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High diversity of *Bradyrhizobium* strains isolated from several legume species and land uses in Brazilian tropical ecosystems

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ABSTRACT

The genus *Bradyrhizobium* stands out among nitrogen-fixing legume-nodulating bacteria because it predominates among the efficient microsymbionts of forest, forage, and green manure legume species, as well as important species of grain legumes, such as soybean, cowpea, and peanut. Therefore, the diversity of *Bradyrhizobium* strains is a relevant resource from environmental and economic perspectives, and strains isolated from diverse legume species and land uses in Brazilian tropical ecosystems were assessed in this study. To accomplish this, sequences of four housekeeping genes (*atpD*, *dnaK*, *gyrB*, and *recA*) were individually analysed, with the first three also being considered using multilocus sequence analysis (MLSA). The sensitivity of the strains to different antibiotics, their tolerance to different levels of salinity, and their ability to nodulate soybean plants were also measured. The phylogenetic trees based on each individual gene, and on the concatenated housekeeping genes, revealed several strain clusters separated from any currently described species. The *Bradyrhizobium* strains studied were generally resistant to antibiotics. All strains were able to grow at salinity levels of up to 0.5% NaCl, whereas only strains UFLA03-142, UFLA03-143, UFLA03-145, and UFLA03-146 grew in the presence of 1% NaCl. Together, the results indicated that some of the strains studied were potential novel species, indicating that the various soils and ecosystems in Brazil may harbour an as yet unknown diversity of rhizobia.

Introduction

Biological nitrogen fixation, performed by a group of prokaryotes known as diazotrophic bacteria, is one of the most important processes mediated by soil microorganisms. These bacteria can be free-living organisms, endophytes or symbionts of certain plant families, especially legumes.

The genus *Bradyrhizobium* predominates among the efficient microsymbionts that nodulate legume species with diverse economic uses. Currently, this genus has 27 described species: *B. japonicum* [18], *B. elkanii* [21], *B. liaoningense* [52], *B. yuanmingense* [53], *B. betae* [35], *B. canariense* [46], *B. denitrificans* [45], *B. pachyrhizi* and *B. jicamae* [33], *B. iriomotense* [16], *B. cytisi* [2], *B. lablabi* [4], *B. daqingense* [49], *B. huanghuaihaiense* [55], *B. oligotrophicum* [32], *B. rifense* [3], *B. retamae* [14], *B. arachidis* [50], *B. diazoefficiens* [6], *B. ottawaense* [54], *B. ganzhouense* [23], *B. paxllaeri* and *B. icense* [7], *B. manausense* [37], *B. ingae* [38], *B. neotropicale* [56], and *B. valentinum* [8].

The analysis of 16S rRNA gene sequences has been used for over 20 years as the standard in taxonomic studies for the identification of most of the nitrogen-fixing legume-nodulating bacterial species [13]. However, identification and classification of *Bradyrhizobium* species through analysis of 16S rRNA gene sequences are limited due to the high level of conservation of this gene among these species [48,47,51]. Thus, the sequencing of housekeeping genes, such as *atpD*, *recA*, *dnaK* and *gyrB*, as well as combined analysis of several housekeeping genes (MLSA – multilocus sequence analysis), has been successfully used as an alternative approach for the taxonomic study of *Bradyrhizobium* strains [5,34,42,41,43,46,48].

Previous studies [10,15,17,29,36,39] using 16S rRNA gene sequence analysis classified several Brazilian tropical strains as

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members of the genus *Bradyrhizobium*. Most of these strains were studied regarding their symbiotic efficacy with diverse hosts and some of them are already or are intended to be approved as inoculants by the Brazilian government. The taxonomic positions of these strains, as well as the identification of useful symbiotic and phenotypic traits, are requirements for their potential use as inoculants and/or for inoculant quality control.

Therefore, in this study, the diversity of *Bradyrhizobium* strains isolated from several legume species and land uses in Brazilian tropical ecosystems was assessed. To evaluate the taxonomy of these strains, phylogenetic analyses of housekeeping genes (i.e. *atpD*, *gyrB*, *dnaK*, and *recA*) were performed individually and by using multilocus sequence analysis. The ability of these strains to nodulate soybean (*Glycine max*), resist antibiotics, and tolerate saline conditions was also analysed.

Materials and methods

Origin of strains

The 50 strains had already been identified as *Bradyrhizobium* based on their partial 16S rRNA sequences [15,17,26,29,30,36,39] (Moreira et al., unpublished results; Neves et al., unpublished results; Nóbrega et al., unpublished results) (Table 1).

Forty-eight strains belonged to the SBMPBS/UFLA (Setor de Biologia, Microbiologia e Processos Biológicos do Solo/Universidade Federal de Lavras) collection (code: UFLA and INPA) and two strains belonged to the collection of the National Centre of Agrobiological Research at EMBRAPA (Centro Nacional de Pesquisa de Agrobiologia – EMBRAPA; code: BR). The two strains from the EMBRAPA collection were used as references because some housekeeping gene sequences were already available for them [25]. Of these strains, four were approved as inoculants of legumes in Brazil: UFLA03-84 and INPA03-11B for *Vigna unguiculata* [39], and BR2801 and BR2003 for *Cajanus cajan*. Two other strains (UFLA03-153 and UFLA03-164) are currently undergoing selection tests in order to determine their effectiveness as inoculants for *Vigna unguiculata* under field conditions. The symbiotic efficiencies of all strains with their original host and/or with other species were tested previously (Table 1).

DNA extraction

The strains were grown in duplicate in liquid 79 medium [11] under constant agitation for five days. The genomic DNA was obtained using a ZR Fungal/Bacterial DNA Kit™ (Zymo Research Corp., CA, US) according to the manufacturer's instructions. Subsequently, the quality and concentration of the extracted DNA were verified using a NanoDrop device.

Amplification and sequencing of housekeeping genes (gyrB, dnaK, atpD, and recA)

Four housekeeping genes (*gyrB*, *dnaK*, *atpD*, and *recA*) were amplified and sequenced as described previously [34]. Table S1 shows all primers and cycling conditions used during the procedure. The PCR reaction was performed on a Bio-Rad Thermo Cycler using a 25-μL reaction volume containing 2.5 μL of DNA, 2.5 μL of 2 mM deoxyribonucleotide triphosphates (dNTP), 2.5 μL of 10X PCR buffer, 0.25 μL of 50 μM each primer, and 0.5 μL of 1 U μL^{-1} Taq. The quality and concentration of the PCR products were verified on a 1% (w/v) agarose gel stained with ethidium bromide. The products were then purified using the NucleoFast® 96 PCR clean-up kit under vacuum, rinsed, and resuspended in ultrapure water. They were sequenced using the dideoxynucleotide chain termination method with fluorescent ddNTPs (Applied Biosystems) on an ABI Prism 3130xl capillary sequencer according to the manufacturer's instructions (Applied Biosystems). Prior to sequencing, the PCR products were purified again using SAM™ and a BigDye XTerminator™ in a MicroAmp™ optical 96-well reaction plate.

Phylogenetic analysis

The quality of the housekeeping gene (*atpD*, *gyrB*, *dnaK*, and *recA*) sequences was inspected using BioNumerics 6.5 (Applied Maths, Sint-Martens-Latem, Belgium). ClustalW was used for the sequence alignment. Subsequently, the phylogeny was determined by the neighbour-joining method for the 16S rRNA, *atpD*, *gyr*B, *dnaK*, and *recA* genes, and MLSA for the three housekeeping genes (*dnaK*, *gyrB*, and *atpD*). The construction of the phylogenetic trees was performed using the Kimura 2-parameter model [19] in the molecular evolutionary genetic analysis software (MEGA, version 5) [44], using a bootstrap confidence analysis with 1000 replications. For comparison, the alignment included sequences of *Bradyrhizobium* genospecies [1,22,31] and type species available in GenBank (National Center for Biotechnology Information, NCBI).

Sensitivity to antibiotics

Ten antibiotics were tested at the following concentrations: 10 μg mL^{-1} ampicillin (AMP), 30 μg mL^{-1} cefuroxime (CRX), 5 μg mL^{-1} ciprofloxacin (CIP), 30 μg mL^{-1} chloramphenicol (CLO), 30 μg mL^{-1} doxycycline (DOX), 15 μg mL^{-1} erythromycin (ERI), 10 μg mL^{-1} gentamicin (GEN), 30 μg mL^{-1} kanamycin (KAN), 30 μg mL^{-1} neomycin (NEO), and 10 μg mL^{-1} penicillin G (PEN). The choice of these antibiotics was based on previous work describing new species of *Bradyrhizobium* [2,3,16,33,35,46,49,53].

The strains were grown in liquid 79 medium [11] under constant agitation for five days, which was a sufficient period of time for the cells to reach the logarithmic growth phase. Subsequently, the cells were rinsed with 0.85% saline solution to remove any residual culture medium from the inoculum, which could have resulted in a false positive growth effect. This procedure comprised the transfer of 1.0 mL aliquots of each bacterial culture (approximately 10^9 cells) to 1.5 mL sterile microtubes and centrifugation at 12,768 × *g* and 4 °C for 10 min. The supernatants were discarded, and the cells were re-suspended in 1.0 mL 0.85% sterile saline solution and re-centrifuged. This process was repeated three times. Then, 0.1 mL aliquots of the bacterial cell suspensions were inoculated and streaked on 79 medium in Petri dishes using a Drigalski spatula [11]. For each strain, the ten antibiotics were tested in different dishes. On each dish, three disks of the same antibiotic were inserted, and they comprised three biological replicates. *Bradyrhizobium japonicum* LMG 6138^T, *B. elkanii* LMG 6134^T, and *B. canariense* LMG 22265^T were used as controls and they were compared with the 50 strains studied. Since it would have been too laborious to include all the *Bradyrhizobium* type strains, *B. japonicum* and *B. elkanii* were included because they were the first species described in the genus, and *B. canariense* because it was isolated from a completely different ecosystem. *B. japonicum* and *B. elkanii* are also widespread in Brazil. Strain LMG 22265^T was used as a negative control in the test of soybean symbiosis. All treatments were incubated at 28 °C for seven days, and were subsequently assessed by measuring the diameter of the growth inhibition halo.

Salinity tolerance

The cell suspensions were prepared as described in the previous section. The suspensions were incubated and streaked on 79 medium in Petri dishes using a Drigalski spatula [11], which was