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Diazotrophic *Burkholderia* species isolated from the Amazon region exhibit phenotypical, functional and genetic diversity

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ABSTRACT

Forty-eight *Burkholderia* isolates from different land use systems in the Amazon region were compared to type strains of *Burkholderia* species for phenotypic and functional characteristics that can be used to promote plant growth. Most of these isolates ($n = 46$) were obtained by using siratro (*Macroptilium atropurpureum* – 44) and common bean (*Phaseolus vulgaris* – 2) as the trap plant species; two isolates were obtained from nodules collected in the field from *Indigofera suffruticosa* and *Pithecellobium* sp. The evaluated characteristics were the following: colony characterisation on "79" medium, assimilation of different carbon sources, enzymatic activities, solubilisation of phosphates, nitrogenase activity and antifungal activity against *Fusarium oxysporium* f. sp. *phaseoli*. Whole cell protein profiles, 16S rRNA, *gyrB*, and *recA* gene sequencing and multilocus sequence typing were used to identify the isolates. The isolates showed different cultural and biochemical characteristics depending on the legume species from which they were obtained. Except for one isolate from *I. suffruticosa*, all isolates were able to solubilise calcium phosphate and present nitrogenase activity under free-living conditions. Only one isolate from common beans, showed antifungal activity. The forty four isolates from siratro nodules were identified as *B. fungorum*; isolates UFLA02-27 and UFLA02-28, obtained from common bean plants, were identified as *B. contaminans*; isolate INPA89A, isolated from *Indigofera suffruticosa*, was a close relative of *B. caribensis* but could not be assigned to an established species; isolate INPA42B, isolated from *Pithecellobium* sp., was identified as *B. lata*. This is the first report of nitrogenase activity in *B. fungorum, B. lata* and *B. contaminans*.

Introduction

The genus *Burkholderia* of the subclass β-proteobacteria was originally created to accommodate seven species from the *Pseudomonas* genus [52]. Currently, more than 60 *Burkholderia* species have been validly named (http://www.bacterio.cict.fr/b/burkholderia.html), and new species are frequently discovered. This genus has revealed a great diversity of species and ubiquity, as isolates have been isolated from human, animal and environmental sources [12].

In recent years, there has been considerable interest in using *Burkholderia* as plant-growth-promoting rhizobacteria (PGPR), because several mechanisms to promote plant growth were detected in this genus. They are described as having the ability to fix N_2 as free-living or endophytic bacteria [20] or as symbiotic bacteria with leguminous species [18,19]. Previous reports described some *Burkholderia* strains as phosphate solubilising bacteria [11,40], and several strains have been used as biocontrol agents because of their ability to inhibit many fungi that cause plant diseases [31,40]. Strains of this genus are also able to produce phytohormones and secrete siderophores, among other abilities [15]. Despite the useful properties those organisms may offer to in

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agriculture, commercial application has been questioned because the risk that some strains may pose to human health, mainly those of *Burkholderia cepacia* complex (Bcc) strains.

The Amazonian forest is known throughout the world for its high diversity of plant and animal species. However, little is known about its microbial diversity. By partial 16S rRNA sequencing of 88 isolates that were representative of 1890 isolates from Western Amazonas State soil under diverse land use system, six different genera were found that were related to the well-known α- and β-rhizobia *Azorhizobium*, *Rhizobium, Bradyrhizobium, Mezorhizobium, Sinorhizobium* and *Burkholderia* [32], demonstrating the high diversity in this region. When common bean plants (*Phaseolus vulgaris*) were used as the trap species, isolates similar to *Burkholderia cepacia* were also captured [5]. In another study, isolates from different land use systems in Acre, Eastern Amazonas, and Rondonia States soil had partial 16S rRNA gene sequences similar to *B. caribensis*, *B. cepacia* and *Burkholderia* spp. accessions [37]. However, the reliable identification and environmental role of these *Burkholderia* isolates are not clear.

The identification of species in the genus *Burkholderia* is complex, as the 16S rRNA gene sequence used in the taxonomy of prokaryotes cannot be used for reliable identification. Housekeeping genes showed better separation of related species, including those of the *Burkholderia* genus, and they must be used in identification and species definition [45]. The *B. cepacia* complex (Bcc) is a group of close related species that share a high (>97.5%) level of 16S rRNA gene sequence similarity and moderate (30–60%) DNA–DNA hybridisation values [13,49], thus, for reliable identification other techniques are necessary, such as *rec*A gene sequencing and multilocus sequence analysis [47].

Understanding the ecological role of *Burkholderia* associated with legume plants and its phenotypical and functional characteristics to improve plant growth is important for a potential use in agriculture. On the other hand, knowing the potential pathogens among such isolates is also relevant both for the safe use of *Burkholderia* strains in agriculture and for etiological studies. The aims of the present study were to identify the *Burkholderia* isolates obtained from Amazonian soils accurately to the species level and to evaluate their functional characteristics that may be used to promote plant growth.

Materials and methods

Origin of isolates

Forty isolates were obtained from "Conservation and Sustainable Management of Below-Ground Biodiversity" (CSM-BGBD) project, the Brazilian component of which is called BiosBrasil (www.biosbrasil.ufla.br). The studied area was located in the Benjamin Constant Municipality, Northwest Amazonas state, on the triple border of Brazil, Colombia and Peru, within the geographic coordinates 4°20′ and 4°26′ South and 69°36′ and 70°2′ West. The sampling grids were characterised [22], and the respective sampling points and land use systems (LUS) in the area using six categories (Table S1). The isolates were obtained from Amazon soils using siratro (*Macroptilium atropurpureum*) [32] and *Phaseolus vulgaris* [5] as the trap species. Six isolates used in the present study were obtained in the course of the project "Alternatives to Slash-and-Burn/International Centre for Research in Agroforestry" (ASB/ICRAF) carried out in 1997. These isolates were obtained from the Amazonian States of Rondônia and Acre by using siratro as trap species from soil samples taken at 0–20 cm depth in four land use systems: pasture, agriculture, agroforestry and fallow. A preliminary identification by comparison of their partial 16S rRNA sequences indicated they belonged to the genus *Burkholderia* [37] (Table S1). Finally, another two isolates (INPA89A and INPA42B) were obtained from root nodules collected in the field from *Indigofera suffruticosa* (Fallow-Rondônia State) and *Pithecellobium* sp. roots (Pristine forest-Amazonas State) [33,39] (Table S1).

Cultural and biochemical characterisation

At first, the cultural (morphological and physiological) characterisation of 48 isolates from the Amazon region and type strains of *Burkholderia* species was performed on culture medium 79 [23] and the following variables were analysed: change in the pH of the culture medium after growth (acid, alkaline, neutral); time needed for the appearance of isolated colonies (fast, 2–3 days; intermediate, 4–5 days; slow, 6–10 days); elevation; colour; exopolysaccharide production (few, moderate and abundant); consistency; indicator absorption and light transmission (opaque, brilliant and translucent) [27].

APIZYM and API20 NE micro test systems were performed according to the recommendations of the manufacturer (bioMérieux).

SDS-PAGE of whole-cell proteins

Burkholderia isolates were grown on nutrient agar (CM3; Oxoid) supplemented with 0.04% (w/v) KH_2PO_4 and 0.24% (w/v) $Na_2HPO_4{\cdot}12H_2O$ (pH 6.8) and incubated at 28 °C for 48 h. Whole-cell protein preparations and SDS-PAGE were performed as previously described [39,41]. Densitometric analysis, normalisation and interpolation of the protein profiles and numerical analysis using Pearson's product-moment correlation coefficient were performed using the gelcompar 4.2 software package (Applied Maths). The protein profiles were compared with the data base of the LM-UGent (Laboratory of Microbiology, Ghent University) research group which consists of whole cell protein profiles of all established *Burkholderia* species [13].

16S rRNA sequencing

Six isolates obtained from siratro root nodules (UFLA04-53, UFLA04-130, UFLA04-136, UFLA04-155, UFLA04-219 and UFLA04-223) and the isolate from *Indigofera suffruticosa*, INPA89A, were chosen for 16S rRNA sequencing based in whole cell protein profiles. Nearly full-length 16S rRNA genes were amplified with the primer pair 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) [30] from log phase cultures in 79 medium. Isolated colonies were removed, placed in 1.5-mL microtubes with 1 mL sterile ultrapure water, and heated to 95 °C for 10 min. An aliquot of 5 μL of lysed cells was used for PCR reaction. The PCR products were purified with Microcon™ filters (Millipore). Single pass sequencing of PCR amplified rDNAs was performed with the 27F and 1492R primers in a 3730xl sequencer.

The sequences were assembled using DNASTAR SeqMan Version 5.0. Sequences were selected based on the Phred value being equal to or greater than 20 and were compared with GenBank sequences using the Basic Local Alignment Search Tool (NCBI). Only sequences with more than 1300 bp were used in the phylogenic analyses. Novel sequences and selected sequences of reference strains were aligned using ClustalW [26], and the phylogenetic tree was inferred by the neighbour-joining method using Kimura's 2-parameter model [28] as implemented in the MEGA 4.1 package [44]. A bootstrap confidence analysis was performed with 1000 replicates.

gyrB gene sequencing

Apart from isolates utilised for 16S rDNA sequencing (UFLA04-53, UFLA04-130, UFLA04-136, UFLA04-155, UFLA04-219, UFLA04-223 and INPA89A), an additional five isolates from siratro root nodules (UFLA04-138, UFLA04-148, UFLA04-218, UFLA04-239 and UFLA04-240) and 17 type and reference strains of *B. fungorum* and close relatives and *B. caribensis* were utilised for *gyrB* sequencing. To prepare genomic DNA, the isolates were grown in trypticase soy agar for 2 days at 28 °C. Then, the DNA was obtained using the alkaline lysis procedure [3]. The PCR was performed using the primers gyrB-20-F 5′-GAC AAY GGB CGY GGV RTB CC-3′ and gyrB-21-R 5′-ACR CCR TTR TTC AGG AAY GA-3′, which were redesigned from primers designed in Ref. [45]. The obtained PCR products were purified in the Robot Sample Preparation System (Tecan, Switzerland). Sequencing was performed using an Applied Biosystems 3100 DNA sequencer following the manufacturer's protocols (Perkin-Elmer). The sequencing primers for *gyr*B were the same as those used for the amplification. Alignment and phylogenetic tree was inferred as described in 16S rRNA sequencing.

recA gene sequence analysis and multilocus sequencing typing (MLST)

Two isolates obtained from root nodules of common bean plants (UFLA02-27 and UFLA02-28) and one from *Pithecellobium* sp. nodule (INPA42B) showed the similarity with Bcc strains in previous work [5,37,39]. The *recA* gene sequences of these isolates were determined as described [34]. In addition, MLST analysis was performed as previously described [4]. Alignment and phylogenetic tree of *rec*A gene and concatenated sequences of MLST was constructed as described in 16S rRNA sequencing. The sequences of each allele type at all seven loci are available at http://pubmlst.org/bcc/.

Nucleotide sequence accession number

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences obtained in this study are detailed in Supplementary Table S2.

Phosphate solubilisation assay

For the phosphate solubilisation assay used on all of the isolates, we used GL medium [43] modified: 0.5% (w/v) yeast extract; 1% (w/v) glucose and 2% (w/v) agar. The phosphates used were 0.26% (w/v) $CaHPO_4$ and 0.43% (w/v) $Fe(PO_4)_3$ [42], both at pH 6.8. Aluminium phosphate was made by mixing 6% (w/v) K_2HPO_4 and 5,34% (w/v) $AlCl_3$, then adding to the medium at pH 4.5 [25]. The isolates were maintained for 15 days by incubating at 28°C. Bacterial colonies forming clear zones were considered to be phosphate solubilisers. We also included for comparison *Burkholderia tuberum* LMG 21444^T, *Burkholderia nodosa* LMG 23741^T, *Burkholderia mimosarum* LMG 23256^T, *Burkholderia cepacia* LMG 1222^T, *Burkholderia caribensis* LMG 18531^T, *Burkholderia silvatlantica* LMG 23149^T, *Burkholderia tropica* LMG 22274^T, *Burkholderia unamae* LMG 22722^T, *Burkholderia vietnamiensis* LMG 10929^T and *Burkholderia xenovorans* LMG 21463^T.

Nitrogenase activity

All 48 *Burkholderia* isolates were evaluated for their ability to grow by fixing N_2 in nitrogen-free LO medium [17]. Growth of these isolates was also evaluated in LO medium modified by replacing lactate by mannitol or fructose in the same concentration. After the formation of typical thick pellicle near the medium surface, nitrogenase activity was confirmed using the acetylene reduction assay (ARA) [16]. The production of ethylene was verified by gas chromatography (Varian Star 3400 c*x*). We also tested the ten *Burkholderia* type strains listed above in the phosphate solubilisation assay. *Azorhizobium* spp. type strains (*A. doebereinerae* BR 5401^T and *A. caulinodans* ORS 571^T) were used as positive controls [17,38] in the media with lactate and frutose.

Antifungal-activity of Burkholderia isolates

Some isolates (UFLA04-155, UFLA04-236, UFLA04-237, UFLA04-239, INPA42B, INPA89A and UFLA02-27) that were representative of the groups obtained by the morphological and physiological characterisation on 79 medium were chosen for the antifungal activity test. The antifungal activities of *Burkholderia* isolates were tested against *Fusarium oxysporum* f. sp. *phaseoli* using a method modified from Ref. [40]. Discs (6 mm) of *Fusarium* mycelium growing in PDA (Potato Dextrose Agar) were transferred to plates of the same medium, to which three different bacteria had been streaked. The plates were incubated for 3 days at 28 °C. After the incubation, the isolates that inhibited fungal growth were verified. Each isolate was tested in triplicate. The ten *Burkholderia* type strains listed above in phosphate solubilisation assay were also tested.

nodC and nifH genes amplification and nodulation test

Burkholderia isolates (UFLA04-53, UFLA04-130, UFLA04-136, UFLA04-155, UFLA04-219, UFLA04-223, UFLA04-138, UFLA04-148, UFLA04-218, UFLA04-239, UFLA04-240, UFLA02-28, INPA89A and INPA42B) were examined for the presence of *nod*C and *nif*H gene using the primers nodCF and nodCI [29] and 19F and 407R [46]. The *nod*C and *nif*H gene were also examined using the primers for betaproteobacteria [8]. *Burkholderia tuberum* LMG 21444^T was used as positive control both for *nod*C and *nif*H genes. Nodulation test with siratro (*Macroptilium atropurpureum*), cowpea (*Vigna unguiculata*) and common beans (*Phaseolus vulgaris*) in axenic conditions were realised by using recycled 350 mL "long neck" beer bottles containing 300 mL Jensen solution was reported previously [32]. Based on results of identification, the isolates UFLA02-28, UFLA04-53, UFLA04-155, UFLA04-239 and INPA42B were utilised for nodulation test.

Results and discussion

In the present study, we investigated the phenotypic and functional characteristics of bacterial isolates obtained from Amazonian soils trapped with different legume hosts (*M. atropurpureum*, *P. vulgaris*, *Indigofera suffruticosa* and *Pithecellobium* sp.). The 48 isolates isolated from Amazonian soils presented different morphological and physiological characteristics depending on the legume species from which they were isolated (Table 1). The isolates obtained using siratro as the trap species presented similar characteristics on 79 medium, and they were different from those isolated from *Indigofera suffruticosa* and *Pithecellobium* sp. (INPA89A and INPA42B) and common bean plants (UFLA02-27 and UFLA02-28) (Table 1). All isolates showed fast growth on 79 medium at 28 °C, only taking 2–3 days for the appearance of isolated colonies. However, other characteristics, such as pH reaction on culture medium, exopolysaccharide production, colour, elevation, consistency, indicator absorption and light transmission were isolate dependent.

Only isolate INPA42B was able to assimilate all 12 carbon sources tested (Table 1). For the enzymatic activity, all isolates presented a positive reaction for catalase, C_4-esterase, C_8-ester lipase, leucine aminopeptidase, alkaline phosphatase and acid phosphatase activities. The following characteristics were absent

in all of the investigated isolates: nitrite reduction; indole production; arginine dihydrolase; valine aminopepetidase; trypsin; chymotrypsin; α-galactosidase; β-galactosidase; β-glucuronidase; α-glucosidase; β-glucosaminidase and α-fucosidase.

The cultural and biochemical characterisation showed that isolates presented similar traits depending on the trap plant utilised. This also occurred regarding the whole cell protein profiles. All isolates captured from *M. atropurpureum* showed very similar of protein profiles (Fig. 1). When the cell protein of all 48 *Burkholderia* isolates were compared in the database, 44 isolates obtained using siratro as trap species were indistinguishable from *B. fungorum* reference strains (Fig. 1), with more than 85% of similarity. The isolate from *I. suffruticosa* (INPA89A) had a protein profile that was unique (Fig. 1). Finally, the remaining three isolates, (UFLA02-27, UFLA02-28 and INPA42B) had whole cell protein profiles that resembled those of *B. cepacia* complex (Bcc) strains but unequivocal species assignment was not possible (data not shown).

Based on results of whole cell proteins, we used different genotypic methods to determine the phylogenetic position and accurate species status of isolates. For those captured from siratro plants (very close to *B. fungorum*) and the isolate from *I. suffruticosa* (INPA89A), were utilised 16S rDNA and *gyr*B sequencing. By 16S rRNA sequencing, six representative isolates (UFLA04-130, UFLA04-155, UFLA04-53, UFLA04-223, UFLA04-219 and UFLA04-136) presented 100% identity with rRNA gene sequences of *B. fungorum* reference strains (Fig. 1); the 16S rRNA gene of isolate INPA89A was 99% similar to that of the *B. caribensis* type strain with which it was clustered supported by a bootstrap value of 100 (Fig. 2). A dendrogram based on the *gyrB* sequences of the isolates analysed is shown in Fig. 3. The isolates from siratro nodules clustered with *B. fungorum* type and reference strains. The isolate INPA89A, which was obtained from an *Indigofera suffruticosa* nodule, occupied a unique position in the tree. After protein profiling and 16S rRNA and *gyrB* gene sequencing, we could conclude that

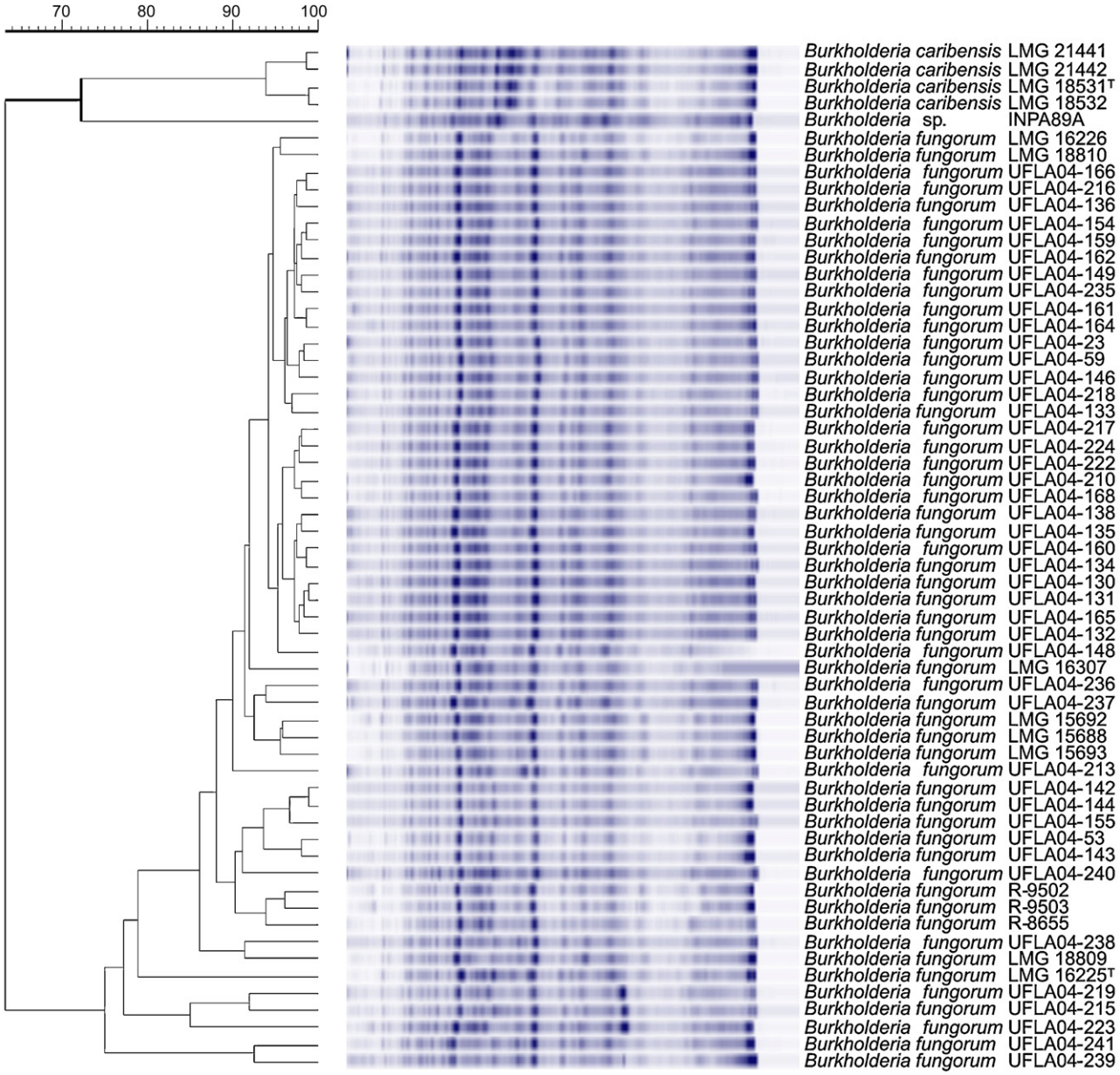

Fig. 1. Dendrogram derived from UPGMA of correlation coefficients between the whole-cell protein patterns of *Burkholderia* isolates obtained from Amazonian soils using siratro (*Macroptilium atropurpureum*) as the trap species and *B. fungorum* and *B. caribensis* type and reference strains.

Table 1
Cultural and biochemical characteristics of *Burkholderia* isolates obtained from Amazonian soils by using different plant species as trap.

Characteristics	Siratro isolates (*M. atropurpureum*)	INPA89A (*I. suffruticosa*)	INPA42B (*Pithecellobium* sp.)	UFLA02-27 (*P. vulgaris*)
Cultural characteristics on 79 medium:				
Medium pH	Neutral	Acid	Alkaline	Acid
Exopolysaccharide production[a]	M/A	A	M	A
Color	Cream	Cream	White	Yellow
Elevation	Convex	Convex	Flat	Convex
Consistency	Gum	Gum	Butter	Gum
Indicator absorption	+/−	+	−	+
Light transmission	Translucent	Brilliant	Opaque	Brilliant
Assimilation of:				
Maltose	−	−	+	−
Caprate	−	−	+	+
Adipate	+	−	+	+
Malate	+	−	+	+
Citrate	+	−	+	+
Phenyl-acetate	+	−	+	+
Activity of:				
Nitrate reduction	+	−	+	−
Oxidase	+	−	−	+
β-Glucosidase	−	−	+	+
Gelatinase	−	−	+	+
Urease	+	+	−	−
C_{14}-Lipase	−	−	+	+
Phosphoamidase	−	+	+	+
Cystine aminopeptidase	+	−	−	−
α-Mannosidase	−	−	−	+

Characteristics are scored as: + positive, − negative, +/− variable, depending on the isolate utilised. The following features are present in all strains investigated: fast growth (2–3 days for the appearance of isolated colonies); assimilation of glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine and gluconate; catalase, C4-esterase, C8-esterase lipase, leucine aminopeptidase and alkaline and acid phosphatase activity.

[a] A: abundant; M: moderate.

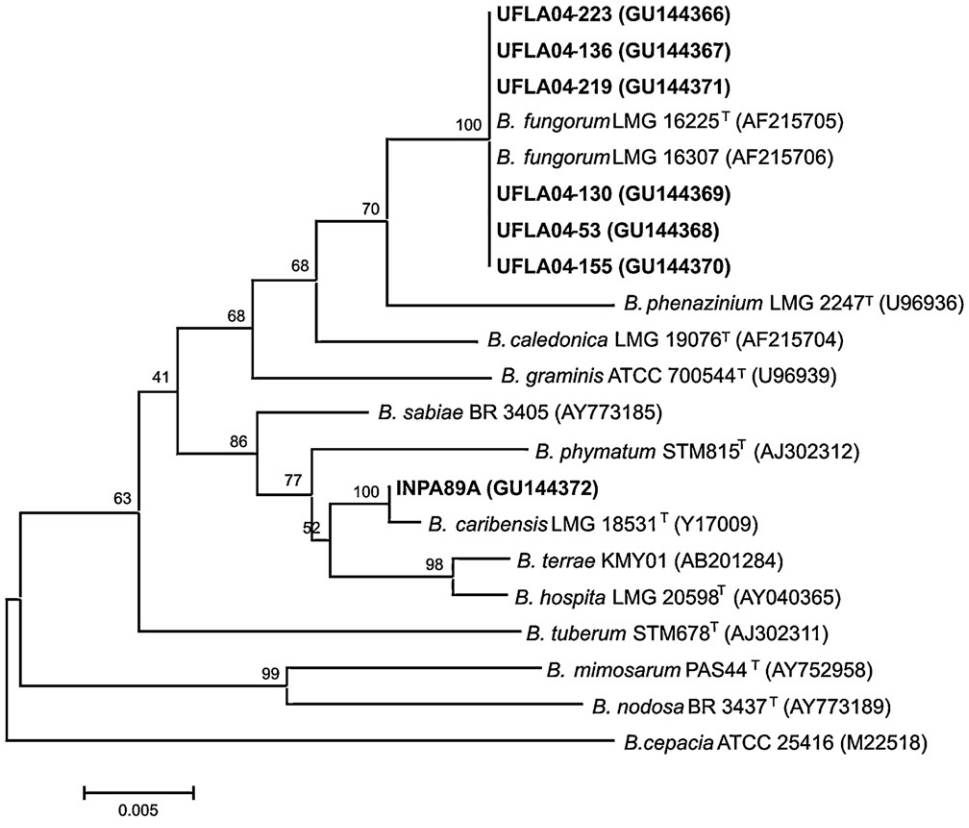

Fig. 2. Phylogenetic tree of isolates obtained from Amazonian soils and *Burkholderia* type strains based on 16S rDNA sequences. Phylogenies were estimated by the neighbour-joining method. Bootstrap values were based on 1000 trials.

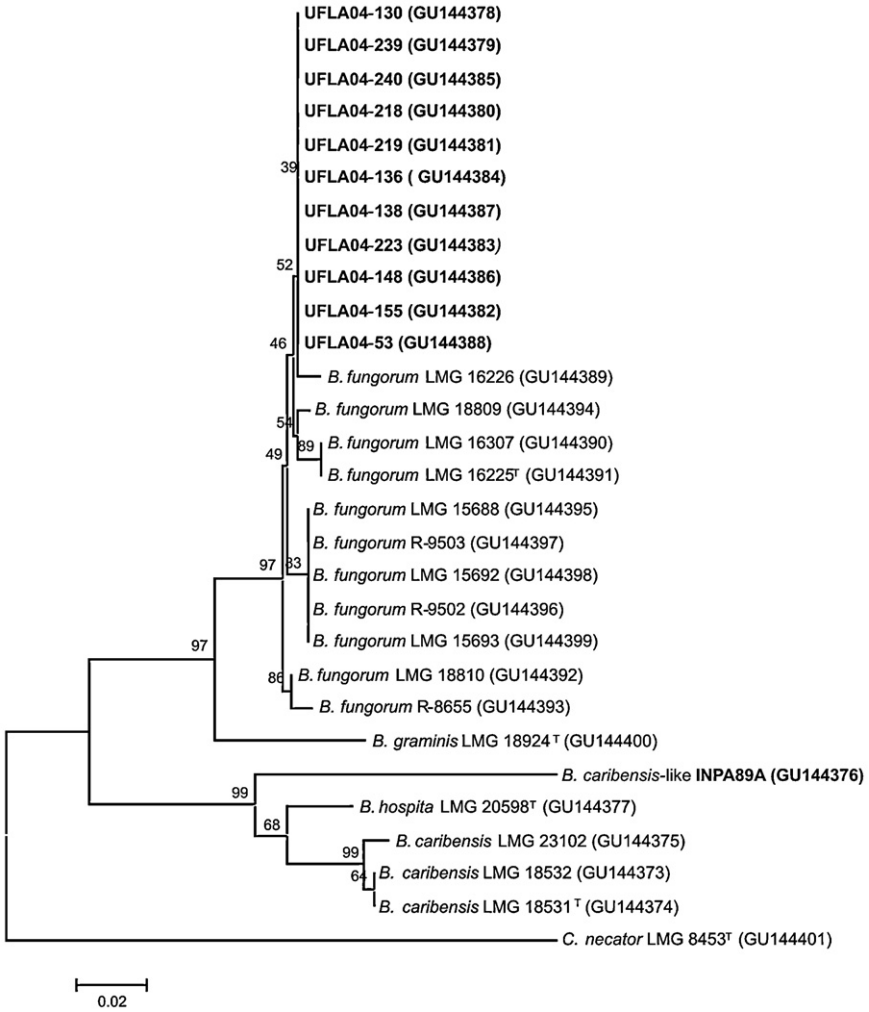

Fig. 3. Phylogenetic tree of isolates obtained from Amazonian soils and *Burkholderia* type strains based on *gyr*B sequences. Phylogenies were estimated by the neighbour-joining method. Bootstrap values were based on 1000 trials.

the isolates from siratro nodules belong to *B. fungorum*. Similarly we concluded that the isolate INPA89A from *Indigofera suffruticosa* nodules represents a species closely related but distinct from *B. caribensis*.

Isolates were identified as *B. fungorum* just by comparing their whole cell protein profiles with those from type and reference strains in the data base LM-UGent. A high level of whole cell protein pattern similarity correlates with a high DNA–DNA hybridisation level, and the use of this method for accurate species level identification of many bacterial species has been validated [50]. Identification to species level of *B. fungorum* isolates was confirmed with 16S rRNA and *gyr*B gene sequences, as for the *gyr*B sequences there is a divergence less than 4% between different *Burkholderia* species [45]. However, to INPA89, isolated from *Indigofera suffruticosa*, these techniques were useful to identification as a species closely to *B. caribensis* but other methods are necessary to confirm the classification.

The comparison of whole cell protein profiles and 16S rRNA sequencing did not enable reliable identification of isolates belonging to Bcc. *rec*A gene and multilocus sequence analysis are indicated for identification of the large majority of *B. cepacia* complex [47]. Thus, the other three isolates (UFLA02-27, UFLA02-28 and INPA42B) close to Bcc were analysed by *recA* gene sequencing and MLST. Sequence analysis of *rec*A gene revealed that all three isolates belonged to the so-called taxon K [51] within the Bcc with the *recA* sequences of isolates UFLA02-27 and UFLA02-28 being more than 99% similar to those of *B. contaminans* and *B. metallica* reference strains; the *recA* sequence of isolate INPA42B being more than 98% similar to those of *B. lata* and *B. contaminans* reference strains. The phylogenetic tree (Fig. 4) showed ULA02-27 and UFLA02-28 clustered close to *B. contaminans* strains and INPA42B to *B. lata* strains. For the *rec*A sequences, there is generally a gap of 4–5% between different species [34] except for the taxon k species, where our isolates belong. Some studies demonstrated that the *rec*A genes of Bcc showed 98–99% similarity within species [34,51]. In the MLST analysis, new sequences (alleles) were found for five loci of isolates UFLA02-27 and UFLA02-28 (*glt*B, *gyr*B, *rec*A, *lep*A, and *trp*B) and for all loci of isolate INPA42B (Table S3). The taxonomic position of the isolates was confirmed by the tree (Fig. 5). The isolates UFLA02-27 and UFLA02-28 represented a single novel sequence type belonging to *B. contaminans* and INPA42B also represented a novel sequence type which was identified as a member of *B. lata*. Vanlaere et al. [51] demonstrated that for species belonging to this group MLST is required and there is typically less than 3% divergence within Bcc species and more than 3% between different species.

Thus, phenotypic characteristics and different genotypic methods were necessary to allow the identification between different species of *Burkholderia*, including Bcc that is a group very close of bacteria making their differentiation quite difficult. For all isolates obtained in this work, is the first report showing the occurrence

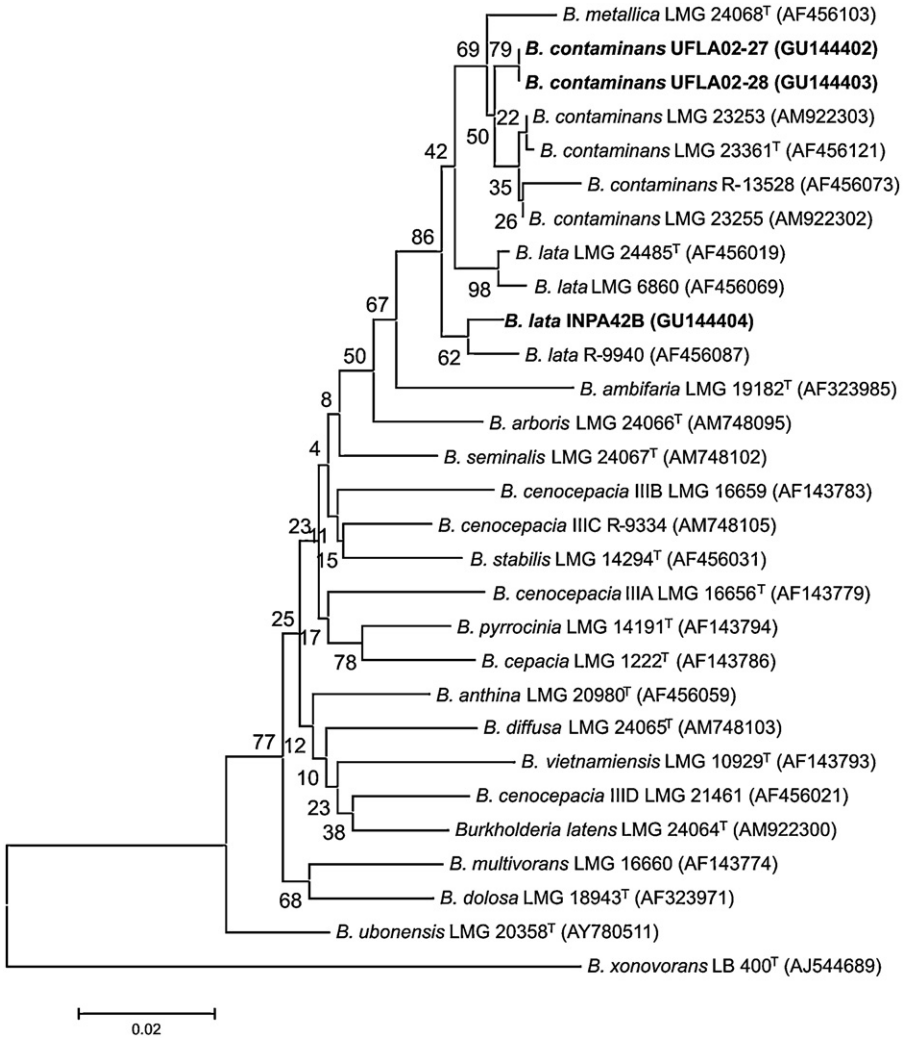

Fig. 4. Phylogenetic tree derived from the *rec*A gene sequences of Bcc isolates from Amazon soils and type and reference strains. Phylogenies were estimated by the neighbour-joining method. Bootstrap values were based on 1000 trials.

of these species in Amazonian soils. *B. fungorum* has been isolated from human, animal, fungal and environmental samples [14]. *B. caribensis* is a soil bacterium isolated from vertisol microaggregates in Martinique (the French West Indies) [1]. *B. lata* has been isolated from environmental and human samples and *B. contaminans* from human and animal samples [51].

Regarding plant growth promoting traits, the isolates presented diverse mechanisms. All of the isolates except UFLA04-158 and INPA89A were able to solubilise calcium phosphate (Table 2). The type strains *B. cepacia* LMG 1222^T, *B. tropica* LMG 22274^T, *B. silvatlantica* LMG 23149^T and *B. vietnamiensis* LMG 10929^T were also able to solubilise calcium phosphate. None of the isolates or type strains was able to solubilise iron and aluminium phosphates. All *B. fungorum* isolates trapped using siratro, the isolates *B. contaminans* UFLA02-27 (common beans) and *B. lata* INPA42B (*Pithecellobium* sp.) and some *Burkholderia* type strains (*B. unamae* LMG 22722^T, *B. tropica* LMG 22274^T, *B. silvatlantica* LMG 23149^T, *B. vietnamiensis* LMG 10929^T, *B. xenovorans* LMG 21463^T, *B. nodosa* LMG 23741^T, *B. tuberum* LMG 21444^T, and *B. caribensis* LMG 18531^T) presented positive ARA results on media with fructose as sole carbon source indicating the presence of nitrogenase, the enzyme responsible for N_2-fixation (Table 2). Only one isolate (INPA89A) and two type strains (*B. cepacia* LMG 1222^T and *B. mimosarum* LMG 23256^T) did not reduce acetylene (ARA) on the N-free media tested. The strains of *Azorhizobium doebereinerae* BR 5401^T and *A. caulinodans* ORS 571^T presented positive ARA in media with fructose and lactate as sole carbon sources, but not in medium containing mannitol, what was expected because they do not use mannitol as sole carbon source [17,38]. Most of the isolates were ARA negative on mannitol medium (Table 2). The antifungal-activity of the *Burkholderia* isolates and type strains were tested against *Fusarium oxysporum* f. sp. *phaseoli*, but only one isolate (*B. contaminans* UFLA02-27) trapped by common bean plants, and two type strains (*B. cepacia* LMG 1222^T and *B. vietnamiensis* LMG 10929^T) both belonging to the Bcc, presented this characteristic (Table 2), that is well known for Bcc strains [7,9,10,24,36,40].

All *Burkholderia* studied showed a diversity of cultural and biochemical characteristics, that is typical for members of the genus *Burkholderia*. Several studies have reported the ability of *Burkholderia* strains to solubilise inorganic phosphates [2,11,35,40]. This characteristic can be used to increase the available phosphate in soil and thus is a characteristic useful for plant growth promotion. In our collection of *Burkholderia* isolates this characteristic was widespread. Some *Burkholderia* strains are known for their ability to fix N_2 as free-living bacteria [20]. Other strains are known to nodulate and also fix N_2 on media, such as *B. phymatum* [18]; in our study *B. tuberum*, *B. nodosa* and *B. caribensis* type strains also possess this characteristic, however, we used a medium different from the one

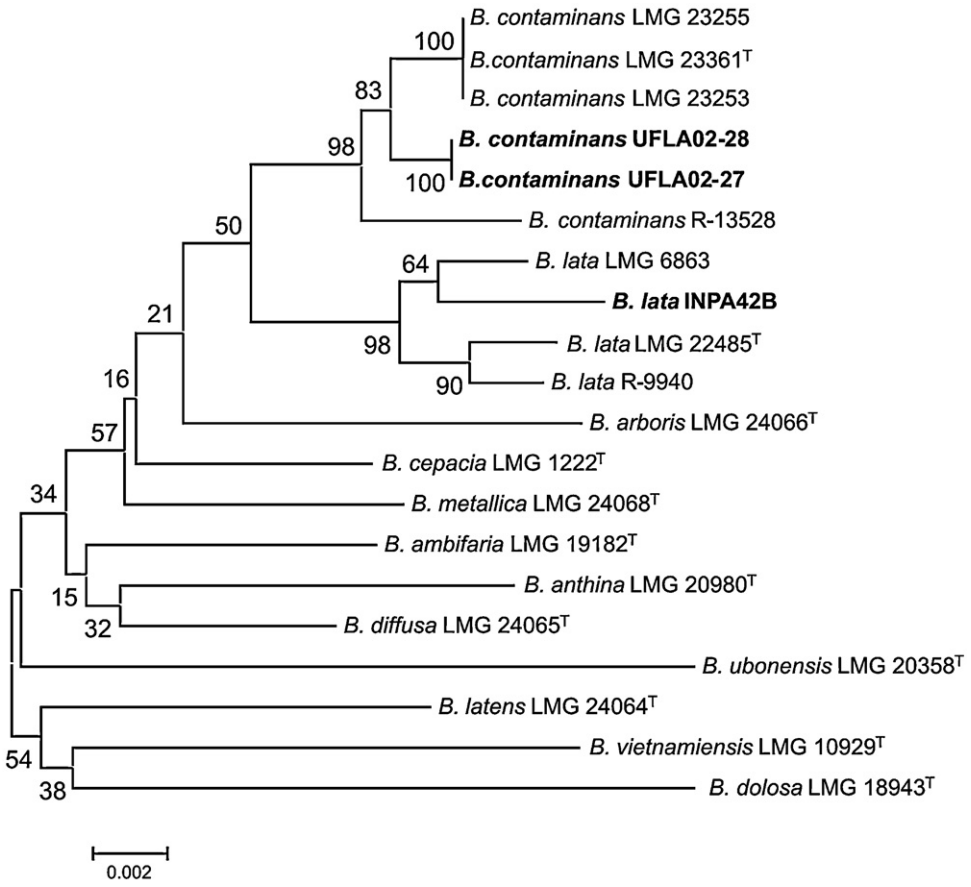

Fig. 5. Unrooted phylogenetic tree of concatenated nucleotide sequences from seven loci of Bcc isolates from Amazon soils and some type and reference. Phylogenies were estimated by neighbour-joining method. Bootstrap values were based on 1000 trials.

Table 2
Plant growth promotion traits of *Burkholderia* isolates obtained from Amazonian soils and type and reference strains.[a]

Isolates and strains of *Burkholderia*	Phosphate solubilisation	ARA[b]			Antifungal activity
		Fructose	Lactate	Mannitol	
Siratro strains (*M. atropurpureum*)	+	+	+	+/–	–
UFLA02-27 (*P. vulgaris*)	+	+	+	+	+
INPA42B (*Pithecellobium* sp.)	+	+	+	+	–
B. tuberum LMG 21444^T	–	+	+	–	–
B. nodosa LMG 23741^T	–	+	+	+	–
B. cepacia LMG 1222^T	+	–	–	–	+
B. caribensis LMG 18531^T	–	+	–	+	–
B. silvatlantica LMG 23149^T	+	+	+	–	–
B. tropica LMG 22274^T	+	+	+	–	–
B. unamae LMG 22722^T	–	+	+	–	–
B. vietnamiensis LMG 10929^T	+	+	+	+	+
B. xenoverans LMG 21463^T	–	+	+	+	–
A. doebereinerae BR 5401^T	ne	+	+	–	ne
A. caulinodans ORS 571^T	ne	+	+	–	ne

[a] Characteristics are scored as: +, positive; –, negative; ne, not evaluated.
[b] Detection of nitrogenase by measuring acetyle reduction activity in N-free semi-solid media with different sole carbon sources.

used by Elliot et al. [18] and with different carbon sources, explaining the different results obtained. Our results showed for the first time that *B. fungorum* is able to solubilise calcium phosphate and fix N_2. It could be an adaptative trait for rhizosphere inhabitants to be plant growth promoting bacteria.

The *nodC* genes were not detected in these isolates when we used primers that were designed for α-proteobacteria or for β-proteobacteria. The *nif*H gene was not detected using primers for β-proteobacteria; when using universal primers only nonspecific amplicon was detected. That is, an amplicon of 800 bp instead the usual found of 400 bp. When this nonspecific amplicon was sequenced, no similarity was found with *nif*H genes. The *nod*C and *nif*H of the positive control utilised, *B. tuberum* LMG 21444^T, was successfully amplified with primers designed for α-proteobacteria and with universal primers, respectively. However, amplification of both *nod*C and *nif*H of LMG 21444^T was not successful with the primers designed for β-proteobacteria, even after several attempts. The isolates of *B. fungorum* from siratro nodules did not nodulate when re-inoculated in this legume. The *B. caribensis*-like isolate INPA89A, *B. contaminans* UFLA02-27, UFLA02-28 and *B. lata*

INPA42B also did not form nodules in siratro and common bean plants (data not shown). However, some *B. fungorum* strains isolated from siratro plants were able to nodulate [21]. Therefore, it seems nodulating ability is not a common trait to all *B. fungorum* isolates. There are other reports of strains closely related to *B. fungorum* isolated from the root nodules of legume species [6,48]. However the nodulating capability and/or nitrogen fixation was not confirmed. The *B. fungorum* isolates of our study were recovered from Amazonian soils at different places (States, land uses) and times and were obtained from the root nodules of siratro plants formed after inoculation with soil suspensions diluted from 10^{-1} to 10^{-4}, indicating that *B. fungorum* is abundant in these soils. More isolates were captured from soils of old second forest and young second forest, following by agriculture and agroforestry, and less in pristine forest and pasture.

In conclusion, this study showed that different methods were necessary for the species identification of all isolates. Members of the genus *Burkholderia* occur in a variety of environmental habitats, including the very acid Amazon soils. The ability to nodulate seems not to be a common trait for all isolates; but they can be rhizospheric bacteria which can also have ability to colonise host plants. These *Burkholderia* have a potential to increase plant growth in diverse ways. In our study they were able to fix nitrogen as free-living organisms, solubilise calcium phosphate and possess antifungal activity, but their potential as plant growth promoting rhizobacteria has to be viewed with caution due to the widespread occurrence of *Burkholderia* species as opportunistic pathogens [48].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2012.04.001.

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