Burkholderia silvatlantica sp. nov., a diazotrophic bacterium associated with sugar cane and maize

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In a previous study, nitrogen-fixing isolates were recovered from the rhizosphere of maize and from surface-sterilized leaves of sugar cane cultivated in Rio de Janeiro, Brazil. On the basis of 16S rRNA gene sequence similarities, these isolates were identified as belonging to the genus *Burkholderia*, and whole-cell-protein profiles demonstrated that they are closely related to each other. In the present study, novel isolates were recovered from the roots of different sugar-cane varieties cultivated in diverse geographical regions of Brazil. Twenty-one nitrogen-fixing isolates were analysed using polyphasic taxonomy criteria, including DNA–DNA relatedness, 16S rRNA gene sequence similarities, fatty acid profiles, whole-cell-protein patterns and multilocus enzyme electrophoresis profiles, as well as morphological, physiological and biochemical characterization. The analysis confirmed that these isolates belong to a novel species within the genus *Burkholderia*, for which the name *Burkholderia silvatlantica* sp. nov. is proposed. The type strain, SRMrh-20^T (=LMG 23149^T=ATCC BAA-1244^T), was isolated from the rhizosphere of maize var. Avantis A2345 cultivated in Seropédica, Rio de Janeiro.

The genus *Burkholderia* was created in 1992 to include seven species transferred from *Pseudomonas*, with *Burkholderia cepacia* as the type species (Yabuuchi *et al.*, 1992). Coenye & Vandamme (2003) reported that the genus *Burkholderia* included over 30 species, but N_2-fixing species were poorly represented in the genus until recently. Nitrogen fixation, the reduction of atmospheric N_2 to ammonia, was reported only for *Burkholderia vietnamiensis* (Gillis *et al.*, 1995) of all the species of the genus. Nevertheless, a study by Estrada-de los Santos *et al.* (2001) showed that *Burkholderia* could be a genus rich in plant-associated nitrogen-fixers. In that study, *Burkholderia kururiensis* (Zhang *et al.*, 2000) was identified as a diazotrophic species, and many N_2-fixing isolates recovered from different plants (maize, coffee and sorghum) or their rhizospheres have subsequently been classified within novel *Burkholderia* species, including *Burkholderia unamae* (Caballero-Mellado *et al.*, 2004), *Burkholderia xenovorans* (Goris *et al.*, 2004) and *Burkholderia tropica* (Reis *et al.*, 2004). Concomitantly, two nodulating N_2-fixing strains recovered from legume plants were assigned to the genus *Burkholderia* according to their 16S rRNA gene sequences (Moulin *et al.*, 2001), and later they were formally classified as *Burkholderia phymatum* and *Burkholderia tuberum* (Vandamme *et al.*, 2002). The ability of several as yet unnamed strains to form nodules on *Macroptilium atropurpureum*, on *Mimosa* species (including *Mimosa pigra*) and on other mimosoid legumes probably indicates that these micro-organisms represent novel *Burkholderia* species (Barrett & Parker, 2005, 2006; Chen *et al.*, 2005). It is worth noting that the diazotrophic *Burkholderia* species, with the exception of *B. vietnamiensis*, comprise a group of closely related species that are distant from the group of opportunistic pathogens referred to as the *Burkholderia cepacia* complex, which includes *B. vietnamiensis* (Coenye & Vandamme, 2003).

In the present study, a polyphasic approach was undertaken to determine the taxonomic status of diazotrophic isolates tentatively designated as the *Burkholderia* NAR group. These include isolates from sugar-cane and maize plants reported by Perin *et al.* (2006) together with novel strains isolated from other sugar-cane varieties grown in different geographical regions of Brazil. A detailed analysis confirmed that these isolates belong to a novel species within the genus *Burkholderia*.

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Abbreviations: ARDRA, amplified rDNA restriction analysis; MLEE, multilocus enzyme electrophoresis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains SRMrh-20^T, SRCL-18, SRMrh-85 and PPCR-2 are AY965240–AY965243, respectively.

A dendrogram derived from MLEE and SDS-PAGE showing whole-cell protein profiles for the novel strains, N_2-fixing *Burkholderia* species and *B. sacchari* are available as supplementary figures in IJSEM Online.

The sources of the 21 N_2-fixing *Burkholderia* isolates that were analysed are shown in Table 1. Eleven of these strains were described previously (Perin *et al.*, 2006); they correspond to those tentatively named as *Burkholderia* NAR isolates. In a previous study, diazotrophic isolates were obtained using both nitrogen-free semi-solid BAz and JMV media for enrichment, and BAc agar plates for the isolation and purification of isolates as described previously (Perin *et al.*, 2006). In the present study, nitrogen-free semi-solid JMV (pH 5·2) and LGI (pH 6·0) media (Perin *et al.*, 2006) were used for enriching diazotrophic bacteria, and the strains were purified in JMV and LGI agar plates containing yeast extract (100 and 20 mg l^{-1}, respectively). Nitrogenase activity (N_2 fixation) was confirmed in pure cultures by the acetylene-reduction method (Burris, 1972). The presence of *nifH* genes was determined with primers IGK (Poly *et al.*, 2001) and NDR-1 (Valdés *et al.*, 2005), using PCR-amplification conditions as described by Perin *et al.* (2006). Although many novel N_2-fixing *Burkholderia* isolates from each sample (rhizosphere or plant tissues) were obtained, only one representative strain, based on amplified rDNA restriction analysis (ARDRA) as described below, from each sample was included in the present study. Earlier, we had reported that the *Burkholderia* NAR isolates reduce acetylene to ethylene and also that *nifH* genes were present in several of these isolates recovered from maize and sugarcane plants (Perin *et al.*, 2006). These characteristics are confirmed in the novel isolates examined in the present study (data not shown), thereby confirming that they all possess the ability to fix nitrogen.

Total DNA isolation, amplification of the 16S rRNA genes by PCR and ARDRA assays were performed as described by Estrada-de los Santos *et al.* (2001). Each isolate was assigned to one ARDRA genotype as described previously (Estrada-de los Santos *et al.*, 2001). Many isolates had an ARDRA profile that was identical (data not shown) to those of micro-organisms designated as *Burkholderia* NAR strains (Perin *et al.*, 2006), thus confirming the prevalence of this N_2-fixing group in association with sugar cane in Brazil. Previously, 16S rRNA gene sequences were obtained from strains SRMrh-20^T, SRMrh-85 and SRCL-318 (Perin *et al.*, 2006) and deposited in the GenBank/EMBL database (accession numbers are shown in Fig. 1). In the present study, the 16S rRNA gene sequence was obtained from a novel isolate, strain PPCR-2. Remarkably, the 16S rRNA gene sequence of strain PPCR-2, isolated from the roots of sugar cane var. RB 85-5113, grown in Paraná State (1100 km from Rio de Janeiro), showed 100 % similarity with those of strains SRMrh-20^T and SRMrh-85, both of which had been isolated from maize cultivated in Rio de Janeiro (Perin *et al.*, 2006), and with the sequence from an unidentified strain designated AB48 (GenBank accession no. AF164043), originally isolated from pineapple (*Ananas comosus*) in Bahia (Cruz *et al.*, 2001). This confirmed that all of these strains belong to the same species. In addition, this result suggests

Table 1. Source and locality of isolation of the strains investigated in this study

Leaves from which isolates were obtained were surface-sterilized.

Strain	Plant and cultivar	Source	Locality	Reference
SRMrh-20^T (=LMG 23149^T=ATCC BAA-1244^T)	Maize, Avantis 2345	Rhizosphere	Rio de Janeiro	Perin *et al.* (2006)
SRMrh-85	Maize, Avantis 2345	Rhizosphere	Rio de Janeiro	Perin *et al.* (2006)
SRMrh-3	Maize, Avantis 2345	Rhizosphere	Rio de Janeiro	Perin *et al.* (2006)
SRMrh-77	Maize, Avantis 2345	Rhizosphere	Rio de Janeiro	Perin *et al.* (2006)
SRMrh-109	Maize, Avantis 2345	Rhizosphere	Rio de Janeiro	Perin *et al.* (2006)
SRMrh-114	Maize, Avantis 2345	Rhizosphere	Rio de Janeiro	Perin *et al.* (2006)
SRCL-318 (=LMG 23150=ATCC BAA-1246)	Sugar cane, RB 72-454	Leaves	Rio de Janeiro	Perin *et al.* (2006)
SRCL-319	Sugar cane, RB 72-454	Leaves	Rio de Janeiro	Perin *et al.* (2006)
SRCL-321	Sugar cane, RB 72-454	Leaves	Rio de Janeiro	Perin *et al.* (2006)
SRCL-327	Sugar cane, RB 72-454	Leaves	Rio de Janeiro	Perin *et al.* (2006)
PPCR-2 (=LMG 23151=ATCC BAA-1245)	Sugar cane, RB 85-5113	Roots	Paraná	This work
PPCR-3	Sugar cane, RB 85-5113	Roots	Paraná	This work
PPCR-7	Sugar cane, RB 72-454	Roots	Paraná	This work
PPCrh-15	Sugar cane, RB 72-454	Rhizosphere	Paraná	This work
PSCR-88	Sugar cane, SP 80-3280	Roots	Sao Paulo	This work
PSCR-95	Sugar cane, SP 80-3280	Roots	Sao Paulo	This work
CRCrh-25	Sugar cane, AKBAR	Rhizosphere	Rio de Janeiro*	This work
TPCrh-75	Sugar cane, SP 78-4764	Rhizosphere	Pernambuco	This work
TPCrh-89	Sugar cane, SP 78-4764	Rhizosphere	Pernambuco	This work
TPCrh-85	Sugar cane, SP 87-344	Rhizosphere	Pernambuco	This work
AB48	Pineapple, Perolera	Roots	Bahia	Cruz *et al.* (2001)

*Plants were collected in the campus of Universidade Federal Rural do Rio de Janeiro.

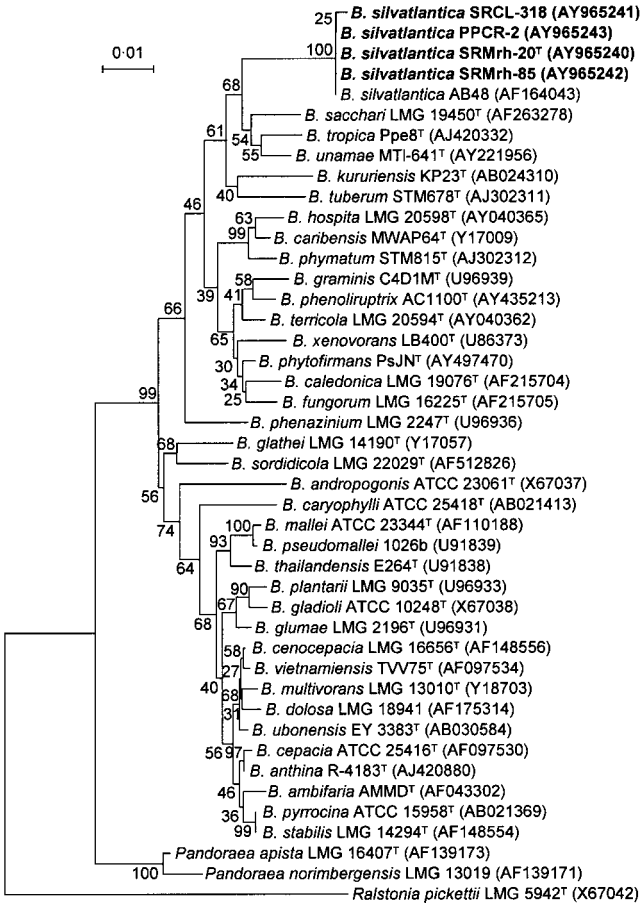

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences of *Burkholderia* species. The multiple alignments of the sequences were performed with CLUSTAL W, version 1.8 (Thompson *et al.*, 1994). A neighbour-joining tree, based on 1329 sites, was constructed (Saitou & Nei, 1987), and a distance matrix was generated according to Jukes & Cantor (1969) by using the program MEGA, version 2.1 (Kumar *et al.*, 2001). Numbers at branch points indicate percentages of bootstrap support based on 1000 replications (Kumar *et al.*, 1993). Bar, 1 substitution per 100 nucleotide positions.

that ARDRA profiles can be used successfully to differentiate the novel isolates from other *Burkholderia* species. A neighbour-joining phylogenetic tree based on available 16S rRNA gene sequences from *Burkholderia* species is shown in Fig. 1. It illustrates the position of five of the novel strains recovered during different seasons and from diverse plants and geographical regions of Brazil. *Burkholderia sacchari*, a non-diazotrophic species, and *B. unamae* and *B. tropica*, two N_2-fixing bacteria, were closely related to the cluster constituted by the novel strains (97·1, 96·9 and 96·8 % identity, respectively). The novel strains constituted a cluster that is significantly distant from the cluster formed by the diazotroph *B. vietnamiensis* (<96 % identity) and other species included in the *B. cepacia* complex (Fig. 1).

A total of 21 novel isolates and type strains of N_2-fixing and other related taxa were analysed by means of classical phenotypic tests, multilocus enzyme electrophoresis (MLEE) and SDS-PAGE of whole-cell proteins. Colony morphology was examined on JMV agar plates and growth was recorded after 4 days incubation at 29 °C. Bacteriological and biochemical characterization was carried out by growing isolates in BSE medium (Estrada-de los Santos *et al.*, 2001) for 12 h at 29 °C. Thereafter, the cultures were centrifuged and resuspended in fresh medium and the cell number was adjusted as described previously (Caballero-Mellado *et al.*, 2004). An aliquot (10 µl) from each culture was streaked on solid medium and incubated for 72 h at 29 °C, unless otherwise indicated. The temperature effects on growth were determined on BSE agar medium. Growth on BAc agar plates (Estrada-de los Santos *et al.*, 2001), MacConkey agar (Difco) plates and on *B. cepacia* selective agar medium (Henry *et al.*, 1997) was also determined. Growth of *Burkholderia* isolates using 1-aminocyclopropane-1-carboxylic acid as the sole nitrogen source was examined on Az-Acc medium as described previously (Caballero-Mellado *et al.*, 2004). In addition, phenotypic tests to determine nitrate reduction, gelatin liquefaction, aesculin hydrolysis and urease activity were performed with the API 20NE system, according to the instructions of the manufacturer (bioMérieux). The oxidase reaction was assayed as described by Caballero-Mellado *et al.* (2004). The assimilation of 49 carbon sources was determined with the API 50 CH system and CHB medium (bioMérieux), and the results were obtained after 5 days at 29 °C. Acetylene-reduction activity with succinate, benzoate and propionate as single carbon sources was assayed as described previously (Estrada-de los Santos *et al.*, 2001). Two replicates were used in each test. MLEE and SDS-PAGE assays 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 at 29 °C.

Colonies produced by the novel isolates on JMV agar plates were round, cream-coloured with yellow in the centre, smooth, convex with entire margins and were 2–3 mm in diameter. Phenotypic characteristics useful for differentiating the novel strains from the most closely related species, *B. sacchari*, and from diazotrophic *Burkholderia* species are summarized in Table 2. The novel strains differ from *B. sacchari* in their ability to reduce acetylene to ethylene and they differ from all diazotrophic *Burkholderia* species by their poor ability to fix nitrogen when using propionate as a carbon source. All of the novel strains showed the same assimilation profile for 49 carbon sources. Differences in the utilization of carbon sources among N_2-fixing *Burkholderia* species and *B. sacchari* are summarized in Table 3. Notably, the novel strains differ from all diazotrophic *Burkholderia* species (except *B. vietnamiensis*) by their ability to use sucrose as a carbon source; they differ from *B. vietnamiensis* by their ability to use adonitol and rhamnose.

Table 2. Phenotypic characteristics for the differentiation of strains of *B. silvatlantica* sp. nov. from diazotrophic *Burkholderia* species and *B. sacchari*

Taxa: 1, *B. silvatlantica* sp. nov. ($n=21$); 2, *B. sacchari* LMG 19450^T; 3, *B. tropica* Ppe8^T; 4, *B. unamae* MTl-641^T; 5, *B. kururiensis* KP23^T; 6, *B. xenovorans* LMG 21463^T; 7, *B. vietnamiensis* TVV75^T. Data were obtained in this study unless indicated. Symbols: +, good growth or activity; ±, poor growth or activity; −, no growth; ND, no data available; NI, not identified; NK, proportion not known. Surface pellicle formation and acetylene reduction activity were determined in nitrogen-free semi-solid BAz medium.

Characteristic	1	2	3	4	5	6	7
Surface pellicle formation	+	−	+	+	+	+	+
Acetylene reduction activity (N_2 fixation) without azelate in the presence of:							
Succinate	+	−	+	+	+	+	+
Propionate	±	−	+	+	+	+	+
Benzoate	±	−	−	−	+	+	+
Growth on:							
BAc agar	+	±	+	+	−	+	+
BSE agar at 42 °C	−	−	−	−	+	−	+
MacConkey agar at 29 °C	−	−	+	±	−	±	+
MacConkey agar at 37 °C	−	−	−	−	−	−	+
B. cepacia selective agar	−	−	−	−	−	−	+
Az-Acc agar	−	−	−	+	−	±	−
Fatty acid content (%)*							
16:0	24·8	17·7	ND	18·0	18·2	18·2	19·5
17:0 cyclo	14·1	3·7	ND	7·4	4·8	5·1	14·0
18:1ω7*c*	16·5	34·0	ND	32·6	33·9	27·3	NK
19:0 cyclo ω8*c*	9·4	NI	ND	4·0	4·4	3·6	5·8
Summed feature 3†	7·5	23·4	ND	14·9	13·2	19·1	9·8

*Data for reference strains were taken from Caballero-Mellado *et al.* (2004) (*B. unamae* MTl-641^T), Goris *et al.* (2004) (*B. xenovorans* LB400^T), Brämer *et al.* (2001) (*B. sacchari* IPT101^T and *B. kururiensis* KP23^T) and Coenye *et al.* (2001a, b) (several *B. vietnamiensis* strains).
†Summed feature 3 was reported to consist of the following: 16:1ω7*c* and/or 16:1ω6*c* for *B. silvatlantica* strains, 16:1ω7*c* and/or 15:0 iso 2-OH for *B. unamae*, *B. xenovorans* and *B. vietnamiensis* and 16:1ω7*c* for *B. sacchari* IPT101^T and *B. kururiensis* KP23^T.

The combination of alleles for 13 enzyme loci, analysed in MLEE assays, was common to all of the novel strains (data not shown), and thus a single electrophoretic type was present. The genetic relationships among N_2-fixing *Burkholderia* species and *B. sacchari* based on the MLEE results are illustrated in a dendrogram (see Supplementary Fig. S1 in IJSEM Online). The novel isolates diverged, at genetic distances ranging from 0·560 to 0·820, from other diazotrophic *Burkholderia* species, and they diverged from *B. sacchari* at a genetic distance of 0·890. The fact that the coefficients of genetic distance are above 0·5 strongly supports the argument that the isolates analysed represent a novel *Burkholderia* species. In other studies, estimates of genetic relatedness of bacterial species obtained by MLEE were strongly correlated with estimates of divergence obtained from DNA–DNA reassociation experiments (for a review see Martínez-Romero & Caballero-Mellado, 1996).

The SDS-PAGE protein patterns of some representative novel strains isolated from different plants and geographical regions are shown in Supplementary Fig. S2 (available in IJSEM Online). The 21 strains analysed showed almost identical protein profiles, but strains AB-48 and SRCL-318 (having identical profiles) showed slight differences with respect to the other strains. However, the protein profiles of the novel strains were clearly distinct from those of the N_2-fixing *Burkholderia* species examined and from that of their closest relative, *B. sacchari* (Supplementary Fig. S2). It is well established that bacteria with identical or very similar protein patterns possess high levels of genome similarity (Vandamme *et al.*, 1996). On this basis, the SDS-PAGE results confirm that the novel isolates represent a novel diazotrophic species.

Three strains were analysed for their cellular fatty acid compositions. The strains were grown in trypticase soy broth agar at 28 °C for 24 h and the analysis was performed by Microbial ID, Inc. (Newark, DE, USA). Strains SRMrh-20^T, SRCL-318 and PPCR-2 had almost identical fatty acid profiles, but slight quantitative variations were observed. The major fatty acid components (means and standard deviations based on the profiles of three strains) were as follows: 14:0 (4·6±0·4 %), 16:0 (26·5±1·9 %), 16:0 2-OH (2·7±0·1 %), 16:0 3-OH (4·9±0·7 %), 16:1 2-OH (1·9±0·3 %), 17:0 cyclo (17·2±3·5 %), 18:1ω7*c* (14·0±4·5 %), 18:1 2-OH (1·0±0·2 %), 19:0 cyclo ω8*c* (11·7±2·0 %), summed feature 2 (5·8±0·7 %) and summed feature 3 (5·3±2·0 %). Summed feature 2

Table 3. Differences in the utilization of carbon sources by strains of *B. silvatlantica* sp. nov., diazotrophic *Burkholderia* species and *B. sacchari*

Taxa: 1, *B. silvatlantica* sp. nov. ($n=21$); 2, *B. sacchari* LMG 19450^T; 3, *B. tropica* ($n=10$); 4, *B. unamae* ($n=20$); 5, *B. kururiensis* KP23^T; 6, *B. xenovorans* LMG 21463^T; 7, *B. vietnamiensis* TVV75^T. Data for *B. unamae* and *B. tropica* strains, including the respective type strains, are from Caballero-Mellado *et al.* (2004); other data were obtained in this study. Strains of *B. silvatlantica* sp. nov. assimilated *N*-acetylglucosamine, adonitol, D- and L-arabinose, D-arabitol, cellobiose, erythritol, D-fructose, L-fucose, galactose, glucose, glycerol, inositol, mannitol, mannose, rhamnose, ribose, sorbitol, sucrose and D-xylose. None of the *B. silvatlantica* strains assimilated amygdalin, L-arabitol, arbutin, dulcitol, aesculin, D-fucose, β-gentiobiose, gluconate, methyl α-D-glucoside, 2-ketogluconate, 5-ketogluconate, glycogen, inulin, lactose, D-lyxose, maltose, melibiose, melezitose, methyl α-D-mannoside, methyl β-xyloside, D-raffinose, salicin, starch, L-sorbose, D-tagatose, trehalose, D-turanose, xylitol or L-xylose. Symbols: +, good growth; −, no growth; V, variable (55–70 % of the strains gave a positive reaction).

Carbon source	1	2	3	4	5	6	7
Adonitol	+	+	+	+	+	−	−
L-Arabitol	−	−	−	−	+	−	−
Cellobiose	+	−	+	+	−	+	+
β-Gentiobiose	−	−	+	−	−	−	+
Lactose	−	−	V	−	−	−	−
D-Raffinose	−	+	−	−	−	−	+
Rhamnose	+	−	+	+	+	+	−
Ribose	+	−	+	−	−	+	+
Salicin	−	−	V	−	−	−	+
Sucrose	+	+	−	−	−	−	+
Trehalose	−	−	V	+	−	−	+
Xylitol	−	−	−	−	+	−	−

corresponds to 14 : 0 3-OH, 16 : 1 iso I (an unknown fatty acid with an equivalent chain-length of 10·947) or 12 : 0 alde or any combination of these fatty acids. Summed feature 3 corresponds to 16 : 1ω7*c* and/or 16 : 1ω6*c*. These profiles are similar to the fatty acids reported for other *Burkholderia* species (Vandamme *et al.*, 1997; Coenye *et al.*, 2001b), except for the component 16 : 1ω6*c*. The fatty acid profile of the novel strains showed clear quantitative differences from those of N_2-fixing *Burkholderia* species, and from that of the phylogenetically closest species, *B. sacchari* (Table 2). For example, the novel isolates contain relatively small amounts of 18 : 1ω7*c* and summed feature 3 and relatively large amounts of 19 : 0 cyclo ω8*c* in comparison with diazotrophic *Burkholderia* species and *B. sacchari*. This could be useful for identification of the species represented by the novel strains.

DNA–DNA reassociation experiments were based on relative levels of hybridization to ^{32}P-labelled DNA from novel strain SRMrh-20^T, as described previously (Estrada-de los Santos *et al.*, 2001). DNA–DNA relatedness assays were performed with eight novel strains as well as with the type strain of *B. sacchari*, the most closely related *Burkholderia* species (as indicated by 16S rRNA gene sequence data), and other N_2-fixing *Burkholderia* species. The DNA–DNA reassociation values between strain SRMrh-20^T and other novel strains were in the range 75–102 % (SRMrh-85, 102 %; SRCL-327, 99 %; PPCrh-15, 96 %; SRMrh-77, 86 %; AB48, 83 %; SRCL-18, 79 %; PPCR-2, 78 %; PPCR-3, 75 %), indicating a relationship at the species level (Stackebrandt *et al.*, 2002; Vandamme *et al.*, 1996). In contrast, low reassociation values (below 30 %) were obtained in hybridizations of strain SRMrh-20^T with *B. sacchari* LMG 19450^T (=IPT101^T) (30 %) and N_2-fixing *Burkholderia* species such as *B. unamae* MTl-641^T (28 %), *B. tropica* Ppe8^T (27 %), *B. kururiensis* KP23^T (22 %), *B. xenovorans* LMG 21463^T (=LB-400^T) (21 %), *B. vietnamiensis* TVV75^T (19 %), *B. tuberum* STM678^T (14 %) and *B. phymatum* STM815^T (13 %). These DNA–DNA reassociation data, together with the 16S rRNA gene sequence analyses, SDS-PAGE protein patterns, MLEE assay data and fatty acid profiles, support the notion that diazotrophic isolates previously and tentatively named as the *Burkholderia* NAR group (Perin *et al.*, 2006) belong to a novel species within the genus *Burkholderia*, for which we propose the name *Burkholderia silvatlantica* sp. nov.

Description of *Burkholderia silvatlantica* sp. nov.

Burkholderia silvatlantica (sil.vat.lan′ti.ca. L. n. *silva* wood, forest; L. fem. adj. *Atlantica* pertaining to the Atlas Mountains and by extension to the Atlantic Ocean; N.L. fem. adj. *silvatlantica* pertaining to the Atlantic forest of Brazil).

Cells are straight rods (1·6–1·9 μm long and 0·8–0·9 μm wide), each having a polar tuft of flagella. Isolates are Gram-negative and oxidase- and catalase-positive. Growth and nitrogenase activity are observed with different carbon sources in nitrogen-free LGI, JMV and BAz semi-solid media. Strains grown on JMV agar plates produce colonies that are round, cream-coloured with yellow in the centre, smooth, convex with entire margins and that are 2–3 mm in diameter. Grows on BSE medium at 29 and 37 °C, but not at 42 °C. Does not grow on MacConkey agar or *B. cepacia* selective agar medium at 29 or 37 °C. Phenotypic characteristics useful for differentiation from other N_2-fixing *Burkholderia* species and the most closely related species, *B. sacchari*, are shown in Table 2. Nitrate is reduced to nitrite but not to N_2; there is urease activity and aesculin hydrolysis, but not liquefaction of gelatin or indole production. Additional phenotypic characteristics are listed in Table 3. Can be differentiated phenotypically from all diazotrophic *Burkholderia* species and from *B. sacchari* on the basis of SDS-PAGE protein profiles, the electrophoretic mobility patterns of metabolic enzymes and fatty acid profiles. Strains SRMrh-20^T, SRMrh-85, PPCR-2 and AB48 show 100 % similarity among their 16S rRNA gene

sequences and show 99·77 % similarity to strain SRCL-318. Can also be differentiated genomically from all diazotrophic *Burkholderia* species, and from *B. sacchari*, by ARDRA profiles.

The type strain, SRMrh-20^T (= LMG 23149^T = ATCC BAA-1244^T), was isolated from the rhizosphere of maize var. Avantis A2345, cultivated in the Experimental Campus of Embrapa Agrobiology in Seropédica, Rio de Janeiro, Brazil. The phenotypic and genomic characteristics of the type strain are the same as those described above for the species.

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References

Barrett, C. F. & Parker, M. A. (2005). Prevalence of *Burkholderia* sp. odule symbionts on four mimosoid legumes from Barro Colorado Island, Panama. *Syst Appl Microbiol* **28**, 57–65.

Barrett, C. F. & Parker, M. A. (2006). Coexistence of *Burkholderia*, *Cupriavidus*, and *Rhizobium* sp. nodule bacteria on two *Mimosa* spp. in Costa Rica. *Appl Environ Microbiol* **72**, 1198–1206.

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

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

Caballero-Mellado, J., Fuentes-Ramirez, L. E., Reis, V. M. & Martinez-Romero, E. (1995). Genetic structure of *Acetobacter diazotrophicus* populations and identification of a new genetically distant group. *Appl Environ Microbiol* **61**, 3008–3013.

Caballero-Mellado, J., Martínez-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.

Chen, W. M., de Faria, S. M., Straliotto, R. & 10 other authors (2005). Proof that *Burkholderia* strains form effective symbioses with legumes: a study of novel *Mimosa*-nodulating strains from South America. *Appl Environ Microbiol* **71**, 7461–7471.

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

Coenye, T., LiPuma, J. J., Henry, D., Hoste, B., Vandemeulebroecke, K., Gillis, M., Speert, D. P. & Vandamme, P. (2001a). *Burkholderia cepacia* genomovar VI, a new member of the *Burkholderia cepacia* complex isolated from cystic fibrosis patients. *Int J Syst Evol Microbiol* **51**, 271–279.

Coenye, T., Mahenthiralingam, E., Henry, D., LiPuma, J. J., Laevens, S., Gillis, M., Speert, D. P. & Vandamme, P. (2001b). *Burkholderia ambifaria* sp. nov., a novel member of the *Burkholderia cepacia* complex including biocontrol and cystic fibrosis-related isolates. *Int J Syst Evol Microbiol* **51**, 1481–1490.

Cruz, L. M., de Souza, E. M., Weber, O. B., Baldani, J. I., Döbereiner, J. & Pedrosa, F. de 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.

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.

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.

Goris, J., De Vos, P., Caballero-Mellado, J., Park, J.-H., Falsen, E., Quensen, J. F., III, Tiedje, J. M. & Vandamme, P. (2004). Classification of the PCB- and biphenyl-degrading strain LB400 and relatives as *Burkholderia xenovorans* sp. nov. *Int J Syst Evol Microbiol* **54**, 1677–1681.

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.

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

Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001). MEGA2: molecular evolutionary genetics analysis software. Tempe, AZ: Arizona State University.

Martínez-Romero, E. & Caballero-Mellado, J. (1996). *Rhizobium* phylogenies and bacterial genetic diversity. *Crit Rev Plant Sci* **15**, 113–140.

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.

Perin, L., Martínez-Aguilar, L., Castro-González, R., Estrada-de los Santos, P., Cabellos-Avelar, T., Guedes, H. V., Reis, V. M. & Caballero-Mellado, J. (2006). Diazotrophic *Burkholderia* species associated with field-grown maize and sugarcane. *Appl Environ Microbiol* **72**, 3103–3110.

Poly, F., Jocteur-Monrozier, L. & Bally, R. (2001). Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. *Res Microbiol* **152**, 95–103.

Reis, V. M., Estrada-de los Santos, P., Tenorio-Salgado, S. & 10 other authors (2004). *Burkholderia tropica* sp. nov., a novel nitrogen-fixing, plant-associated bacterium. *Int J Syst Evol Microbiol* **54**, 2155–2162.

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

Stackebrandt, E., Frederiksen, W., Garrity, G. M. & 10 other authors (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**, 1043–1047.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment

through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Valdés, M., Pérez, N. O., Estrada-de los Santos, P., Caballero-Mellado, J., Peña-Cabriales, J. J., Normand, P. & Hirsch, A. M. (2005). Non-*Frankia* actinomycetes isolated from surface-sterilized roots of *Casuarina equisetifolia* fix nitrogen. *Appl Environ Microbiol* **71**, 460–466.

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.

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.