Microvirga vignae sp. nov., a root nodule symbiotic bacterium isolated from cowpea grown in semi-arid Brazil

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16S rRNA gene sequence analysis of eight strains (BR 3299T, BR 3296, BR 10192, BR 10193, BR 10194, BR 10195, BR 10196 and BR 10197) isolated from nodules of cowpea collected from a semi-arid region of Brazil showed 97% similarity to sequences of recently described rhizobial species of the genus Microvirga. Phylogenetic analyses of four housekeeping genes (gyrB, recA, dnaK and rpoB), DNA–DNA relatedness and AFLP further indicated that these strains belong to a novel species within the genus Microvirga. Our data support the hypothesis that genes related to nitrogen fixation were obtained via horizontal gene transfer, as sequences of nifH genes were very similar to those found in members of the genera Rhizobium and Mesorhizobium, which are not immediate relatives of the genus Microvirga, as shown by 16S rRNA gene sequence analysis. Phenotypic traits, such as host range and carbon utilization, differentiate the novel strains from the most closely related species, Microvirga lotononidis, Microvirga zambiensis and Microvirga lupini. Therefore, these symbiotic nitrogen-fixing bacteria are proposed to be representatives of a novel species, for which the name Microvirga vignae sp. nov. is suggested. The type strain is BR3299T (=HMBI 3457T).

In Brazil, great efforts have been made to enhance cowpea (Vigna unguiculata) productivity via biological nitrogen fixation (BNF), using the same strategy as that applied to soyabeans. Therefore, many studies have been conducted to screen diazotrophic bacteria isolated from cowpea nodules, looking for highly efficient strains. In contrast to the results observed for African and Asian soils, in which slow-growing isolates from the genus Bradyrhizobium predominate (Pule-Meulenberg et al., 2010; Zhang et al., 2008), in semi-arid regions of Brazil, up to 60% of the bacteria isolated from cowpea nodules showed fast-growth in YMA (Fred & Waksman, 1928) medium, which is not characteristic of the genus Bradyrhizobium (Leite et al., 2009; Martins et al., 1997). Amplified rDNA restriction analysis (ARDRA) showed low similarity between those isolates and type strains of rhizobia, indicating that some of them may actually belong to undescribed species (Leite et al. 2009).

In spite of their great biotechnological potential, most of the bacterial strains isolated from Brazilian soils remain uncharacterized. However, more knowledge about the taxonomy of these strains is required prior to their use as inoculants. In addition, this information may help to understand rhizobial ecology in tropical ecosystems. Therefore, in the present study we describe eight bacterial strains isolated by Martins et al. (1997), including strain BR3299T, which showed a high potential to fix nitrogen in association with cowpea (Gualter et al., 2011), but did not exhibit characteristics of slow-growing bradyrhizobia,

Abbreviations: ALFP, amplified length fragment polymorphism; JC, Jukes–Cantor; K2, Kimura two-parameter; ML, maximum-likelihood; NJ, neighbour-joining; T92, Tamura 3-parameter.

The GenBank/EMBL/DDBJGenBank accession numbers for the 16S rRNA and partial sequences of nifH, nodA, dnaK, recA, rpoB and gyrB genes of strain BR3299T are JX504804, KC768856, KC768859, JX504820, JX504812, JX504828, JX504836, respectively.

Three supplementary tables and four supplementary figures are available with the online version of this paper.
which are usually found in the nodules of this legume. To clarify the taxonomic status of these strains, we applied a polyphasic approach normally used for the characterization of rhizobia, which included analysis of housekeeping genes (16S rRNA, gyrB, rpoB, recA and dnaK) and functional genes (nifH, nodA), DNA–DNA relatedness, amplified fragment length polymorphism (ALFP), fatty acid profiles and phenotypic characteristics.

The strains described in this study (BR3299T, BR3296, BR10192, BR10193, BR10194, BR10195 and BR10196) were isolated from Vigna unguiculata nodules collected from an area known as the Drought Polygon located at the Municipality of Canindé de São Francisco, in the North-east Region of Brazil. The soil in this area was classified as luvisol and had a pH of 7.1. The annual precipitation and mean temperature in this region are 483.9 mm and 25–26 °C, respectively. Type strain BR3299T was deposited in the Diazothrophic Microbial Culture Collection – CRB-Johanna Döbereiner (Embrapa Agrobiologia, Rio de Janeiro, Brazil) and HAMBI Culture Collection (University of Helsinki, Finland). All strains were cultured in yeast mannitol agar (YMA) or tryptone yeast (TY) medium (Beringer, 1974) at 28 °C and 35 °C, respectively. For long-term storage, cultures were lyophilized and also kept at −80 °C.

DNA of pure cultures was extracted using a commercial kit (Wizard DNA Clean up; Promega) and used as a template for the amplification of 16S rRNA, gyrB, rpoB, recA, dnaK, and nifH genes. Primers and annealing temperatures are described in Table S1 available in IJSEM Online. All sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank) and the accession numbers are listed in Table S1.

Partial sequences of the 16S rRNA genes (1398 bp) were aligned with related sequences held in GenBank using CLUSTAL W (Thompson et al., 1994) and manually edited using the BioEdit Sequence Alignment Editor. Only good quality sequences (http://rdp.cme.msu.edu/hierarchy/hb_intro.jsp) were used for the alignments. Distances were calculated using the Kimura two-parameter (K2), Tamura 3-parameter (T92) and Jukes–Cantor (JC) models. Phylogenetic inferences were made by the neighbour-joining (NJ) and maximum-likelihood (ML) methods using MEGA 5.0 (Tamura et al., 2011). The strength of each topology was checked using 1000 bootstrap replications. No differences between the ML and NJ tree topologies were observed, hence, only the NJ tree is presented in Fig. 1. As shown in Table 1, the strains investigated in the present study showed more than 99 % similarity to each other. Based on the 97 % similarity cut-off established for the definition of bacterial species (Amann et al., 1992), these strains belong to a single species. They were grouped in the genus Microvirga with a very high bootstrap value (100 %), and are most closely related to Microvirga lotononidis and Microvirga lupini.

As phylogeny of rhizobial strains based only on 16S rRNA gene sequences might be misleading (van Berkum et al., 2003; Fox et al., 1992), the phylogenetic relationship of the strains was confirmed using further housekeeping loci (gyrB, recA, dnaK and rpoB). Sequence similarities are listed in Table 1. Trees were reconstructed using the NJ and ML methods based on JC, K2 and T92 distance matrices. The best-fit models for ML analysis were defined using MEGA 5.0. As differences in the topology were observed, the data of the four loci were not concatenated. Fig. 2 shows the phylogeny based on partial recA sequences (444 bp). Housekeeping loci confirmed the 16S rRNA results and showed a high similarity among the investigated strains (99–100 %), clearly differentiating them from other species of the genus Microvirga. Furthermore, they formed a group within the genus Microvirga with very high bootstrap values. For recA, the most closely related organism was Microvirga lotononidis WSM3557T (93 %), followed by Microvirga lupini Lut6T (92 %), corroborating the 16S rRNA data (Table 1).

Based on the analyses of housekeeping genes, strain BR3299T could be clearly differentiated from the type strains of the genus Microvirga. However, to attest that this strain actually belongs to a novel species, DNA–DNA hybridization was performed as described by Gonzalez & Saiz-Jimenez (2005). This method estimates the DNA–DNA relatedness between bacterial strains based on the thermal denaturation temperatures of hybrid and homologous genomic DNA. As shown by Moreira et al. (2011), this approach can be successfully used for differentiation of bacterial species. According to these studies a 4–5 % ΔTₘ can be applied as a cut-off for species differentiation, corresponding to the 70 % relatedness determined by the traditional DNA–DNA hybridization method. Table 1 shows the ΔTₘ of strains BR3299T, BR3296, Microvirga lotononidis WSM3557T, Microvirga lupini Lut6T, Microvirga zambiensis WSM3693T and Microvirga subterranea DSM 14364T. DNA–DNA relatedness analysis reinforced the results observed for the housekeeping genes as strain BR3299T was clearly distinguished from type strains of species of the genus Microvirga, except for Microvirga zambiensis for which the ΔTₘ of 3.7 was slightly below the cut-off for the differentiation of bacterial species.

To confirm the taxonomic status of strain BR3299T, AFLP analyses were carried out as described elsewhere (Vos et al., 1995), including the following modifications: 500 ng DNA was digested with a combination of enzymes PsiI/TaqI, then ligated to the respective adapters. Selective amplification was carried out with three primer combinations, PsiI+GC/TaqI+AG, PsiI+CC/TaqI+AG and PsiI+CC/TaqI+TG. The amplification products were separated on a 6 % polyacrylamide denaturing gel and silver stained using a method described elsewhere (Creste et al., 2001). The data showed a high similarity between strains BR3299T and BR3296 (>85 %). Besides, comparisons with others type strains strengthened the classification of BR3299T into a novel species as similarity values were below 50 % (Fig. S1), being within the cut-off established for strains of the same genus but different species (Savelkoul et al., 1999).
Even though investigation of functional genes might not give information on the taxonomic status of a bacterium, analyses of the genes involved in the establishment of symbiosis or in the process of nitrogen fixation are essential to the characterization of rhizobial strains. Phylogenetic analysis of the nifH gene revealed that strain BR 3299<sup>T</sup>...
formed a group with the genera *Mesorhizobium*, *Ensifer* and *Rhizobium* (Fig. S2). Similar results were observed for other rhizobial strains within the genus *Microvirga* (Ardley et al., 2012). This supports the hypothesis that horizontal gene transfer had occurred among the genera *Rhizobium* and *Microvirga*. For *nodA* genes, the sequences of strains BR3299T and BR3296 were identical and most closely related to *Microvirga lotononidis* and *Microvirga zambiensis*, which formed a cluster separated from *Microvirga lupini* (Fig. S3).

In order to discern and describe strain BR3299T, phenotypic features were determined in comparison to other *Microvirga* type strains (Table S2). The utilization of carbon sources was assessed using Biolog GN2 microplates (Biolog) and API 20NE (bioMérieux) according to the manufacturers’ instructions. Tolerance to NaCl, pH and temperature were evaluated in TY agar. The antibiotic resistance profile was determined in TY agar using the SENSIBIODISC-CECON Gram-negative Series kit (CECON), being (mg): amicacina (30), ampicilina (10), cefalotina (30), cefazolina (30), cefotaxima (30), cefoxitina (30), ciprofloxacina (5), clorafenicol (30), gentamicina (120), kanamivina (30), sulfazotrim (25) and tetraciclina (30). Phospholipid fatty acid analysis of strain BR3299T was performed according to the MIDI protocol, as described by Ardley et al. (2012). The major cellular fatty acids were 18:1ω7c (71.86%), 16:0 (8.21%), 19:0 CYCLO ω8c (5.92%) and 18:0 (5.43%). Those were identified using the TSBA database version 6.10 (Microbial Identification System; MIDI). The fatty acid composition was very similar to that observed for other strains belonging to the genus *Microvirga* (Table S3).

The host range is a very important trait of rhizobial strains. Therefore, nodulation tests were carried out for strain BR3299T under axenic conditions using modified Leonard jars containing a mixture of sand and vermiculite (2:1) supplied with nitrogen-free nutrient solution (Norris & Mannetje, 1964). Surface-sterilized seeds were inoculated with 1 ml of a suspension containing 10⁹ cells. Non-inoculated controls were included in the assays. Plants were grown in a greenhouse under conditions of natural sunlight and temperature. Nodulation was evaluated 39 days after inoculation. Nodulation experiments confirmed that this strain actually forms nodules in association with cowpea. Moreover, nodule formation was detected for *Arachis hypogaea*, *Crotalaria spectabilis*, *Macroptillium artropurpureum*, *Mucuna deeringiana* and *Tephrosia sinapou*, but not for *Glycine max*, *Phaseolus vulgaris*, *Leucaena*...
leukocephala, Mimosa caesalpinifolia or Chamaecrista nictitans. Differences between strain BR3299T and other closely related rhizobial strains are summarized in Table 2.

According to the current criteria used for the description of rhizobial species [Graham et al., 1991 and the ICSP – Subcommittee on the taxonomy of Rhizobium and Agrobacterium (http://edzna.ccg.unam.mx/rhizobial-taxonomy/node/12)], the strains described in the present study represent a novel species within the genus Microvirga. This statement is clearly supported by results obtained for the analysis of housekeeping genes (16S rRNA, recA, rpoB, gyrB, dhaK), DNA–DNA relatedness and AFLP patterns as well as differences in phenotypic characteristics, such as host range. The name Microvirga vignae sp. nov. is proposed for this novel bacterial species.

Description of the Microvirga vignae sp. nov.

Microvirga vignae (vi’gna.e) N.L. gen. n. vignae of Vigna, referring to the fact that all strains were isolated from cowpea nodules, Vigna unguiculata).

Cells are aerobic, Gram-stain-negative rods (approx. 2.0 × 0.87 µm), motile, with four to eight flagella (Fig. S4). Grows well on YMA and TY media. Colonies grown at 28 °C on YMA medium are circular, beige, opaque, and 2 mm in diameter. After 5 days of growth a brown spot is observed at the centre of the colony. Optimum growth occurs at pH 7.0–8.0 (range 6.0 to 9.0). Tolerates 1% NaCl (w/v) and is susceptible to amikacin, ampicillin, cephalothin, cephotaxime, cephoxitin, ciprofloxacin, chloramphenicol, gentamicin and kanamycin, while it is resistant to cephalolin. Utilizes D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, maltose, potassium gluconate, adipic acid and malic acid as a carbon source. Cells do not exhibit urease or nitrate reductase activity. The major cellular fatty acids are 18:1ω7c, 16:00, 19:0 CYCLO ω8c and 18:0. Can be distinguished from other rhizobial species of the genus Microvirga due to its capacity to effectively nodulate cowpea, its inability to metabolize D-fructose and absence of urease and nitrate reductase activity.

The type strain is BR3299T (=HABMI 3457T). It was isolated from cowpea nodules that had been cultivated in soil collected in a semi-arid region in the North-east of Brazil.

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**References**


