Burkholderia unamae sp. nov., an N₂-fixing rhizospheric and endophytic species

Jesús Caballero-Mellado, Lourdes Martínez-Aguilar,
Guadalupe Paredes-Valdez and Paulina Estrada-de los Santos

Correspondence

Jesús Caballero-Mellado
jesuscab@cifn.unam.mx

Programa de Ecología Molecular y Microbiana, Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Apdo. Postal 565-A, Cuernavaca, Morelos, México

It was shown recently that the genus *Burkholderia* is rich in N₂-fixing bacteria that are associated with plants. A group of these diazotrophic isolates with identical or very similar 16S rDNA restriction patterns [designated amplified rDNA restriction analysis (ARDRA) genotypes 13, 14 and 15] was selected and a polyphasic taxonomic study was performed, which included new isolates that were recovered from rhizospheres, rhizoplanes or internal tissues of maize, sugarcane and coffee plants. Morphological, physiological and biochemical features, as well as multi-locus enzyme electrophoresis profiles and whole-cell protein patterns, of 20 strains were analysed. In addition, analysis of cellular fatty acid profiles, 16S rDNA sequence analysis and DNA–DNA reassociation experiments were performed with representative strains. The taxonomic data indicated that the strains analysed belong to a novel diazotrophic *Burkholderia* species, for which the name *Burkholderia unamae* sp. nov. is proposed. Strain MTI-641^T (=ATCC BAA-744^T = CIP 107921^T), isolated from the rhizosphere of maize, was designated as the type strain. *B. unamae* was found as an endophyte of plants grown in regions with climates ranging from semi-hot subhumid to hot humid, but not from plants grown in regions with semi-hot or hot dry climates. Moreover, *B. unamae* was isolated from rhizospheres and plants growing in soils with pH values in the range 4.5–7.1, but not from soils with pH values higher than 7.5.

Presently, the genus *Burkholderia* includes 30 species with validly published names (Coenye & Vandamme, 2003), with *Burkholderia cepacia* as the type species (Yabuuchi *et al.*, 1992). *Burkholderia* species of environmental origin that have been described in the last 5 years include *Burkholderia graminis* (Viallard *et al.*, 1998), *Burkholderia thailandensis* (Brett *et al.*, 1998), *Burkholderia caribensis* (Achouak *et al.*, 1999), *Burkholderia kururiensis* (Zhang *et al.*, 2000), *Burkholderia uboniae* (Yabuuchi *et al.*, 2000a), which has subsequently been corrected to *Burkholderia ubonensis* (Yabuuchi *et al.*, 2000b), *Burkholderia caledonica* and *Burkholderia fungorum* (Coenye *et al.*, 2001a), *Burkholderia*

ambifaria (Coenye *et al.*, 2001b), *Burkholderia sacchari* (Brämer *et al.*, 2001), *Burkholderia anthina* (Vandamme *et al.*, 2002b), *Burkholderia terricola* and *Burkholderia hospita* (Goris *et al.*, 2002). Recently, two nodulating strains recovered from legume plants were assigned to the genus *Burkholderia* as two novel species, *Burkholderia tuberum* and *Burkholderia phymatum* (Vandamme *et al.*, 2002a).

For a long time, N₂-fixing ability in bacteria of the genus *Burkholderia* was recognized only in the species *Burkholderia vietnamiensis* (Gillis *et al.*, 1995). Recently, analysis of maize, sorghum and coffee plants grown under field conditions revealed the richness of the genus *Burkholderia* in unknown diazotrophs and the N₂-fixing ability of *B. kururiensis* (Estrada-de los Santos *et al.*, 2001). These unknown N₂-fixing bacteria showed different amplified 16S rDNA restriction analysis (ARDRA) profiles that corresponded to 14 of the ARDRA genotypes identified, which suggested the existence of at least five novel N₂-fixing *Burkholderia* species. An abundant group of these N₂-fixing *Burkholderia* sp. isolates, identified as ARDRA genotypes 13, 14 and 15, showed almost-identical whole-cell protein patterns, as well as other common phenotypic and genomic features, such as nitrogenase activity with different carbon sources and identical *nifHDK* hybridization patterns. Very recently,

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Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ARA, acetylene reduction activity; ARDRA, amplified rDNA restriction analysis; MLEE, multi-locus enzyme electrophoresis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of strains MCo-762, MTI-641^T and SCCu-23 are AY221955, AY221956 and AY221957, respectively.

A table of restriction patterns of *B. unamae* genotypes, a fuller phylogenetic tree and a dendrogram to illustrate the genetic relatedness of strains of *B. unamae* are available as supplementary material in IJSEM Online.

N₂-fixing isolates recovered from maize and teosinte plants grown in Mexico, as well as from sugarcane plants cultivated in Brazil and South Africa, were described as '*Burkholderia tropica*' (Reis *et al.*, 2004).

In the present study, previously identified diazotrophic isolates that corresponded to 16S rDNA genotypes 13, 14 and 15 (Estrada-de los Santos *et al.*, 2001), along with new isolates recovered from diverse plant species, were subjected to a detailed phenotypic and genomic analysis to determine their taxonomic status.

Sources of the 20 *Burkholderia* isolates that were analysed are shown in Table 1. Seven of these strains, corresponding to ARDRA genotypes 13, 14 and 15, were from a previously described collection (Estrada-de los Santos *et al.*, 2001). New isolates were recovered from the rhizosphere, rhizoplane and surface-sterilized roots and stems of maize and sugarcane plants cultivated in Mexico. Rhizosphere soil and plants were treated as described previously (Estrada-de los Santos *et al.*, 2001). N-free, semi-solid BAZ medium was

used as an enrichment culture for N₂-fixing *Burkholderia* and BAC agar plates were used for isolation and pure cultures (Estrada-de los Santos *et al.*, 2001). Single colonies were inoculated in N-free, semi-solid BAZ medium and assayed for acetylene reduction activity (ARA) as described previously (Estrada-de los Santos *et al.*, 2001). Acetylene-reducing colonies were further verified for culture purity on BAC agar plates. N₂-fixing isolates were maintained in 50 % (v/v) glycerol at -80 °C prior to analysis.

Total DNA isolation, amplification of 16S rRNA genes by PCR and ARDRA were performed by using the conditions described by Estrada-de los Santos *et al.* (2001). Each isolate was assigned to one of the ARDRA genotypes described previously (Estrada-de los Santos *et al.*, 2001). For ribotyping, a Southern blot of total *EcoRI* DNA digest was hybridized by using the conditions described by Caballero-Mellado *et al.* (1995). In order to obtain the 16S rRNA gene sequence, PCR products were cloned into the vector pCRII (Invitrogen) according to the manufacturer's instructions. 16S rRNA genes were restricted in small fragments

Table 1. Representative strains corresponding to ARDRA genotypes 13, 14, 15 and 15a, associated with plants

Each strain was isolated from a different rhizosphere or plant sample. *B. unamae* strains were deposited in the American Type Culture Collection (ATCC) and Collection de l'Institut Pasteur (CIP) with the following numbers: strain MTL-641^T (=ATCC BAA-744^T=CIP 107921^T), strain MCo-762 (=ATCC BAA-745=CIP 107922) and strain SCCu-23 (=ATCC BAA-746=CIP 107923). Climate class codes: 1, semi-hot subhumid; 2, semi-hot humid; 3, hot subhumid; 4, hot humid.

ARDRA genotype and strain	Plant	Cultivar	Source	Locality	Climate class	Reference
13:						
MCo-762	Maize	Criollo	Roots	Coatepec, Veracruz	2	This work
M2Tu-2319	Maize	Criollo	Rhizosphere	Tuxtla Chico, Chiapas	4	This work
SCZa-39	Sugarcane	Mex 57-473	Stem	Zacatepec, Morelos	3	This work
SCZa-211	Sugarcane	Mex 57-473	Stem	Zacatepec, Morelos	3	This work
SCZa-452	Sugarcane	Mex 57-473	Stem	Zacatepec, Morelos	3	This work
CAC-98	Coffee*	Arabiga	Rhizosphere	Coatepec, Veracruz	2	Estrada-de los Santos <i>et al.</i> (2001)
CAC-382	Coffee	Arabiga	Rhizoplane	Coatepec, Veracruz	2	Estrada-de los Santos <i>et al.</i> (2001)
14:						
M2Cy-626	Maize	H-910	Rhizoplane	Cocoyoc, Morelos	4	This work
CGC-321	Coffee	Garnica	Rhizosphere	Coatepec, Veracruz	2	Estrada-de los Santos <i>et al.</i> (2001)
15:						
MTL-641 ^T	Maize	Landrace	Rhizosphere	Tlayacapan, Morelos	1	Estrada-de los Santos <i>et al.</i> (2001)
M2Cy-711	Maize	H-910	Rhizosphere	Cocoyoc, Morelos	3	This work
MEd-9573	Maize	Criollo	Stem	El Eden, Chiapas	4	This work
SCCu-22	Sugarcane	My 55-14	Rhizosphere	Cuernavaca, Morelos	1	This work
SCCu-23	Sugarcane	My 55-14	Roots	Cuernavaca, Morelos	1	This work
SCZa-121	Sugarcane	Mex 57-473	Stem	Zacatepec, Morelos	3	This work
CAC-112	Coffee	Arabiga	Rhizoplane	Coatepec, Veracruz	2	Estrada-de los Santos <i>et al.</i> (2001)
CGC-72	Coffee	Garnica	Rhizoplane	Coatepec, Veracruz	2	Estrada-de los Santos <i>et al.</i> (2001)
CGC-316	Coffee	Garnica	Rhizosphere	Coatepec, Veracruz	2	Estrada-de los Santos <i>et al.</i> (2001)
C2RT-2811	Coffee	Robusta	Rhizosphere	Tapachula, Chiapas	4	This work
15a:						
M2Cy-717	Maize	H-910	Rhizosphere	Cocoyoc, Morelos	3	This work

*Coffee cultivars are *Coffea arabica*, whereas cv. Robusta is *Coffea canephora*.

(0.6–0.9 kb) by using *EcoRI* and subcloned into the vector pUC18 (Invitrogen). 16S rRNA gene sequencing was performed by Medigenomix GmbH (Martinsried, Germany). DNA–DNA reassociation was based on relative levels of hybridization to ^{32}P -labelled DNA, as described previously (Estrada-de los Santos *et al.*, 2001).

Multi-locus enzyme electrophoresis (MLEE) and SDS-PAGE of whole-cell proteins were performed as described previously (Caballero-Mellado *et al.*, 1995; Estrada-de los Santos *et al.*, 2001), except that each isolate was grown for 18 h in BSE medium (Estrada-de los Santos *et al.*, 2001) at 29 °C. For determination of cellular fatty acid composition, strains were grown in trypticase soy broth agar at 28 °C for 24 h; the analysis was performed by Microbial ID, Inc. (Newark, DE, USA).

Bacteriological and biochemical characterization was carried out by growing isolates in BSE medium for 12 h at 29 °C (Estrada-de los Santos *et al.*, 2001). Cultures were centrifuged twice and resuspended each time in 10 mM MgSO_4 , and then adjusted to an OD_{450} of 0.2 (3×10^6 c.f.u. ml^{-1}). An aliquot (10 μl) from each culture was streaked onto solid medium and incubated for 72 h at 29 °C unless otherwise indicated, to determine phenotypic features. Colony morphology was examined on BAc agar plates (Estrada-de los Santos *et al.*, 2001) and growth was recorded after 4 days incubation. Effects of temperature and pH on growth were determined in BSE agar medium. Growth on MacConkey agar (Difco) plates and on *B. cepacia*-selective agar (BCSA) medium, as well as on BCSA/vancomycin (2.5 mg l^{-1}) medium (Henry *et al.*, 1997), was determined. Growth of N_2 -fixing *Burkholderia* isolates with 1-aminocyclopropane-1-carboxylic acid (ACC) as the sole nitrogen source was tested on Az–Acc medium (0.2 % azelaic acid, 0.003 % ACC, 0.04 % K_2HPO_4 , 0.04 % KH_2PO_4 , 0.02 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1.6 % agarose). The pH was adjusted to 5.7 and the medium was sterilized at 121 °C for 20 min prior to the addition of filter-sterilized ACC (pore size, 0.22 μm). In addition, phenotypic features were assayed with the API 20NE and API 50CH systems, according to the manufacturer's instructions (bioMérieux). The API 20NE system was used to determine nitrate reduction, gelatin liquefaction, aesculin hydrolysis and urease activity. Two colonies grown on BAc agar plates were harvested to determine oxidase reaction, which is a complementary test in the API 20NE system. Results of carbon source assimilation using the API 50CH system were obtained after 4 days incubation at 29 °C. At least two replicates were used for each characteristic examined. Single colonies were inoculated in a modified BAZ medium (which lacked azelaic acid, but was supplemented with either 0.5 % succinate or 0.2 % benzoate or propionate) and assayed for ARA as described previously (Estrada-de los Santos *et al.*, 2001). Fresh culture medium with benzoate as the carbon source was used to avoid variable ARA results. Siderophores were detected by using universal chemical assays on chromeazurol-S agar plates (Schwyn & Neilands, 1987).

In the present study, diazotrophic isolates that corresponded to 16S rDNA genotypes 13, 14 and 15 were recovered repeatedly from the rhizospheres and rhizoplanes of maize, sugarcane and coffee (*Coffea arabica* and *Coffea canephora*) plants, as well as from the endophyte environment of maize and sugarcane, by using the culture media BAZ and BAC. All isolates that corresponded to ARDRA genotypes 13, 14 or 15 formed very thin and fine pellicles at a depth of 5–6 mm below the surface in N-free, semi-solid BAZ medium after 24 h. After 72 h, the pellicles become whitish, thick but slightly diffuse and moved up to the surface. Colonies of isolates that corresponded to genotypes 13, 14 or 15, when growing on BAC medium plates, were slightly yellowish, round, smooth and convex, 1–2 mm in diameter with entire margins and turned the colour of the culture medium from green to deep blue after incubation for 4 days at 29 °C.

ARDRA genotypes 13, 14 and 15 showed identical profiles with enzymes *AluI*, *DdeI*, *HaeIII*, *HhaI*, *MspI* and *RsaI*, but they were distinguished by using the enzyme *HinfI* (see Supplementary Table in IJSEM Online). One isolate showed the same profile as genotype 15 except with enzyme *HaeIII*; it was therefore designated ARDRA genotype 15a (Table 1). Genotypes 13, 14, 15 and 15a could be differentiated from other N_2 -fixing *Burkholderia* species, as well as from non-diazotrophic *Burkholderia* species, by their ARDRA profiles (see Supplementary Table in IJSEM Online). For instance, ARDRA profiles obtained with restriction enzymes *AluI*, *DdeI*, *HaeIII* and *RsaI* can differentiate genotypes 13, 14, 15 and 15a from *B. sacchari* IPT 101^T, a non-diazotrophic species, and the restriction enzymes *HinfI* and *RsaI* differentiate genotypes 13, 14, 15 and 15a from '*B. tropica*' Ppe8. In addition, strains corresponding to genotypes 13, 14, 15 and 15a can be differentiated both from '*B. tropica*' and *B. sacchari* by their distinct ribotypes obtained with *EcoRI* (Fig. 1).

As isolates corresponding to ARDRA genotypes 13 and 15 were the most common among all strains analysed (Table 1), strains MTI-641^T and SCCu-23, both of which correspond to ARDRA genotype 15, and strain MCo-762 (genotype 13) were chosen for 16S rRNA gene sequencing. The 16S rRNA gene sequences of these strains were compared with available 16S rDNA sequences from *Burkholderia* species. The phylogenetic tree shown in Fig. 2 illustrates the position of strains MTI-641^T, SCCu-23 and MCo-762 relative to the nearest *Burkholderia* species, as well as to other N_2 -fixing *Burkholderia* species. Strains MTI-641^T, SCCu-23 and MCo-762 constituted a single, homogeneous cluster with similarity between their 16S rDNA sequences in the range of 99.2–99.6 %. *B. sacchari* and '*B. tropica*' were the most closely related species to the cluster that comprised strains MTI-641^T, SCCu-23 and MCo-762 (98.5 and 98.2 % sequence similarity, respectively). The N_2 -fixing strains MCo-762, MTI-641^T and SCCu-23 constituted a cluster that was clearly separated from the cluster formed by the diazotrophic species *B. kururiensis* and *B. tuberum*, as well as

Fig. 1. Ribotype profiles for selected strains corresponding to ARDRA genotypes 13, 14, 15 and 15a (*B. unamae*), as well to other N_2 -fixing *Burkholderia* species and the most closely related species, *B. sacchari*. Strains: 1, M2Cy-717; 2, CGC-321; 3, MTI-641^T; 4, SCCu-23; 5, MCo-762; 6, *B. sacchari* IPT 101^T; 7, '*B. tropica*' PPe8; 8, '*B. tropica*' MTo-293; 9, *B. vietnamiensis* TVV75^T; 10, *B. kururiensis* KP23^T.

from the diazotroph *B. vietnamiensis* (95.7% similarity), which belongs to the *B. cepacia* complex (Vandamme *et al.*, 1997). A phylogenetic tree based on available 16S rRNA gene sequences of all *Burkholderia* species is available as supplementary material (see Supplementary Fig. A in IJSEM Online). For clarifying the taxonomic status of strains corresponding to ARDRA genotypes 13, 14, 15 and 15a, a polyphasic taxonomic approach was undertaken, as suggested by Vandamme *et al.* (1996).

In total, 20 isolates that corresponded to ARDRA genotypes 13, 14, 15 and 15a were examined by MLEE. Distinctive combinations of alleles for 12 enzyme loci were found to be monomorphic (data not shown); therefore, a single electrophoretic type was identified. The genetic relationships

among N_2 -fixing and related *Burkholderia* species are illustrated by a dendrogram (see Supplementary Fig. B in IJSEM Online). Isolates corresponding to ARDRA genotypes 13, 14, 15 and 15a largely diverged at a genetic distance of 0.870 from *B. sacchari* and at 0.780 from '*B. tropica*'. These coefficients of genetic distances at levels >0.5 suggested strongly that genotypes 13, 14, 15 and 15a represent a novel *Burkholderia* species. In other cases, estimates of genetic relatedness of bacterial strains obtained by MLEE correlate strongly with estimates of divergence that are obtained from DNA–DNA hybridization experiments (for a review, see Martínez-Romero & Caballero-Mellado, 1996).

Whole-cell protein extracts were prepared from all 20 isolates corresponding to genotypes 13, 14, 15 and 15a and from several related species. SDS-PAGE protein patterns of some representative strains, corresponding to ARDRA genotypes 13 and 15, are shown in Fig. 3. All 20 strains showed almost-identical protein profiles between themselves, but their protein patterns were clearly different from those of other N_2 -fixing and non- N_2 -fixing *Burkholderia* species (Fig. 3). It is well-known that bacteria with identical or very similar protein patterns possess high genome similarity (Vandamme *et al.*, 1996). On this basis, the SDS-PAGE results suggest strongly that genotypes 13, 14, 15 and 15a represent a novel N_2 -fixing *Burkholderia* species.

Strains MTI-641^T, MCo-762, SCCu-23 and CGC-72 showed almost-identical fatty acid profiles. Fatty acid profiles (mean $\pm$ SD) for the four strains consisted of: 14:0 (4.3 $\pm$ 0.1%), 16:0 (17.5 $\pm$ 0.5%), 16:0 2-OH (2.4 $\pm$ 0.2%), 16:0 3-OH (4.7 $\pm$ 0.2%), 16:1 ω 5c (0.46 $\pm$ 0.06%), 16:1 2-OH (3.41 $\pm$ 0.3%), 17:0 cyclo (6.6 $\pm$ 1.0%), 18:0 (0.3 $\pm$ 0.7%), 18:1 ω 7c (34.2 $\pm$ 1.7%), 18:1 2-OH (1.29 $\pm$ 0.07%), 19:0 ω 8c cyclo (3.6 $\pm$ 0.5%) and summed features 2 (5.5 $\pm$ 0.1%) and 3 (15.6 $\pm$ 0.9%). Summed feature 2 could correspond to 14:0 3-OH, iso-16:1 I, an unknown fatty acid with equivalent chain-length of 10.928, 12:0 ALDE or any combination of these fatty acids, and summed

Fig. 2. Phylogenetic tree showing the relationships of strains MTI-641^T, SCCu-23 and MCo-762 (*B. unamae*) to the nearest *Burkholderia* species, as well as to other N_2 -fixing *Burkholderia* species, based on 16S rDNA sequence comparisons. 16S rRNA gene sequences were aligned by using CLUSTAL W, version 1.8 (Thompson *et al.*, 1994). The tree topology was inferred by the neighbour-joining method (Saitou & Nei, 1987) and the phylogenetic analysis was conducted by using MEGA version 2.1 (Kumar *et al.*, 2001). The alignment included 1358 DNA sites.

Fig. 3. Protein electrophoretograms (SDS-PAGE) of selected isolates corresponding to ARDRA genotypes 13 and 15 (*B. unamae*) and *Burkholderia* reference strains. Strains: 1, SSCu-23; 2, M2Cy-711; 3, MCo-762; 4, MEd-9573; 5, MTI-641^T; 6, CGC-72; 7, SZa-211; 8, CAC-98; 9, *B. sacchari* IPT 101^T; 10, '*B. tropica*' PPe8; 11, '*B. tropica*' MTo-293; 12, *B. vietnamiensis* TVV75^T; 13, *B. vietnamiensis* CCE-201; 14, *B. kururiensis* KP23^T.

feature 3 could correspond to 16:1 ω 7c or iso-15:0 2-OH or both, similar to those fatty acids reported in other *Burkholderia* species (Vandamme *et al.*, 1997; Coenye *et al.*, 2001b). A comparison of some fatty acids found in strain MTI-641^T and other *Burkholderia* species is shown in Table 2. Strain MTI-641^T was identified by the Microbial

Identification system as *B. cepacia* (identification score of 0.568), followed by *Burkholderia glathei* (identification score of 0.317) and *Burkholderia caryophylli* (identification score of 0.314). There were only very slight differences in the identification indices between strain MTI-641^T and strains MCo-762, SCCu-23 and CGC-72.

Table 2. Phenotypic characteristics that are useful for the differentiation of *B. unamae* from other N₂-fixing *Burkholderia* and related species

Species: 1, *B. unamae* (n=20); 2, '*B. tropica*' (n=10); 3, *B. kururiensis* KP23^T; 4, *B. vietnamiensis* (n=10); 5, *B. sacchari* IPT 101^T; 6, *B. cepacia* ATCC 25416^T. +, Good growth; $\pm$, poor growth; –, no growth; v, variable; ND, not determined.

Characteristic	1	2*	3	4†	5	6
Surface pellicle formation in N-free, semi-solid BAz medium	+	+	+	+	–	–
C ₂ H ₂ reduction activity (N ₂ fixation) in N-free, semi-solid BAz medium without azelate but with:						
Succinate	+	+	+	+	–	–
Propionate	+	v	+	+	–	–
Benzoate	–	–	+	+	–	–
Growth on:						
BAC agar medium	+	+	–	+	$\pm$	+
BSE agar medium at 29 and 37 °C	+	+	+	+	+	+
BSE agar medium at 42 °C	–	–	+	+	–	+
MacConkey medium at 29 °C	$\pm$	+	–	+	–	+
MacConkey medium at 37 °C	–	–	–	+	–	+
BCSA medium	–	–	–	+	–	+
BCSA/vancomycin medium	–	–	–	+	–	+
Az–Acc agar medium	+	–	–	–	–	–
Fatty acid content (%)‡:						
16:0	18.0	ND	18.2	19.5	17.7	26.8
17:0 cyclo	7.4	ND	4.8	14.0	3.7	17.9
Summed feature 3§	14.9	ND	13.2	9.8	23.4	4.6

*Including strain Ppe8.

†Including type strain TVV75^T.

‡Data for strains IPT 101^T and KP23^T are from Brämer *et al.* (2001) and those for *B. cepacia* and *B. vietnamiensis* are from Coenye *et al.* (2001b).

§Summed feature 3 comprises 16:1 ω 7c or iso-15:0 2-OH or both for either *B. unamae* or for *B. cepacia* and *B. vietnamiensis* (Coenye *et al.*, 2001b), and comprises 16:1 ω 7c for strains IPT 101^T and KP23^T.

DNA–DNA reassociation experiments that were carried out between strain MTL-641^T and strains SSCu-23, CGC-321 and MCo-762 showed reassociation values of 97, 89 and 75 %, respectively. DNA–DNA reassociation levels between strain MTL-641^T and type strains of other *Burkholderia* species were in the range 20–40 %, including *B. sacchari* IPT 101^T (40 %), ‘*B. tropica*’ Ppe8 (36 %), *B. kururiensis* KP23^T (36 %), *B. phymatum* STM815^T (32 %), *B. tuberum* STM678^T (29 %), *B. vietnamiensis* TVV75^T (24 %) and *B. cepacia* ATCC 25416^T (20 %).

On the basis of the SDS-PAGE protein profiles, electrophoretic mobility patterns of metabolic enzymes and fatty acid profiles, as well as the genomic data described here, we propose the name *Burkholderia unamae* sp. nov. for the isolates corresponding to ARDRA genotypes 13, 14 and 15, as well as for genotype15a, which was identified in the present work.

All *B. unamae* strains were Gram-negative and motile in N-free, semi-solid media at 29 °C. Transmission electron microscopy revealed that strain MTL-641^T was a straight rod with a single polar flagellum or a tuft of polar flagella (data not shown). The ability of *B. unamae* strains to grow on different culture media and at different temperatures is reported in the species description (below). *B. unamae* strains exhibited good growth on BSE medium at pH 4.5–6.5, but poor growth was observed at pH 7.0–7.5. Phenotypic characteristics for the differentiation of *B. unamae* from other N₂-fixing *Burkholderia* species, as well as from *B. sacchari* and from the type species of the genus *Burkholderia*, are shown in Table 2.

Results of the API 20NE biochemical tests for *B. unamae* strains are reported in the species description (below). These biochemical tests identified the isolates as *B. cepacia* (67.3 % confidence limits, based on the API analytical profile index), followed by *Pseudomonas aureofaciens* (confidence limits of 29.9 %). All *B. unamae* isolates grew and showed the ability to reduce acetylene in N-free, semi-solid BAz medium, as well as with succinate and propionate as carbon sources (Table 2). Hitherto, N₂-fixing ability had been described only in *B. vietnamiensis* (Gillis *et al.*, 1995), *B. kururiensis* (Estrada-de los Santos *et al.*, 2001) and, recently, in ‘*B. tropica*’ (Reis *et al.*, 2004), among all known species of the genus *Burkholderia*. Previously, we reported the ability of *Burkholderia* isolates corresponding to ARDRA genotypes 13, 14 and 15 to reduce acetylene to ethylene with different carbon sources, and also showed the presence of *nifHDK* genes in several of these isolates that were recovered from coffee plants (Estrada-de los Santos *et al.*, 2001). These characteristics have been confirmed with 13 new isolates (Table 1) tested in the present study (data not shown), which confirms that the ability to fix nitrogen is a typical feature of the species *B. unamae*. In this framework, N₂-fixing ability can be used as a distinctive feature for the delineation of *Burkholderia* species. On this basis, *B. unamae* can be differentiated from the most closely related species, *B. sacchari*, and from the type species, *B. cepacia*, by its

ability to fix nitrogen. *B. unamae* can be differentiated from other diazotrophic *Burkholderia* species, except ‘*B. tropica*’, by its inability to fix nitrogen using benzoate as a carbon source. The assimilation profile of 49 carbon sources was identical among *B. unamae* strains (Table 3). *B. unamae* can be differentiated from the most closely related N₂-fixing species, ‘*B. tropica*’, by its inability to assimilate β-gentiobiose and ribose, and from *B. sacchari* by its ability to assimilate cellobiose, rhamnose and trehalose. Differences in the usage of carbon sources by *B. unamae* strains and other related *Burkholderia* species are shown in Table 3.

Description of *Burkholderia unamae* sp. nov.

Burkholderia unamae [u.na'mae. N.L. gen. fem. n. *unamae* arbitrary name formed from UNAM, acronym for Universidad Nacional Autónoma de México. This name was chosen to commemorate the 450th anniversary of the University, which occurred in the same year as the first isolation of the species (2001)].

Cells are straight rods (1.8–2.5 μm long and 0.5–0.8 μm wide) with a single flagellum or a polar tuft of four to eight

Table 3. Differences in the utilization of carbon sources by *B. unamae* and related *Burkholderia* species

Species: 1, *B. unamae* (n=20); 2, ‘*B. tropica*’ (n=10); 3, *B. kururiensis* KP23^T; 4, *B. vietnamiensis* (n=10); 5, *B. sacchari* IPT 101^T; 6, *B. cepacia* ATCC 25416^T. All strains of *B. unamae* assimilated N-acetylglucosamine, adonitol, D-arabinose, L-arabinose, D-arabitol, cellobiose, D-fructose, L-fucose, galactose, gluconate, 2-ketogluconate, D-glucose, glycerol, inositol, mannitol, D-mannose, rhamnose, sorbitol, trehalose and D-xylose. None of the strains assimilated amygdalin, L-arabitol, arbutin, dulcitol, erythritol, D-fucose, β-gentiobiose, methyl α-D-glucoside, 5-ketogluconate, glycogen, inulin, lactose, D-lyxose, maltose, melibiose, melezitose, methyl α-D-mannoside, methyl β-xyloside, D-raffinose, ribose, salicin, starch, L-sorbose, sucrose, D-tagatose, D-turanose, xylitol or L-xylose. +, Good growth; –, no growth; V, variable.

Carbon source	1	2*	3	4†	5	6
Adonitol	+	+	+	–	+	+
L-Arabitol	–	–	+	–	–	+
Cellobiose	+	+	–	+	–	+
β-Gentiobiose	–	+	–	+	–	–
Lactose	–	V‡	–	–	–	–
D-Raffinose	–	–	–	+	+	–
Rhamnose	+	+	+	–	–	–
Ribose	–	+	–	+	–	+
Salicin	–	V‡	–	+	–	+
Sucrose	–	–	–	+	+	+
Trehalose	+	V‡	–	+	–	+
Xylitol	–	–	+	–	–	–

*Including strain Ppe8.
†Including type strain TVV75^T.
‡Between 55 and 70 % of strains gave a positive reaction.

flagella. Isolates are Gram-negative and oxidase- and catalase-positive. Growth and acetylene reduction to ethylene is observed with different carbon sources in N-free, semi-solid media. *B. unamae* strains grow on BAC plates, forming colonies that are yellowish, round, smooth and convex with entire margins. Isolates grew on agar media at 29 °C, but not at 42 °C. At 37 °C, they grew on BSE medium, but not on MacConkey agar medium. Strains did not grow on BCSA medium with or without vancomycin. Phenotypic characteristics for the differentiation of *B. unamae* from other N₂-fixing *Burkholderia* species and related species of the genus *Burkholderia* are shown in Table 2. Nitrate is reduced to nitrite, but not to N₂; there is urease activity, but not aesculin hydrolysis, liquefaction of gelatin or indole production. Additional phenotypic characteristics are listed in Table 3. All isolates showed the ability to produce siderophores. *B. unamae* can be differentiated phenotypically from all diazotrophic *Burkholderia* species and from *B. sacchari* and *B. cepacia* by its SDS-PAGE protein profile, electrophoretic mobility pattern of metabolic enzymes and fatty acid profile. *B. unamae* comprises strains with four different ARDRA genotypes and their 16S rDNA sequences show >99.2 % similarity. *B. unamae* can also be differentiated genomically from all diazotrophic *Burkholderia* species and from *B. sacchari* by its ARDRA and ribotype profiles.

The type strain is MTI-641^T (= ATCC BAA-744^T = CIP 107921^T). This strain was isolated from the rhizosphere of maize cultivated in the fields in Tlayacapan, Morelos State, Mexico. Phenotypic and genomic characteristics of the type strain are the same as described above for the species.

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