: 1–13, *B. tropica* strains MOc-725, TTe-225, MTe-73523, MTo-293, LM1-376.8, LM2-376.3, MTo-672, TTe-1910, MXo-435, MCh-1057, TSj-832, MSj-8432 and Ppe8^T respectively; 14, *B. vietnamiensis* TVV75^T; 15, *B. kururiensis* KP23^T; 16, '*B. brasilensis*' M130; 17, *B. caribensis* MWAP64^T; 18, *B. cepacia* LMG 1222^T.

corresponding to ARDRA genotype 17, which were greater than 2·0 kb with the *Hin*fI enzyme (see Supplementary Fig. A, lane 8, in IJSEM Online). A possible explanation for the larger size could be the amplification of distinct copies of these genes, some of which lack one of the restriction sites. It is known that bacteria have up to 15 copies of ribosomal operons and that they have internal differences (Klappenbach *et al.*, 2000).

The 16S rRNA gene sequence of strain Ppe8^T as well as sequences for isolates MOc-725, MTo-672, MTo-293 and LM2-376.3, representing different ARDRA genotypes, were compared with available 16S rRNA gene sequences from all of the *Burkholderia* species. The phylogenetic tree shown in Fig. 3 illustrates the position of the novel isolate group, *B. tropica* sp. nov., relative to other *Burkholderia* species. Strains Ppe8^T, MTo-293, LM2-376.3, MOc-725 and MTo-672 were closely related, forming a cluster with strains BM16 and BM273 described in a previous study (Estrada *et al.*, 2002), as well as with strain AB98 (Cruz *et al.*, 2001). The similarity among the 16S rRNA gene sequences of these strains ranged from 99·2 to 99·9 %. The N_2-fixing species *B. tropica* clearly constituted a well-supported cluster separate from the cluster formed by the diazotrophic species *B. kururiensis*/*B. tuberum*. *Burkholderia sacchari*, a non-diazotrophic bacterium, was the closest species to the *B. tropica* cluster (97·2 % similarity). The N_2-fixing species *B. vietnamiensis*, which belongs to the second major lineage of *Burkholderia* comprising the '*Burkholderia cepacia* complex' (Vandamme *et al.*, 1997), appeared distantly related to *B. tropica* with a sequence similarity of <96 % (Supplementary Fig. B). Since 97 % is the threshold 16S rRNA gene similarity level for the delineation of bacterial species (Stackebrandt & Goebel, 1994), *B. tropica* could be clearly differentiated from the closely related species *B. sacchari* and *B. tuberum* (96·2 % similarity).

DNA–DNA relatedness analysis

Strains MOc-725 (genotype 16), MTo-672 (genotype 17) and MTo-293 (genotype 19), as well as strains LM1-376.8, LM2-376.3 and RASC (genotype 19a), were related to strain Ppe8^T with DNA–DNA reassociation values of 74–97·3 %. Type strains of closely related *Burkholderia* species such as *B. sacchari*, *B. tuberum*, *B. phymatum*, *B. kururiensis*, *B. cepacia*, *Burkholderia graminis* and *Burkholderia caribensis* exhibited low DNA–DNA reassociation levels, ranging from 41 to 9·8 %, with strain Ppe8^T.

Species-specific PCR

Results showed that PCR amplification was strictly specific for *B. tropica* strains. An 800 nt amplicon was obtained with *B. tropica* strains (Ppe8^T, SMi-583, MMi-786, MTo-431, BM16, BM273, MTo-293, LM1-376.8 and LM2-376.3) but not with type strains of other related *Burkholderia* species such as *Burkholderia caledonica*, *B. caribensis*, *B. cepacia*, *Burkholderia fungorum*, *B. graminis*, *Burkholderia phenazinium*, *Burkholderia thailandensis* and *B. vietnamiensis*, nor with six strains of *Burkholderia cenocepacia* (data not shown).

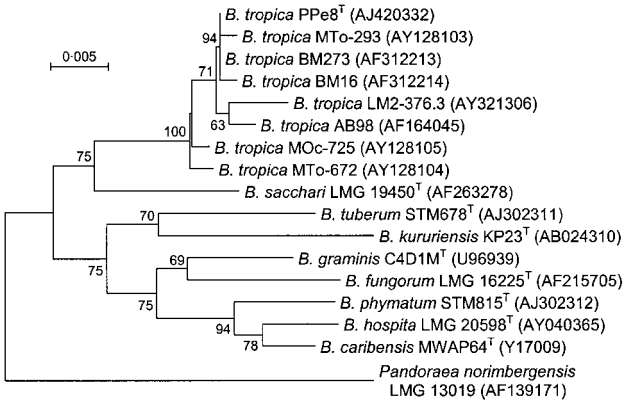

Fig. 3. Phylogenetic tree based on 16S rRNA gene sequences showing the relatedness among *B. tropica* sp. nov. and the nearest *Burkholderia* species. Phylogenetic relationships were estimated according to Jukes & Cantor (1969) and the tree was constructed by the neighbour-joining method (Saitou & Nei, 1987). The alignment included 1349 nt. Bootstrap probabilities (Kumar *et al.*, 1993) are indicated at the branch points. The bar represents 5 nt substitutions per 1000 nt. The GenBank accession number for each strain is shown in parentheses.

Taxonomic considerations

Many phenotypic features and all of the genomic characteristics described above agree with similarity criteria recommended for the delineation of bacterial species (Vandamme *et al.*, 1996). Accordingly, we consider that the strains analysed in this work belong to a novel plant-associated N_2-fixing bacterial species within the genus *Burkholderia* and propose the name *Burkholderia tropica* sp. nov.

Description *of Burkholderia tropica* sp. nov.

Burkholderia tropica (L. fem. adj. *tropica* tropical).

Cells are slightly curved rods, approximately 1·5–1·6 μm long and 0·7–0·8 μm wide. They occur singly and possess a capsule. They are motile by means of one to four polar flagella. Isolates are Gram negative and oxidase and catalase positive. On LGI-P solid medium, small colonies are formed with a yellow centre and white margins. Colonies on BAc plates are yellowish, round, smooth and convex with entire margins. Growth and acetylene reduction to ethylene are observed in nitrogen-free semi-solid media. Strains have an aerobic metabolism but fix nitrogen microaerobically. They grow well with ammonium, and nitrate is reduced to nitrite, but nitrite is not further reduced. Nitrate and ammonium inhibit N_2 fixation, but small amounts of yeast extract (100 mg l^{-1}) enhance N_2 fixation. Several carbon sources support growth, including sugars and organic acids. Growth occurs from 22 to 40 °C and the optimum temperature is 30 °C. No hydrolysis of starch or gelatin is observed, but Tween 40 and Tween 80 are hydrolysed. Characteristics that differentiate *B. tropica* from other N_2-fixing *Burkholderia* species are listed in Tables 2 and 3. *B. tropica* can also be differentiated from other N_2-fixing and non-N_2-fixing *Burkholderia* species by 16S rRNA gene PCR primers. The species *B. tropica* comprises four different ARDRA genotypes. It has a G+C content of 63·5 mol%.

The type strain is strain Ppe8^T (=ATCC BAA-831^T=LMG 22274^T=DSM 15359^T), isolated from sugarcane var. SP 71-1406 grown in the fields of the Cruangi Sugar factory located in Pernambuco State, Brazil.

ACKNOWLEDGEMENTS

This study is dedicated to J. Döbereiner, in memoriam. This work was funded in part by Pronex II/CNPq (grant 661309/97-5), which supported the Brazilian partnership, and Conselho Nacional de Pesquisa e Desenvolvimento (CNPq)-Brazil for a PhD fellowship. Thanks also are due to the German Brazilian Scientific & Technological co-operation (grant BRA ENV 57) by the BMBF-Berlin and CNPq-Brazil. This research was also partially funded by Consejo Nacional de Ciencia y Tecnología (CONACyT)-México (grant 33576-V) and by grants from BGR (Bureau des Ressources Génétiques) and PICS 1061 from CNRS-France. We thank Dr M. Dunn for constructive English corrections, as well as G. Paredes-Valdez and L. Martínez-Aguilar for technical assistance. We are indebted to Pr. R. Rohr (University of Lyon 1) for electron microscopy and J. H. Omarjee for 16S rRNA gene sequences of South African strains LM1-376.8 and LM2-376.3. We also acknowledge the help of I. Caballero-Mellado (Instituto Nacional de Estadística, Geografía e Informática, México) for detailed information on climate class, and M. Carcaño, J. Leyva and A. Morett for plant collection. Thanks are also due to CONACyT and to Dirección General de Estudios de Posgrado (DGEP)-UNAM for a PhD fellowship to P. E.-de los S.

REFERENCES

Ausubel, F. M., Brent, R., Kingston, R. E., More, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1987). *Current Protocols in Molecular Microbiology*. New York: Wiley.

Brämer, C. O., Vandamme, P., da Silva, L. F., Gomez, J. G. C. & Steinbüchel, A. (2001). *Burkholderia sacchari* sp. nov., a polyhydroxyalkanoate-accumulating bacterium isolated from soil of a sugar-cane plantation in Brazil. *Int J Syst Evol Microbiol* **51**, 1709–1713.

Burbage, D. A. & Sasser, M. (1982). A medium selective for *Pseudomonas cepacia*. *Phytopathology* **76**, 706.

Burris, R. H. (1972). Nitrogen fixation assay – methods and techniques. *Methods Enzymol* **24B**, 415–431.

Coenye, T. & Vandamme, P. (2003). Diversity and significance of *Burkholderia* species occupying diverse ecological niches. *Environ Microbiol* **5**, 719–729.

Cruz, L. M., Maltempi-de Souza, E., Weber, O. B., Baldani, J. I., Döbereiner, J. & Pedrosa, O. (2001). 16S ribosomal DNA characterization of nitrogen-fixing bacteria isolated from banana (*Musa* spp.) and pineapple (*Ananas comosus* (L.) Merril). *Appl Environ Microbiol* **67**, 2375–2379.

Czàky, T. Z. (1948). On the estimation of bound hydroxylamine in biological materials. *Acta Chem Scand* **2**, 450–454.

Estrada-de los Santos, P., Bustillos-Cristales, R. & Caballero-Mellado, J. (2001). *Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. *Appl Environ Microbiol* **67**, 2790–2798.

Estrada, P., Mavingui, P., Cournoyer, B., Fontaine, F., Balandreau, J. & Caballero-Mellado, J. (2002). A N_2-fixing endophytic *Burkholderia* sp. associated with maize plants cultivated in Mexico. *Can J Microbiol* **48**, 285–294.

Gillis, M., Trân Van, V., Bardin, R. & 7 other authors (1995). Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp. nov. for N_2-fixing isolates from rice in Vietnam. *Int J Syst Bacteriol* **45**, 274–289.

Hartmann, A., Baldani, J. I., Kirchhof, G., Assmus, B., Hutzler, P., Springer, N., Ludwig, G. W., Baldani, V. L. & Döbereiner, J. (1995). Taxonomic and ecological studies of diazotrophic rhizosphere bacteria using phylogenetic probes. In *Azospirillum VI and Related Microorganisms: Genetics, Physiology and Ecology*, pp. 416–427. Edited by I. Fendrick, M. del Gallo, J. Vanderleyden & M. Zamaroczy. Berlin: Springer.

Henry, D. A., Campbell, M. E., LiPuma, J. J. & Speert, D. P. (1997). Identification of *Burkholderia cepacia* isolates from patients with cystic fibrosis and use of a simple new selective medium. *J Clin Microbiol* **35**, 614–619.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K.-H., Pommerening-Röser, A., Koops, H. P. & Wagner, M. (1998). Combined molecular and conventional analyses of nitrifying

bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. *Appl Environ Microbiol* **64**, 3042–3051.

Kirchhof, G., Schloter, M. Aßmus B. & Hartmann, A. (1997). Molecular microbial ecology approaches applied to diazotrophs associated with non-legumes. *Soil Biol Biochem* **29**, 853–862.

Klappenbach, J. A., Dunbar, J. M. & Schmidt, T. M. (2000). rRNA operon copy number reflects ecological strategies of bacteria. *Appl Environ Microbiol* **66**, 1328–1333.

Kumar, S., Tamura, K. & Nei, M. (1993). MEGA: molecular evolutionary genetics analysis, version 1.0. University Park, PA: The Pennsylvania State University.

Liesack, W., Janssen, P. H., Rainey, F. A., Ward-Rainey, N. L. & Stackebrandt, E. (1997). Microbial diversity in soil: the need for a combined approach using molecular and cultivation techniques. In *Modern Soil Microbiology*, pp. 375–439. Edited by J. D. Van Elsas, J. T. Trevors & E. M. H. Wellington. New York: Marcel Dekker.

Minerdi, D., Fani, R., Gallo, R., Boarino, A. & Bonfante, P. (2001). Nitrogen fixation genes in an endosymbiotic *Burkholderia* strain. *Appl Environ Microbiol* **67**, 725–732.

Moulin, L., Munive, A., Dreyfus, B. & Boivin-Masson, C. (2001). Nodulation of legumes by members of the beta-subclass of Proteobacteria. *Nature* **411**, 948–950.

Nogales, B., Moore, E. R. B., Llobet-Brossa, E., Rossello-Mora, R., Amman, R. & Timmis, K. N. (2001). Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. *Appl Environ Microbiol* **67**, 1874–1884.

Pallud, C., Viallard, V., Balandreau, J., Normand, P. & Grundmann, G. (2001). Combined use of a specific probe and PCAT medium to study *Burkholderia* in soil. *J Microbiol Methods* **47**, 25–34.

Reis, V. M., Olivares, F. L. & Döbereiner, J. (1994). Improved methodology for isolation of *Acetobacter diazotrophicus* and confirmation of its endophytic habitat. *World J Microbiol Biotechnol* **10**, 101–104.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Schwyn, B. & Neilands, J. B. (1987). Universal chemical assay for detection and determination of siderophores. *Anal Biochem* **160**, 47–56.

Smibert, R. M. & Krieg, N. R. (1981). General characterization. In *Manual of Methods for General Bacteriology*, pp. 409–443. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costillo, E. W. Nester, W. A. Wood, N. R. Krieg & G. B. Phillips. Washington, DC: American Society for Microbiology.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Suwa, Y., Wright, A. D., Fukimori, F., Nummy, K. A., Hausinger, R. P., Holben, W. E. & Forney, L. J. (1996). Characterization of a chromosomally encoded 2,4-dichlorophenoxiacetic acid/α-ketoglutarate dioxygenase from *Burkholderia* sp. strain RASC. *Appl Environ Microbiol* **62**, 2464–2469.

Trân Van, V., Mavingui, P., Berge, O., Balandreau, J. & Heulin, T. (1994). Promotion de croissance du riz inoculé par une bactérie fixatrice d'azote, *Burkholderia vietnamiensis*, isolée d'un sol sulfaté acide du Viêt-nam. *Agronomie* **14**, 697–707 (in French).

Vandamme, P., Pot, B., Gillis, M., de Vos, P., Kersters, K. & Swings, J. (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol Rev* **60**, 407–438.

Vandamme, P., Holmes, B., Vancanneyt, M. & 8 other authors (1997). Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal of *Burkholderia multivorans* sp. nov. *Int J Syst Bacteriol* **47**, 1188–1200.

Vandamme, P., Goris, J., Chen, W.-M., de Vos, P. & Willems, A. (2002). *Burkholderia tuberum* sp. nov. and *Burkholderia phymatum* sp. nov., nodulate the roots of tropical legumes. *Syst Appl Microbiol* **25**, 507–512.

Weber, O. B., Baldani, V. L. D., Teixeira, K. R. S., Kirchhof, G., Baldani, J. I. & Döbereiner, J. (1999). Isolation and characterization of diazotrophic bacteria from banana and pineapple plants. *Plant Soil* **210**, 103–113.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.

Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T. & Arakawa, M. (1992). Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiol Immunol* **36**, 1251–1275.

Zhang, H., Hanada, S., Shigematsu, T., Shibuya, K., Kamagata, Y., Kanagawa, T. & Kurane, R. (2000). *Burkholderia kururiensis* sp. nov., a trichloroethylene (TCE)-degrading bacterium isolated from an aquifer polluted with TCE. *Int J Syst Evol Microbiol* **50**, 743–749.