GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Genetic diversity of *Burkholderia* (Proteobacteria) species from the Caatinga and Atlantic rainforest biomes in Bahia, Brazil

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Genet. Mol. Res. 12 (1): 655-664 (2013)
Received March 5, 2012
Accepted August 6, 2012
Published March 11, 2013
DOI http://dx.doi.org/10.4238/2013.March.11.13

ABSTRACT. The genus *Burkholderia* (β-Proteobacteria) currently comprises more than 60 species, including parasites, symbionts and free-living organisms. Several new species of *Burkholderia* have recently been described showing a great diversity of phenotypes. We examined the diversity of *Burkholderia* spp in environmental samples collected from Caatinga and Atlantic rainforest biomes of Bahia, Brazil. Legume nodules were collected from five locations, and 16S rDNA and *rec*A genes of the isolated microorganisms were analyzed. Thirty-three contigs of 16S rRNA genes and four contigs of the *rec*A gene related to the genus *Burkholderia* were obtained. The genetic dissimilarity of the strains ranged from 0 to 2.5% based on 16S rDNA analysis, indicating two main branches: one distinct branch of the dendrogram for the *B. cepacia* complex and another

branch that rendered three major groups, partially reflecting host plants and locations. A dendrogram designed with sequences of this research and those designed with sequences of *Burkholderia*-type strains and the first hit BLAST had similar topologies. A dendrogram similar to that constructed by analysis of 16S rDNA was obtained using sequences of the fragment of the *rec*A gene. The 16S rDNA sequences enabled sufficient identification of relevant similarities and groupings amongst isolates and the sequences that we obtained. Only 6 of the 33 isolates analyzed via 16S rDNA sequencing showed high similarity with the *B. cepacia* complex. Thus, over 3/4 of the isolates have potential for biotechnological applications.

Key words: 16S rDNA; *rec*A; *Inga*; *Calliandra*; *Mimosa*; Legume nodule

INTRODUCTION

The genus *Burkholderia* currently comprises more than 60 species, most of them recently described (Suarez-Moreno et al., 2012). They are widely distributed in nature and found in soil, water, rhizosphere and in association with plants. They are also found in clinical samples, infecting patients with cystic fibrosis (Zhang and Xie, 2007; Garau et al., 2009; Marquez-Santacruz et al., 2010; Wong-Villarreal and Caballero-Mellado, 2010) .

Besides habitat diversity, *Burkholderia* species exhibit high genetic and phenotypic diversity, which means that two strains may have very similar 16S rDNA and the rest of the genome may be so different, that it would not allow classification within the same species. The high versatility and plasticity of this genus are due to the presence of replicons in its genetic material, in addition to genome size, ranging from about 5 to 9 megabases (Coenye and Vandamme, 2003; Payne et al., 2005; Zhang and Xie, 2007; Garau et al., 2009).

Thus, 16S rDNA gene sequencing, which has traditionally been used in phylogenetic studies, is often not sufficient to allocate isolates of *Burkholderia* at the species level. The sequencing of housekeeping genes such as *rec*A allows molecular identification of strains with higher resolution (Payne et al., 2005; Procopio et al., 2009).

Several *Burkholderia* species are involved in agronomical and biotechnological applications, such as: i) production of antifungal substances for medical, veterinary and agricultural purposes (Sultan et al., 2008); ii) biodegradation of polluting compounds such as trichloroethene (Zhang et al., 2000); iii) ability to fix nitrogen (Wong-Villarreal and Caballero-Mellado, 2010); iv) phosphate-solubilization (Valverde et al., 2006), or v) production of phytohormones (Shaharoona et al., 2007) promoting plant growth, and direct antagonism or induction of resistance against pathogens (Vandamme et al., 2007). Thus, the study of this genus is relevant for bioprospecting of new species or even strains with new or more pronounced properties with potential application in biotechnology (Chiarini et al., 2004; Kang et al., 2004; Li et al., 2007; O'Sullivan and Mahenthiralingam, 2005; Payne et al., 2005).

It is widely recognized that comparative genetic analysis between novel and previously characterized strains or species may show similarities in phenotypes of interest

(Garau et al., 2009; Wong-Villarreal and Caballero-Mellado, 2010). Therefore, considering the number of species of *Burkholderia* that have recently been described (Suarez-Moreno et al., 2012), the agronomic and biotechnological importance of this microbial genus (Compant et al., 2008), and also the agronomic relevance and environmental conservation in Bahia, Brazil, were motives to carry out this research. The main objectives of this study were: i) to collect and isolate new strains of the genus *Burkholderia* from root nodules of legumes in different regions from Caatinga and Mata Atlantica in Bahia, Brazil, ii) to characterize them genetically by 16S rRNA and *rec*A gene sequencing, and iii) to characterize the diversity between two main biomes and host plants.

MATERIAL AND METHODS

Microorganism isolation

Root nodules were obtained from the following host legume plants: *Inga vera* Willd, *Mimosa ophthalmocentra* Benth, *Mimosa xanthocentra* Mart, *Calliandra germana* Barneby, *Calliandra luetzelburgii* Harms, and *Calliandra hirtiflora* Benth, all belonging to the subfamily Mimosoideae. Samples were collected from two different environmental biomes, Caatinga (Raso da Catarina, Serra do Tromba, Pico do Barbado, and Piatã) and Mata Atlantica (Serra Bonita), both located in the State of Bahia. GPS locations are listed in Table 1. Once collected, the nodules were washed with 90% alcohol for 30 s, followed by 1% aqueous hypochlorite for 2 min, and then washed five times with distilled sterile water. Nodules were aseptically cut transversely, collected with tweezers and plated in SDM-79 medium (Fred and Waksman, 1928). Plates were incubated at 28°C for 5 days.

DNA isolation and PCR amplification of rDNA and *rec*A

Isolated colonies were transferred to 1.5-mL microcentrifuge tubes containing 1 mL TY liquid medium, and incubated at 28°C for 72 h, in a rotary shaker at 130 rpm. DNA was extracted according to Edwards et al. (1991) with modifications in the extraction buffer (200 mM Tris-HCl, pH 8.0, 1.5 M NaCl, 250 mM EDTA, 0.5% SDS and 3 M potassium acetate). DNA was purified with 24:1 chloroform:isoamyl alcohol, treated with 25% ammonium acetate and cold 75% isopropanol, washed with 70% ethanol and resuspended in sterile MilliQ water. 16S rDNA was amplified using universal primers F27 and R1525 (Lane, 1991), while the primers BUR3 and BUR4 (Payne et al., 2005) were used for *rec*A amplification.

The 16S rDNA was amplified using 30 mM $MgCl_2$, 0.2 mM dNTP, 0.2 pmol of each primer (F27 and R1525), 1 U Taq and 1-2 µg/mL genomic DNA. PCRs were carried out as follows: 1 cycle of 94°C for 4 min; 35 cycles of 94°C for 30 s, 61°C for 30 s, and 72°C for 1 min and 30 s, and a final 10-min extension at 72°C.

For *rec*A gene amplification, the same reaction conditions were used as above, except for the use of primers BUR3 and BUR4 and 20 mM $MgCl_2$. PCR products were visualized in a UV transilluminator after electrophoresis at 80 V on a 1% agarose gel stained with ethidium bromide.

DNA sequencing and molecular analysis

The material was sequenced by Macrogen Corp. (http://www.macrogen.com; Rockville, MD, USA), and only sequences with consistent contigs were used (overlapping forward and reverse sequences). The programs MEGA (Tamura et al., 2007), ClustalW (Thompson et al., 2002), Mega BLAST (Altschul et al., 1997), and CAP3 (Huang and Madan, 1999) were used for data analysis. Dendrograms were generated by neighbor-joining method using the Jukes-Cantor algorithm. Trees were subjected to bootstrap analysis with 1000 repetitions using only the sequence contigs generated and identified by Mega BLAST as strains close to the genus *Burkholderia*. The 16s rRNA sequences of 46 *Burkholderia*-type strains previously characterized, as well as those that refer to access returning the first hits (BLASTN) compared to those isolated in this study, were imported from Ribosomal Database Project (Cole et al., 2009) and GenBank (Benson et al., 2011), respectively, and used for the alignments.

RESULTS

Among the 127 isolates from collecting expeditions, 33 isolates were genetically characterized as *Burkholderia* (Table 1). 16S rDNA gene amplification with universal primers F27 and R1525 generated bands of approximately 1500 bp for all strains, as expected. Using the Mega BLAST and CAP3 programs, we obtained and identified 33 contigs belonging to the genus *Burkholderia*: 11 from Mata Atlântica (all from *I. vera*) and 22 from the Caatinga (13 from *C. luetzelburgii*; 4 from *C. germana*; 3 from *M. ophthalmocentra*, and 2 from *M. xanthocentra*). Isolates from *C. hirtiflora* did not generate contigs and were therefore not included in the analysis.

Table 1. Collection sites, hosts and *Burkholderia* isolates from the nodules of leguminous plants in different biomes.

Biome	Site	Host	Altitude (m)	GPS	Isolates*
Caatinga	Pico do Barbado	*Mimosa xanthocentra*	1455	13°17'S 41°53'W	805, 807
	Serra do Tromba	*Calliandra germana*	1495	13°16'S 41°47'W	810, 811, 812, 813
	Piatã	*Calliandra luetzelburgii*; *Calliandra hirtiflora*	1208	13°18'S 41°32'W	815, 817, 818, 819, 823, 824, 826, 829, 831, 832, 836, 838, 840
	Raso da Catarina	*Mimosa ophthalmocentra*	548	9°33'S 38°27'W	927, 929, 935
Mata Atlântica	Serra Bonita	*Inga vera*	832	15°23'S 39°33'W	008, 272A, 272B, 273C, 273D, 287E, 287H, 388L, 388M, 395A, 395D

*We have only considered the isolates to obtain a contig of forward and reverse sequences. No contig was obtained from the isolates from nodules of *Calliandra hirtiflora*, located in Piatã.

Similarity analysis among collected isolates led to the identification of two major branches, based on the highest level of dissimilarity: one large branch composed of groups A, B and C, and a second large branch composed of group D (Figure 1). Dissimilarity between all isolates ranged from zero to 2.5%.

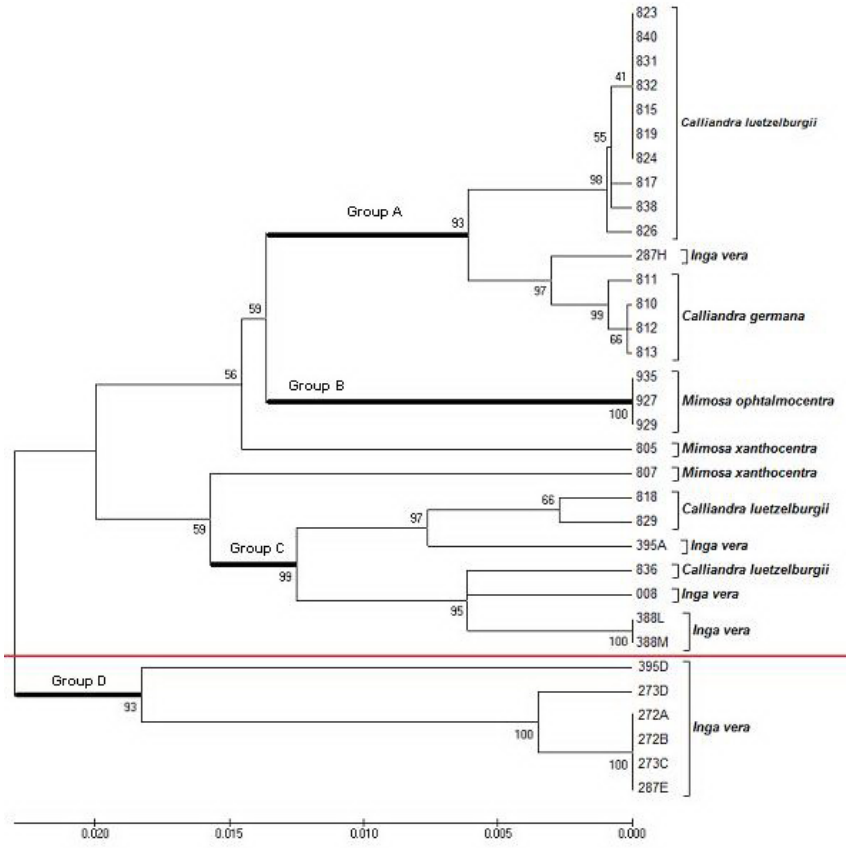

Figure 1. Bootstrap consensus tree (1000X) of the isolates obtained from nodules of legumes from five sites of Bahia and constructed from the neighbor-joining method with the Jukes-Cantor algorithm. The red line shows the division into two main branches, while the branches in bold show the formation of groups A, B, C, and D.

Group A was formed by 15 isolates, with three subgroups, each subgroup reflecting one different host plant (*I. vera*, *C. germana* and *C. luetzelburgii*), with bootstrap values above 93. Three isolates from *M. ophthalmocentra* formed a separate group, with 100% similarity and bootstrap of 100 (group B) and were distinguished from others with dissimilarities above 1.4%. Group C, formed by 7 isolates, could be subdivided into two subgroups composed of strains from *C. luetzelburgii* and *I. vera*. Group C also reflected the relationship of the similarity of the isolates with the host plant or the biome (Figure 1). Group D was composed of only isolates of *I. vera*. Strains 272A, 272B, 273C, and 287E were 100% similar (bootstrap 100).

The first BLAST hit of similarity yielded 2/3 of non-identified *Burkholderia* species (data not shown). Due to difficulties in visualizing the isolates using only the 16S rDNA sequence BLAST as a tool, a genetic distance tree was built from the 33 *Burkholderia* sequences obtained in this study, in addition to the sequences of the first BLAST hit and also the sequences of the *Burkholderia*-type strain species deposited in the Ribosomal Database Project (Figure 2).

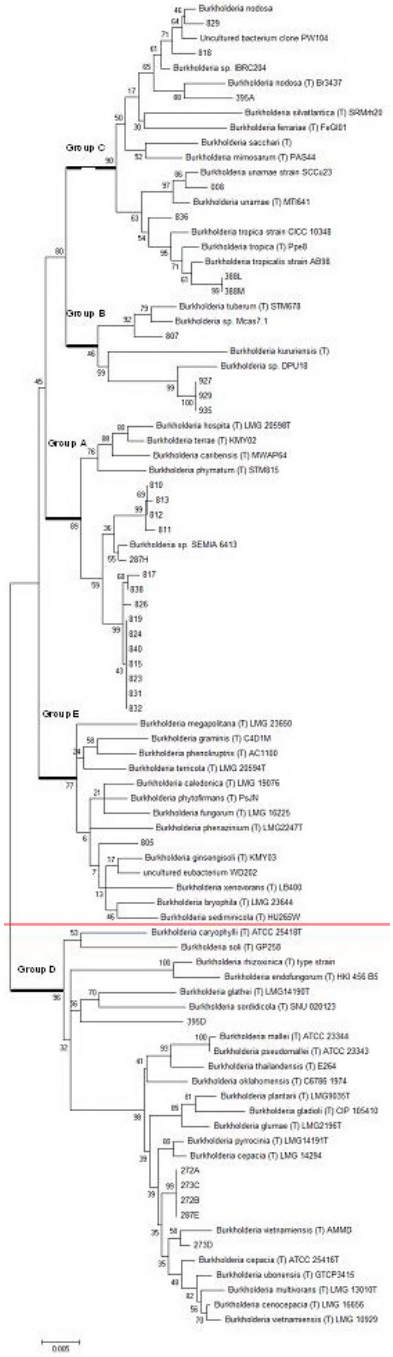

Figure 2. Bootstrap consensus tree (1000X) of the isolate sequences, the strains available in the database and the first BLAST hits constructed from the neighbor-joining method with the Jukes-Cantor algorithm. The red line shows the division into two main branches, while the branches in bold show the formation of groups A, B, C, D, and E.

The tree built with the addition of the database sequences and first BLAST hit (Figure 2) had the same topology as the tree built with only the strains obtained in this study (Figure 1). Isolate 805, from *M. xanthocentra*, separated from the other isolates in the first dendrogram (Figure 1) and formed a new group E (Figure 2). Isolate 807, from *M. xanthocentra*, also separated from the groups in the first dendrogram (Figure 1) and grouped in this second analysis with the isolates of *M. ophthalmocentra* (group B). Genetic distances varied slightly between groups when comparing the two different dendrograms, but a maximum dissimilarity of 2.5% between the two main branches was maintained.

The *rec*A gene amplification with primers BUR3 and BUR4 generated weak bands of expected size (380 bp). The PCR for those primers could not be optimized to yield good-quality products, not permitting reliable sequencing results. Therefore, only four contigs were obtained (272A, 272B, 395A, and 395D). They were used to construct a tree, and the results were consistent with the ones shown in Figures 1 and 2, with dissimilarity values ranging between 0.75 and 6% (data not shown).

DISCUSSION

The newly obtained 16S rDNA sequences, although they are very similar among themselves, allowed the differentiation of the 4 groups (Figure 1). The use of sequences of strains for the generation of a dendrogram, including the isolates under study, allowed a better comparative characterization when compared with the BLAST tool.

The analysis involving multiple alignments of sequences and clusters has been successfully applied to characterize the diversity of *Burkholderia* spp, as it allows the formation of distinct groups that tend to reflect their phenotypical characteristics (Garau et al., 2009; Wong-Villarreal and Caballero-Mellado, 2010). Direct comparison of the isolates (Figure 1) suggested that despite a low overall dissimilarity (given by the scale of the dendrogram branch), 16S rDNA sequencing was sufficient to allow the formation of groups. Both approaches of dendrogram generation yielded similar topology (Figures 1 and 2).

The 16s rDNA sequence analysis was sufficient to identify relevant similarities and groupings between isolates of this study and the strains of the genus. In some cases (e.g., isolates 836, 388L and 395D), strains separated from groups, suggesting the possibility of dealing with new species of *Burkholderia* awaiting more detailed taxonomic description.

In group A, in which most of the isolates of *C. luetzelburgii* were grouped, there are soil species, some of them nodulant and diazotrophic, such as *B. phymatum* (Gyaneshwar et al., 2011), and others producing gluconic acid and exopolysaccharides, such as *B. caribensis* (Achouak et al., 1999). Likewise, all isolates obtained from *M. ophthalmocentra* plus the isolate of *C. luetzelburgii* were grouped with nitrogen-fixing species (group B), nodulating *B. tuberum* (Vandamme et al., 2002) and *B. kururiensis* capable of degrading trichloroethylene (Zhang et al., 2000). Group C was also non-homogeneous concerning biotechnological properties of interest (Figure 1), as we could observe in a) the presence of nitrogen-fixing species (*B. mimosarum*, *B. nodosa*, *B. unamae* and *B. tropica*) (Wong-Villarreal and Caballero-Mellado, 2010), b) phosphate solubilizers (*B. ferriae*) (Valverde et al., 2006), c) producers of biodegradable polymers such as polyhydroxyalcanoate (PHA) from sucrose (*B. sacchari*) (Bramer et al., 2001), and d) even species with activity against fungi and nematodes (*B. tropicalis*) (Mehnaz, 2011).

Group E comprised environmental dye-producing species of industrial and pharmaceutical interest such as phenazine (*B. phenazinium*) (Viallard et al., 1998) and antifungal substances (*B. bryophila* and *B. megapolitana*) (Vandamme et al., 2007). Species involved in bioremediation (*B. phenoliruptrix* and *B. xenovorans*) (Coenye et al., 2004) and present in the rhizosphere with beneficial association with plants (*B. graminis* and *B. caledonia*) (Viallard et al., 1998) are also part of this group. It is worth mentioning, however, that this group also includes a fungal endosymbiont (*B. fungorum*), which although present in the environment is also an opportunistic microbe. This species represents an exception among the isolates of *Burkholderia* that do not belong to the BCC (Coenye et al., 2002). Thus, the commercial use of species closely related to *B. fungorum* should be carefully studied.

Group D was the most genetically distant from others, and is composed of pathogens for humans and animals (BCC, *B. mallei* and *B. pseudomallei*) (Dalmastri et al., 2003; Compant et al., 2008) and for plants (*B. plantarii*, *B. glumae* and *B. gladioli*) (Maeda et al., 2006). Only a few free-living soil species are present in this group: one capable of plant growth promotion (*B. caryophylli*) (Shaharoona et al., 2007), and two others (*B. rhizoxinica* and *B. fungorum*) are endosymbionts of pathogenic fungi (Partida-Martinez et al., 2007). According to these results, 6 of the 11 isolates of *I. vera* from the Mata Atlântica biome are mainly interesting for research related to pathogenicity (Figure 2).

Of the 33 isolates analyzed in this study, only one is relatively close to *B. fungorum*, and six showed high similarity with BCC. Thus, over 78% of the isolates showed a perspective of studies for future biotechnological applications in different areas of interest.

There was congruence of the dendrograms obtained from 16S rDNA and *rec*A gene sequencing data, in a subset of isolates. Therefore, the data obtained in this study from the *rec*A gene sequences are consistent with the literature (Payne, et al., 2005). In-depth studies of species present in Mata Atlântica and Caatinga are necessary to overcome the observed difficulties in the *rec*A gene amplification.

CONCLUSIONS

Sequencing of 16s rRNA was sufficient to identify relevant similarities and groupings among isolates and the sequences deposited. We could not optimize PCR for BUR3 and BUR4 primers for the strains in this study, but the few sequences of the partial *rec*A gene obtained generated a dendrogram congruent with the 16S rDNA tree. Dendrogram groupings partially reflect biomes or host plants. This study showed that 78% of the 33 isolates studied have a good potential for future studies regarding biotechnological applications.

ACKNOWLEDGMENTS

Research supported by Conselho Nacional de Desenvolvimento Científico (CNPq) (#620147/2004-0). We thank José Miguel Ferreira dos Santos for collecting samples, Alinne Batista Ambrósio for providing isolates from Raso da Catarina, Luciano Angelo de Souza Bernardes for suggestions and Martin Brendel for English editing. A doctoral fellowship was provided by CAPES. This paper is dedicated to the memory of Julio Cézar de Mattos Cascardo, who greatly contributed to this research.

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