Antonie van Leeuwenhoek
DOI 10.1007/s10482-013-0028-9

ORIGINAL PAPER

Burkholderia caballeronis sp. nov., a nitrogen fixing species isolated from tomato (*Lycopersicon esculentum*) with the ability to effectively nodulate *Phaseolus vulgaris*

Lourdes Martínez-Aguilar · Corelly Salazar-Salazar · Rafael Díaz Méndez · Jesús Caballero-Mellado · Ann M. Hirsch · María Soledad Vásquez-Murrieta · Paulina Estrada-de los Santos

Received: 18 April 2013 / Accepted: 3 September 2013

Abstract During a survey of *Burkholderia* species with potential use in agrobiotechnology, a group of 12 strains was isolated from the rhizosphere and rhizoplane of tomato plants growing in Mexico (Nepantla, Mexico State). A phylogenetic analysis of 16S rRNA gene sequences showed that the strains are related to *Burkholderia kururiensis* and *Burkholderia mimosarum* (97.4 and 97.1 %, respectively). However, they induced effective nitrogen-fixing nodules on roots of *Phaseolus vulgaris.* Based on polyphasic taxonomy, the group of strains represents a novel species for which the name *Burkholderia caballeronis* sp. nov. is proposed. The type species is TNe-841^T (= LMG 26416^T = CIP 110324^T).

Keywords *Burkholderia caballeronis* · Rhizosphere · Rhizoplane · Tomato · Nodules · Nitrogen fixation

Introduction

Burkholderia is a genus comprised by nearly 70 species with worldwide distribution in diverse environments including soil, water, plant, fungi and insects. Although many species are saprophytes, others are human, animal, or plant pathogens and are or have the potential to be opportunistic pathogens of humans. Recently, a

Electronic supplementary material The online version of this article (doi:10.1007/s10482-013-0028-9) contains supplementary material, which is available to authorized users.

L. Martínez-Aguilar · R. D. Méndez · J. Caballero-Mellado
Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, 565-A Cuernavaca, Morelos, Mexico

C. Salazar-Salazar
Depto. de Biotecnología, Universidad Autónoma Metropolitana-Iztapalapa, San Rafael 186, Col. Vicentina, Mexico, DF, Mexico

A. M. Hirsch
Department of Molecular, Cell and Developmental Biology, Molecular Biology Institute, University of California, Los Angeles, 621 Charles E. Young Drive South, Los Angeles, CA 90095-1606, USA

M. S. Vásquez-Murrieta · P. Estrada-de los Santos (✉)
Instituto Politécnico Nacional, Escuela Nacional de Ciencias Biológicas, Prolongación de Carpio Y Plan de Ayala s/n, Col. Santo Tomás, Delegación Miguel Hidalgo, 11340 Mexico, DF, Mexico
e-mail: pestradadelossantos@gmail.com

Published online: 22 September 2013

number of plant-associated species were described and more are in the pipeline awaiting description (Gyaneshwar et al. 2011; Howieson et al. 2013). Thus in the last few years, *Burkholderia* is steadily growing as a genus with regard to species number. However, as the number of species described increases, it is becoming more obvious that the genus contains two or more lineages (Gyaneshwar et al. 2011; Suarez-Moreno et al. 2012l; Estrada-de los Santos et al. 2013). One distinct lineage contains: (1) members of the *B. cepacia* complex (BCC), which are opportunistic pathogens of humans; (2) the human and animal pathogens of the *B. pseudomallei* group; (3) the phytopathogenic species; and (4) some saprophytic species. The second lineage, which is divided into two sublineages, contains saprophytic and plant-associated species, including several of which nodulate legumes (Vandamme et al. 2002; Chen et al. 2003, 2006, 2007, 2008). *B. andropogonis* and *B. rhizoxinica,* which do not fit into either of the large lineages, may represent new genera.

The genus also includes species with the ability to carry out PGP (Plant Growth Promoting) activities (Balandreau and Mavingui 2007). These species have the potential for agrobiotechnological applications due to such traits as siderophores synthesis, phosphate solubilization, or nitrogen fixation ability (Caballero-Mellado et al. 2007; Angus et al. 2013). Nitrogen fixation was first discovered in *Burkholderia* when *B. vietnamiensis* was described (Gillis et al. 1995). Afterwards, this ability was detected in other newly described species, including *B. unamae, B. tropica, B. xenovorans* and *B. silvatlantica* (Estrada-de los Santos et al. 2001; Caballero-Mellado et al. 2004; Reis et al. 2004; Goris et al. 2004; Perin et al. 2006b). In addition, *Burkholderia* contains 11 species that nodulated legumes effectively; *B. tuberum, B. phymatum, B. caribensis, B. mimosarum, B. nodosa, B. sabiae, B. diazotrophica*, *B. symbiotica, B. phenoliruptrix, B. sprentiae and B. rhynchosiae* (Vandamme et al. 2002; Chen et al. 2003, 2006, 2007, 2008; Sheu et al. 2012, 2013; de Oliveira-Cunha et al. 2012; De Meyer et al. 2013a, b). *B. fungorum* is also able to nodulate legume plants although ineffectively (Ferreira et al. 2012).

Many of the saprophytic *Burkholderia* species degrade xenobiotic compounds (Caballero-Mellado et al. 2007), making them likely bioremediation agents. The different *Burkholderia* species and the substrates they utilize have been extensibely reviewed by Denef (2007). *Burkholderia* species with PGP activities and biodegradation abilities could play a beneficial role in agriculture, but concerns have been expressed about the use of *Burkholderia* species for agriculture because some species are opportunistic pathogens, especially species in the BCC (Hauser et al. 2011). Yet, this may not be a serious problem in the future because evidence is accruing that indicates that the plant-associated *Burkholderia* are not closely related to the BCC, making the plant-associated species useful for both agriculture and bioremediation.

During a survey of *Burkholderia* species with potential for agrobiotechnological applications, several strains were isolated from the tomato rhizosphere and rhizoplane (Caballero-Mellado et al. 2007). Five strains were closely related to the trichloroethylene (TCE) degrading and N-fixing bacteria *B. kururiensis* (Zhang et al. 2000; Estrada-de los Santos et al. 2001). This group was named the Bkr group. A taxonomic analysis of the Bkr strains together with a second group of seven strains isolated from the same area showed that all 12 strains actually belong to a new *Burkholderia* species, for which the name *Burkholderia caballeronis* sp. nov. is proposed.

Materials and methods

Bacterial isolation

Bacteria were isolated from Saladet variety tomato (*Lycopersicon esculentum*) plants, collected in Nepantla, State of Mexico, Mexico (Table 1). All strains

Table 1 *Burkholderia caballeronis* strains isolated from Saladet variety tomato plants cultivated in Nepantla, State of Mexico

Strain	Source	Reference
TNe-841^T	Rhizosphere	Caballero-Mellado et al. (2007)
TNe-878	Rhizoplane	Caballero-Mellado et al. (2007)
TNe-834	Rhizosphere	Caballero-Mellado et al. (2007)
TNe-8682	Rhizosphere	Caballero-Mellado et al. (2007)
TNe-8641	Rhizoplane	Caballero-Mellado et al. (2007)
TNe-835	Rhizosphere	This study
TNe-869	Rhizoplane	This study
TNe-8612	Rhizosphere	This study
TNe-8411	Rhizosphere	This study
TNe-8312	Rhizosphere	This study
TNe-8613	Rhizosphere	This study
TNe-8683	Rhizosphere	This study

were isolated at the same time, but only five were previously described (Caballero-Mellado et al. 2007). The remaining strains were kept in our in-house collection until this description.

Genotypic characterization

The 12 isolates were grouped using Amplified rRNA Restriction Analysis (ARDRA) (Caballero-Mellado et al. 2007). The nearly full 16S rRNA gene sequences (~1,533 nucleotide positions) from the strains were amplified with the universal primers fD1/rD1 (Weisburg et al. 1991), as previously described (Perin et al. 2006a). The 16S rRNA genes were cloned into the pCR2.1 vector according to the manufacturer's instructions (Invitrogen). The sequences of the 16S rRNA genes were obtained from Macrogen (www.macrogen.com) using universal primers. The sequences accession numbers are include in Fig. 1. A multiple alignment was performed with Muscle 3.57 (Edgar 2004) and a phylogenetic analysis was carried out with maximum likelihood (ML) using PhyML program (Guindon and Gascuel 2003). Among-site, rate variation was modeled by a gamma distribution with four rate categories (Yang 1996), with each category being represented by its mean, under the GTR+G model. Tree searches were initiated from a BioNJ seed tree, retaining the best tree among those found with NNI (Nearest Neighbor Interchange). The robustness of the ML topologies was evaluated using a Shimodaira-Hasegawa (SH)-like test (Anisimova and Gascuel 2006). ML trees were visualized with program MEGA version 5 (Tamura et al. 2011). Genomic DNA from strains TNe-841^T, TNe-878, TNe-834, the most closely related type species, *B. cepacia* and other randomly selected *Burkholderia* species was extracted from liquid cultures grown in BSE for the DNA-DNA hybridization (DDH) experiments. These experiments were performed in duplicate as described previously (Estrada-de los Santos et al. 2001). The DNA G+C content determination of strain TNe-841^T was carried out at BCCM/LMG. The strain was cultivated on LMG medium 203. Genomic DNA was extracted according to a modification of the procedure of Wilson (Wilson 1987). The DNA G + C content was determined using the HPLC technique (Mesbah et al. 1989). The given value is the mean of three independent analyses. The novel strains were analyzed using fingerprinting techniques, BOX-PCR (BOX dispersed-repeated motif) (Versalovic et al. 1994). The PCR conditions for the BOX element (BOXA1) were carried out according to a previous description (Estrada-de los Santos et al. 2011). An internal fragment of 320 pb from *nodC* gene was amplified using the primers nodC-F (5′-GAYATGGARTAYTGG CTNGC-3′) and nodC-R (5′-ANGTRCTBCGBGCCC AVC-3′). PCR conditions were carried out with AccuPrime Taq DNA Polymerase HF (Invitrogen), with an initial denaturing step at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 56 °C for 30 s and 68 °C for 30 s; finishing with a final elongation step at 68 °C for 5 min.

The PCR reaction mix was made in 40 µl, with the following proportions : genomic DNA, 1 µl (50 ng); 4 µl Buffer 10X, 2.5 µl each primer (25 µM); water, 30 µl. The amplified fragments were sequenced at Instituto de Biotecnología-UNAM (http://www.ibt.unam.mx/) and deposited to the GenBank, NCBI (National Center for Biotechnology Information).

Phenotypic and biochemical characterization

Phenotypic features for strains TNe-841^T, TNe-878, TNe-834, *B. kururiensis* KP23^T, *B. mimosarum* PAS44^T, *B. sacchari* IPT101^T, *B. ferrariae* FeGI01^T and *B. cepacia* ATCC 25416^T were determined with the API 20NE systems according to the manufacturer's instructions (bioMérieux). For the analysis, bacterial cells were pre-cultured on BSE medium. Additionally, the novel isolates were grown on (1) BSE agar medium at 15, 29 and 37 °C and BSE liquid medium at 42, 46 and 50 °C; (2) MacConkey agar medium at 29, 37 and 42 °C; and (3) LB in the presence of 0.5, 1.0, 1.5, 3.0. 5.0, 7.5, 10, 15 and 20 % NaCl at 29 °C. The similarity among the 12 novel strains was tested by comparison of the whole-cell protein patterns. The bacterial cultures were grown in BSE medium with reciprocal shaking (200 rpm) for 15 h at 29 °C, and 1.0 ml samples were harvested by centrifugation at 12,300×*g* for 10 min. The pellets were resuspended in 70 µl of 0.125 M Tris–HCl, 4 % SDS, 20 % glycerol, and 10 % mercaptoethanol at pH 6.8. Aliquots of 10 µl were used for SDS-PAGE performed as described by Laemmli (1970).

The fatty acid profiles from strains TNe-841^T and TNe-878 was carried out at BCCM/LMG. In short, the cells were grown for 24 h at 28 °C on LMG medium 203 (10 g mannitol, 0.5 g KH_2PO4, 5 g sodium

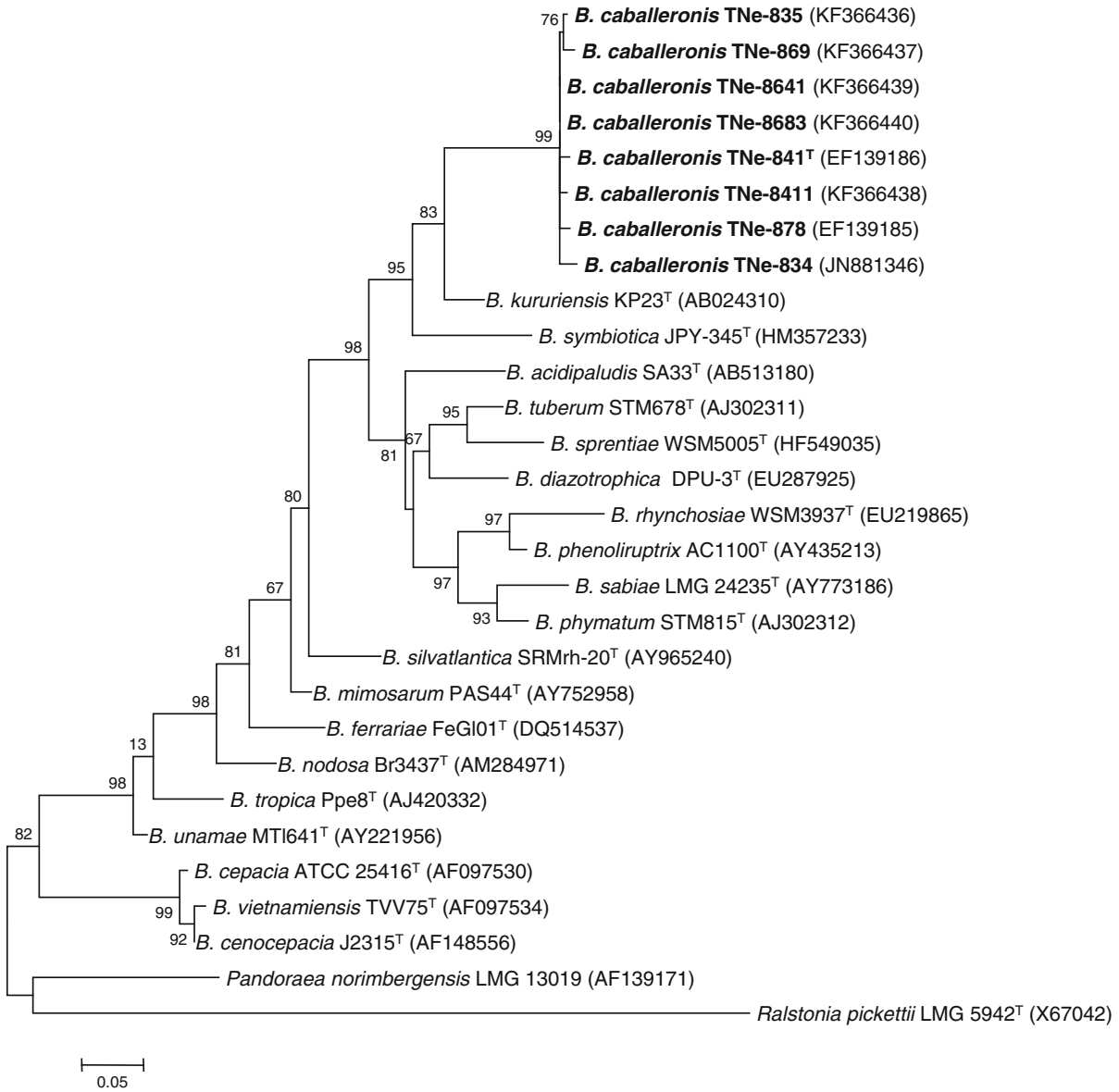

Fig. 1 Maximum likelihood tree based on the 16S rRNA gene sequences of selected *Burkholderia* species. The accession numbers of the sequences used in this analysis are shown *in parenthesis*. The robustness of the ML topologies was evaluated using a Shimodaira-Hasegawa (SH)-like test (Anisimova and Gascuel 2006), which is shown at branches nodes. The bar represents the number of expected substitutions per site under the GTR + G model

glutamate, 50 mg NaCl, 10 ml solution A, 1 ml solution B, 1 ml solution C, 1 g yeast extract, and 20 g agar, per 1 L distillated water, pH 6.8. Solution A consists of 1 % $MgSO_4 \cdot 7H_2O$, solution B is composed of 5.28 % $CaCl_2 \cdot 2H_2O$, and solution C is 0.666 % $FeCl_3.6H_2O$). Inoculation and harvesting of the cells, and extraction and analysis of the fatty acids were performed according to the recommendations of the commercial identification system MIDI (Microbial Identification Systm, Inc., DE, USA). The whole-cell fatty acid composition was determined by gas chromatography. For nodulation tests, *Phaseolus vulgaris* cultivar Negro Jamapa seeds were surface sterilized with 25 % bleach for 25 min and rinsed with sterile water. The seeds were placed on LB agar plates during 3 days in the dark at 30 °C for germination and

sterility before planting. The germinated seeds were transferred to 250 ml aluminumfoil-wrapped Erlenmeyer flasks containing 200 mL of Fahraeus solution (Fahraeus 1957) solidified with 0.8 % agar. The germinated seeds were inoculated with 1 mL of a bacterial suspension adjusted to 0.2 OD_{600} and kept in a plant growth chamber at 26 °C with a photoperiod of 12 h light/12 h dark. After 3 weeks, nodules were tested for acetylene reduction (Mascarua-Esparza et al. 1988). Nodules were surface sterilized with 25 % bleach for 2 min and rinsed with sterile water. The nodules were macerated and streaked on BAc media (Estrada-de los Santos et al. 2001). The isolated bacteria were compared to the original strain by ARDRA. The nodulation experiments were performed in triplicate and repeated in two independent experiments.

Results

Novel species characterization and identification

The percentage of 16S rRNA similarity among the novel strains was 99.8 % and the *Burkholderia* species most closely related to the TNe-841^T strain were *B. kururiensis* KP23^T (97.4 %, AB024310) and *B. mimosarum* PAS44^T (97.1 %, AY752958). Based on an analysis of the 16S rRNA gene sequence in the EzBioCloud server (http://www.ezbiocloud.net/, Kim et al. 2012). Other closest *Burkholderia* species, such as *B. sacchari*, *B. ferrariae*, *B. acidipaludis* or *B. cepacia,* were less than 97.0 % similar to strain TNe-841^T.

The relationship of the novel strains was next determined in a ML tree based on a set of *Burkholderia* species, which not only included the closest species, but also *B. cepacia* (Fig. 1). An additional ML tree was constructed with all the so-far known *Burkholderia* species (Fig. S1). This analysis revealed that the novel strains were integrated into the plant-associated and saprophytic *Burkholderia* lineage we described earlier (Estrada-de los Santos et al. 2013). DDH values between strain TNe-841^T and the other novel strains were higher than the cut-off value used to define a bacterial species (70 %); for example, 90.6 % to TNe-878 and 88.8 % to TNe-834; indicating that these three strains are related at the species level (Tindall et al. 2011). Low DDH values were found relative to other *Burkholderia* species; for example, 12.7 % to *B. kururiensis* KP23^T, 40.5 % to *B. mimosarum* PAS44^T, 13.2 % to *B. sacchari* IPT101^T, 10.1 % to *B. ferrariae* FeGI01^T, 13.8 % to *B. unamae* MTl-641^T and 15.1 % to *B. cepacia* ATCC 25416^T. These results confirm that the novel group of *Burkholderia* species isolated from the tomato rhizosphere and rhizoplane belong to a distinct species.

The DNA G+C content for strain TNe-841^T was 66.0 %. Previously, we showed that the DNA G+C content of certain saprophytic and plant-associated *Burkholderia* species is lower than that of the the BCC and other pathogenic species (Gyaneshwar et al. 2011; Estrada-de los Santos et al. 2013). However, despite the fact that these novel strains are included within the clade of plant-associated bacteria, according to the 16S rRNA gene analysis, the DNA G+C content is higher and more similar to that found in the members of the BCC and the plant and human pathogens *Burkholderia* species. These results strongly suggest that the genus *Burkholderia* deserves a more extensive as well as deeper taxonomical analysis to understand the position of the different lineages in the genus.

The intragenic diversity from the novel species was analyzed with BOX fingerprintings, and compared to related *Burkholderia* species. Although some have indicated that DNA fingerprinting methods have limited value for species description (Tindall et al. 2011), we found in this study that the novel species were very homogeneous in their fingerprinting pattern and that it is significantly different from the patterns of related species (Fig. S2).

The novel strains were also phenotypically characterized. The protein patterns among the novel strains were found to be very similar, but were different from *B. kururiensis* and *B. mimosarum*, the closest related species (Fig. S3). Identical or very similar protein patterns in a group of strains usually correlate with high levels of genome similarity, and when the protein patterns differ, the similarity diminishes (Vandamme et al. 1996). Hence, this result provides additional evidence for the placement of these novel strains into a new *Burkholderia* species.

The biochemical features from the novel strains were also analyzed. The data from strains *B. unamae* MTl-641^T and *B. mimosarum* was collected from the original description (Caballero-Mellado et al. 2004; Chen et al. 2006), which was performed with the same methodology herein. Additionally, the ability of

strains TNe-841^T, TNe-878 and TNe-834 to assimilate different carbon sources was tested with the API 50CH system. The following activities were negative for the novel strains: nitrate and nitrite reduction, β-galactosidase, indole production, esculine and gelatin hydrolisis, D-glucose fermentation and assimilation of D-maltose, citrate, erythritol, methyl-βD-xylopyranoside, dulcitol, methyl-αD-glucopyranoside, amydgaline, arbutine, salicine, D-lactose, inuline, D-melazitose, D-raffinose, amidon, glycogene and xylitol. The following activities were present: arginine dihydrolase, urease, catalase, nitrogen fixation and assimilation of D-glucose, DL-arabinose, D-mannose, D-mannitol, *N*-acetyl glucosamine, gluconate, capric acid, malate, acetate, D-ribose, D-xylose, D-adonitol, D-galactose, D-fructose, L-ramnose, inositol, D-sorbitol, D-cellobiose, D-turanose, D-xylose, D-fucose, D-arabitol, potassium 2-ketogluconate, and postassium 5-ketogluconate. Oxidase activity was weak for strain TNe-841^T and TNe-878, and negative for strain TNe-834. The assimilation of different carbon sources was strain-dependant (adipate, glycerol, L-xylose, methyl-α-D-mannopyranoside, esculine, D-melibiose, D-threalose, gentibiose, D-tagatose and L-arabitol). The twelve novel strains were able to grow on MacConkey agar plates at 29 and 37 °C, but at 42 °C the growth was strain-dependant. All strains grew on LB and BSE agar plates at 15, 29, 37 and 42 °C, and on LB plates at 29 °C with up to 5.0 % NaCl. The distinctive phenotypic features between the novel strains and the closest *Burkholderia* species are displayed in Table 2.

The following fatty acids were detected in strain TNe-841^T and TNe-878, respectively: C14:0 (4.46, 4.66), C16:0 (21.77, 20.14), C16:0 2OH (2.3, 2.5), C16:0 3OH (6.2, 8.0), C16:1 2OH (3.81, 4.5), C17:0 cyclo (12.43, 10.88), C18:1 2OH (1.5, 1.4), C18:1 w7c (16.62, 16.79), C19:0 cyclo w8c (14.89, 11.18), summed feature 2 (5.9, 8.8) and summed feature 3 (8.3, 10.5). Summed feature two corresponds to C14:0 3OH and/or 16:1 ISO I, an unidentified fatty acid with equivalent chain length value of 10.928, 12:0 ALDE or any combination of these fatty acids. Summed feature three corresponds to C16:1 w7c and/or C15:0 ISO 2OH. The large amount of C16:0, C17:0 cyclo and C18:1 w7c is typical for *Burkholderia* species (data not shown).

Because the group of twelve novel *Burkholderia* strains was related to *B. kururiensis*, which can degrade TCE, the novel strains were tested for their ability to grow in the presence of different xenobiotic compounds, including TCE. However, none of the strains grew in liquid mineral media (0.04 % K_2HPO_4, 0.04 % KH_2PO_4, 0.02 % $MgSO_4$-$7H_2O$, 0.5 % NH_4Cl, pH 6.5) supplemented with 4.23 mM of nitrobenzene, nitrotoluene, TCE, cumene, ethylbenzene, o-xylene, toluene, m-xylene or phenol after 15 days of incubation at 29 °C. Approximately 50 % of the strains grew, albeit poorly, with benzene as a carbon source. By contrast, *B. kururiensis* KP23^T grew in the presence of phenol, m-xylene, o-xylene, toluene and ethylbencene.

Given the similarity of the novel species to *B. mimosarum*, strains TNe-841^T and TNe-834 were tested in nodulation experiments on *Phaseolus*

Table 2 Phenotypic differences between *B. caballeronis* and related *Burkholderia* type species

Feature	1	2	3	4	5	6	7
Nitrite reduction	−	+	+	+	+	+	−
Activity of:							
Arginine dihydrolase	+	+	−	+	−	+	+
Urease	+	+	+	+	−	+	+
β-galactosidase	−	+	−	w	+	+	+
Oxidase	sd*	+	+	+	+	+	+
Nitrogenase	+	+	+	−	+	+	−
Hydrolisis of:							
Esculine	−	−	−	−	−	−	+
Gelatine	−	−	−	−	−	−	w
Assimilation of:							
D-Maltose	−	−	−	−	−	+	−
Capric acid	+	+	−	+	+	+	+
Adipic acid	sd**	+	−	+	+	+	+
Citrate	−	+	−	+	+	+	+

Strains *1 B. caballeronis* (TNe-841^T, TNe-878, TNe-834), *2 B. kururiensis* (KP23^T). *3 B. mimosarum* (PAS44^T), *4 B. sacchari* (IPT101^T) *5 B. ferrariae* (FeGl01^T), *6 B. unamae* (MTl-641^T), *7 B. cepacia* (ATCC 25416^T)

+ Positive reaction, − negative reaction, *w* weak reaction, *sd* strain dependent reaction, * strains TNe-841^T and TNe-878 gave a weak reaction, strain TNe-834 was negative, ** strains TNe-841^T and TNe-878 were negative, strain TNe-834 was positive

Phenotypic features from strains *B. unamae* MTl-641^T and *B. mimosarum* were collected from the original description (Caballero-Mellado et al. 2004; Chen et al. 2006). Nitrogenase activity information was gathered from literature (Estrada-de los Santos et al. 2001; Caballero-Mellado et al. 2004, 2007; Chen et al. 2006) and for *B. ferrariae* from Estrada-de los Santos et al. (2013)

vulgaris. *B. mimosarum* PAS44^T, *B. nodosa* Br3437^T and *Rhizobium etli* CFN42^T were included as positive controls and an uninoculated plant served as a negative control. Surprisingly, the two strains and the positive controls nodulated bean roots (Fig. S4), and, the nodules were effectively fixing nitrogen. The inoculated strains corresponded to the original strain (analyzed by ARDRA, data not shown). This result fulfilled the Koch's postulates. Up to now, all nodulating *Burkholderia* species have been isolated from legume nodules, but this novel species was isolated from tomato rhizosphere and rhizoplane. This finding opens up the possibility that other *Burkholderia* species, not recognized as nodulating strains, might harbor the machinery needed to nodulate legume plants. Accordingly, the twelve novel strains were analyzed for the amplification of a 320 pb *nodC* gene fragment using the primers nodC-F/nodC-R. All novel strains were positive for the amplification of *nodC* gene fragment. To corroborate the *nodC* identity, the DNA fragments were sequenced directly from PCR at the Instituto de Biotecnología-UNAM. The analysis of the sequences from strains TNe-841 and TNe-834 revelead the *nodC* gene, 99 % identical to *nodC* of *B. mimosarum*. The sequences were deposited at the GenBank with the following accession numbers, KF484909 for TNe-841 and KF484910 for TNe-834.

Taking together, the phenotypic and genotypic analyses of the group of 12 *Burkholderia* strains presented in this study, it is proposed that they should be considered a new species, for which we have proposed the name *Burkholderia caballeronis* sp. nov.

Description of *Burkholderia caballeronis* sp. nov

Burkholderia caballeronis: ca.bal.le'ro.nis. N.L. gen. masc. n. *caballeronis*, of Caballero, named after the Mexican microbiologist Jesús Caballero Mellado who contributed significantly to the knowledge on plant-associated *Burkholderia* species.

Cells are Gram negative and aerobic. Growth occurs in the range of 15–42 °C and in the presence of up to 5 % NaCl. Oxidase activity and assimilation of adipate is strain dependant. The strains are unable to reduce nitrate and nitrite, produce indol, hydrolyze esculine and gelatine, ferment D-glucose and assimilate D-maltose, citrate, erythritol, methyl-βD-xylopyranoside, dulcitol, methyl-αD-glucopyranoside, amydgaline, arbutine, salicine, D-lactose, inuline, D-melazitose, D-raffinose, amidon, glycogene and xylitol. β-galactosidase activity is negative. The following activities are present: arginine dihydrolase, urease, catalase, nitrogen fixation and assimilation of D-glucose, DL-arabinose, D-mannose, D-mannitol, *N*-acetyl glucosamine, gluconate, capric acid, malate, acetate, D-ribose, D-xylose, D-adonitol, D-galactose, D-fructose, L-ramnose, inositol, D-sorbitol, D-celobiose, D-turanose, D-lyxose, D-fucose, D-arabitol, potassium 2-ketogluconate and postassium 5-ketogluconate. The assimilation of adipate, glycerol, L-xylose, methyl-αD-mannopyranoside, esculine, D-melibiose, D-threalose, gentibiose, D-tagatose and L-arabitol is strain dependant. The major fatty acids are C16:0, C18:1 w7c, C19:0 cyclo w8c and C17:0 cyclo. The novel species is able to effectively nodulate *Phaseolus vulgaris* plants.

The strains TNe-841^T and TNe-878 have been deposited at BCCM/LMG and CIP with the following identifiers: LMG 26416^T = CIP 110324^T and LMG 26417 = CIP 110325, respectively.

The type strain is TNe-841 (=LMG 26416^T = CIP 110324^T) and was isolated from the rhizosphere of tomato plants growing in Nepantla, State of Mexico, Mexico. Phenotypic features are the same as described above for the species. Additionally, the oxidase activity is weak and the assimilation of glycerol, methyl-αD-mannopyranoside, esculine, D-melibiose, D-threalose, gentibiose, L-arabitol and dipate is negative. The assimilation of L-xylose and D-tagatose are positive. Cells are coccoïd rods (0.9 × 1.3–2.0 μm), single or in pairs, non motile. The DNA G+C content of strain TNe-841^T is 66.0 %.

Acknowledgments We are indebted to Dr. Euzeby for the etymological construction of the new bacteria species. We are grateful to Marco Antonio Rogel Hernández (Centro de Ciencias Genómicas, UNAM) for technical support. NSF IOS-0747525, IOS 1201735, and the Shanbrom Family Foundation support *Burkholderia* research in the Hirsch laboratory.

References

Angus AA, Lee A, Lum MR, Shehayeb M, Hessabi R, Fujishige NA, Yerrapragada S, Kano S, Song N, Yang P, Estrada-de los Santos P, de Faria SM, Dakora FD, Weinstock G, Hirsch AM (2013) Nodulation and effective nitrogen fixation of *Macroptilium atropurpureum* (siratro) by *Burkholderia tuberum* a nodulating and plant growth promitng

beta-proteobacterium, are influenced by environmental factors. Plant Soil 369:543–562
Anisimova M, Gascuel O (2006) Approximate likelihood-ratio test for branches: a fast, accurate, and powerful alternative. Syst Biol 55:352–539
Balandreau J, Mavingui P (2007) Beneficial interactions of Burkholderia spp. with plants. In: Coenye T, Vandamme P (eds) *Burkholderia*: molecular microbiology and genomics. Horizon Bioscience, Norfolk, pp 129–151
Caballero-Mellado J, Martnez-Aguilar L, Paredes-Valdez G, Estrada-de los Santos P (2004) *Burkholderia unamae* sp nov., an N_2-fixing rhizospheric and endophytic species. Int J Syst Evol Microbiol 54:1165–1172
Caballero-Mellado J, Onofre-Lemus J, Estrada-de los Santos P, Martinez-Aguilar L (2007) The tomato rhizosphere, an environment rich in nitrogen-fixing *Burkholderia* species with capabilities of interest of agriculuture and bioremediation. Appl Environ Microbiol 73:5308–5319
Chen WM, Moulin L, Bontemps C, Vandamme P, Bena G, Boivin-Masson C (2003) Legume symbiotic nitrogen fixation by b-proteobacteria is widespread in nature. J Bacteriol 185:7266–7272
Chen WM, James EK, Coenye T, Chou JH, Barrios E, de Faria SM, Elliott GN, Sheu SY, Sprent JI, Vandamme P (2006) *Burkholderia mimosarum* sp. nov., isolated from root nodules of *Mimosa* spp. from Taiwan and South America. Int J Syst Evol Microbiol 56:1847–1851
Chen WM, de Faria SM, James EK, Elliott GN, Lin KY, Chou JH, Sheu SY, Cnockaert M, Sprent JI, Vandamme P (2007) *Burkholderia nodosa* sp. nov., isolated from root nodules of the woody Brazilian legumes *Mimosa bimucronata* and *Mimosa scabrella*. Int J Syst Evol Microbiol 57:1055–1059
Chen WM, de Faria SM, Chou JH, James EK, Elliot GN, Sprent JI, Bontemps C, Young JPW, Vandamme P (2008) *Burkholderia sabiae* sp. nov. isolated from root nodules of *Mimosa caesalpiniifolia*. Int J Syst Evol Microbiol 58:2174–2179
De Meyer SE, Cnockaert M, Ardley JK, Maker G, Yates R, Howieson JG, Vandamme P (2013a) *Burkholderia sprentiae* sp. nov. isolated from *Lebeckia ambigua* root nodules from South Africa. Int J Syst Evol Microbiol. doi:10.1099/ijs.0.048777-0
De Meyer SE, Cnockaert M, Ardley JK, Trengove RD, Garau G, Howieson JG, Vandamme P (2013b) *Burkholderia rhynochosiae* sp. nov. isolated from *Rhynchosia ferulifolia* root nodules from South Africa. Int J Syst Evol Microbiol. doi:10.1099/ijs.0.048751-0
de Oliveira-Cunha C, Goda-Zuleta LF, de Almeida LGP, Prioli-Ciapina L, Borges WL, Pitard RM, Baldani JI, Straliotto R, de Faria SM, Hungria M, Sousa-Cavada B, Martinis-Mercante F, de Ribeiro Vasconcelos AT (2012) Complete genome sequence of *Burkholderia phenoliruptrix* BR3459a (CLA1), a heat-tolerant, nitrogen-fixing symbiont of *Mimosa flocculosa*. J Bacteriol 194:6675–6676
Denef V (2007) Biodegradation of organic anthropogenic pollutants by *Burkholderia* species. In: Coenye T, Vandamme P (eds) *Burkholderia*: molecular microbiology and genomics. Horizon Bioscience, Norfolk, pp 177–201
Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797
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-de los Santos P, Vacaseydel-Aceves NB, Martinez-Aguilar L, Cruz-Hernandez MA, Mendoza-Herrera A, Caballero-Mellado J (2011) *Cupriavidus* and *Burkholderia* species associated with agricultural plants that grow in alkaline soils. J Microbiol 49:867–876
Estrada-de los Santos P, Martínez-Aguilar L, Vinuesa P, Hirsch AM, Caballero-Mellado J (2013) Phylogenetic analysis of *Burkholderia* species by Multilocus sequence analysis. Curr Microbiol 67:51–60
Fahraeus G (1957) The infection of clover root hairs by nodule bacteria studied by a simple slide technique. J Gen Microbiol 16:379–381
Ferreira PAA, Bomfeti CA, Lima Soares B, de Souza Moreira FM (2012) Efficient nitrogen-fixing *Rhizobium* strains isolated from amazonian soils are highly tolerant to acidity and aluminium. World J Microbiol Biotechnol 28:1947–1959
Gillis M, Van TV, Bardin R, Goor M, Hebbar P, Willems A, Segers P, Kersters K, Heulin T, Fernandez MP (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
Goris J, de Vos P, Caballero-Mellado J, Park J, Falsen E, Quensen JF III, Tiedje JM, Vandamme P (2004) Classification of the biphenyl- and polychlorinated biphenyl-degrading strain LB400^T and relatives as *Burkholderia xenovorans* sp. nov. Int J Syst Evol Microbiol 54:1677–1681
Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52:696–704
Gyaneshwar P, Hirsch AM, Chen WM, Elliott GN, Bontemps C, Gross E, dos Reis Junior FB, Sprent JI, Young JPW, James EK (2011) Legume nodulating β-proteobacteria: diversity, host range and future prospects. Mol Plant-Microbe Int 24:1276–1288
Hauser AR, Jain M, Bar-Meir M, McColley SA (2011) Clinical significance of microbial infection and adaptation in cystic fibrosis. Clin Microbiol Rev 24:29–70
Howieson JG, De Meyer SE, Vivas-Marfisi A, Ratnayake S, Ardley JK, Yates RJ (2013) Novel *Burkholderia* bacteria isolated from *Lebeckia ambigua*—a perennial suffrutescent lagume of the fynbos. Soil Biol Biochem 6:55–64
Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, Park SC, Jeon YS, Lee JH, Yi H, Won S, Chun J (2012) Introducing EzTaxon-e: a prokaryotic 16S rRNA Gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol 62:716–721
Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
Mascarua-Esparza MA, Villa-Gonzalez R, Caballero-Mellado J (1988) Acetylene reduction and indoleacetic acid production by *Azospirillum* isolates from cactaceous plants. Plant Soil 106:91–95
Mesbah M, Premachandran U, Whitman WB (1989) Precise measurement of the G + C content of deoxyribonucleic

acid by high performance liquid chromathography. Int J Syst Bacteriol 39:159–167

Perin L, Martinez-Aguilar L, Castro-Gonzalez R, Estrada-de los Santos P, Cabellos-Avelar T, Guedes HV, Reis VM, Caballero-Mellado J (2006a) Diazotrophic *Burkholderia* species associated with field-grown maize and sugarcane. Int J Syst Evol Microbiol 72:3103–3110

Perin L, Martinez-Aguilar L, Paredes-Valdez G, Baldani JI, Estrada-de los Santos P, Reis VM, Caballero-Mellado J (2006b) *Burkholderia silvatlantica* sp. nov., a diazotrophic bacterium associated with sugarcane and maize. Int J Syst Evol Microbiol 56:1931–1937

Reis VM, Estrada-de los Santos P, Tenorio-Salgado S, Vogel J, Stoffels M, Guyon S, Mavingui P, Baldani VLD, Schmid M, Baldani JI, Balandreau J, Hartmann A, Caballero-Mellado J (2004) *Burkholderia tropica* sp. nov., a novel nitrogen-fixing, plantassociated bacterium. Int J Syst Evol Microbiol 54:2155–2162

Sheu SY, Chou JH, Bontemps C, Elliott GN, Gross E, James EK, Sprent JI, Young JPW, Chen WM (2012) *Burkholderia symbiotica* sp. nov., isolated from root nodules of *Mimosa* spp. native to north-east Brazil. Int J Syst Evol Microbiol 62:2272–2278

Sheu SY, Chou JH, Bontemps C, Elliot GN, Gross E, dos Reis Junior FB, Meldonian R, Moulin L, James EK, Sprent JI, Young JPW, Chen WM (2013) *Burkholderia diazotrophica* sp. nov., isolated from root nodules of *Mimosa* spp. Int J Syst Evol Microbiol 63:435–441

Suarez-Moreno ZR, Caballero-Mellado J, Coutinho BG, Mendonça-Previato L, James EK, Venturi V (2012) Common features of environmetal and potentially beneficial plant-associated *Burkholderia*. Microb Ecol 63:249–266

Tamura K, Peterson D, Stecher G, Nei M, Kumar S (2011) MEGA 5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distances, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Tindall BJ, Rosello-Mora R, Busse HJ, Ludwing W, Kampfer P (2011) Notes on the characterization of prokaryote strains for taxonomic purposes. Int J Syst Evol Microbiol 60:249–266

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, Goris J, Chen WM, 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

Versalovic J, Schneider M, de Bruijn FJ, Lupski JR (1994) Genomic fingerprinting of bacteria using repetitive sequence based polymerase chain reaction. Meth Mol Cell Biol 5:25–40

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703

Wilson K (1987) Preparation of genomic DNA from bacteria. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) Current protocols in molecular biology. Greene Publishing and Wiley-Interscience, New York, pp 2.4.1–2.4.5

Yang Z (1996) Among-site rate variation and its impact on phylogenetic analyses. Trends Ecol Evol 11:367–372

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