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Azorhizobium doebereinerae sp. Nov. Microsymbiont of *Sesbania virgata* (Caz.) Pers. ☆

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Abstract

Thirty-four rhizobium strains were isolated from root nodules of the fast-growing woody native species *Sesbania virgata* in different regions of southeast Brazil (Minas Gerais and Rio de Janeiro States). These isolates had cultural characteristics on YMA quite similar to *Azorhizobium caulinodans* (alkalinization, scant extracellular polysaccharide production, fast or intermediate growth rate). They exhibited a high similarity of phenotypic and genotypic characteristics among themselves and to a lesser extent with *A. caulinodans*. DNA:DNA hybridization and 16SrRNA sequences support their inclusion in the genus *Azorhizobium*, but not in the species *A. caulinodans*. The name *A. doebereinerae* is proposed, with isolate UFLA1-100 (= BR5401, = LMG9993 = SEMIA 6401) as the type strain.
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Introduction

Sesbania virgata (Caz.) Pers. (syn. *S. marginata* Benthan) is a fast-growing shrub about 2–4 m high, well adapted to flooded conditions, that produces a lot of seeds with long-term viability. Because of these characteristics, it has been used for revegetation of riparian forests, soil erosion control and rehabilitation of degraded areas, firewood and charcoal production. A beverage similar to coffee can be prepared from the

☆ Most of this work was carried out when Fátima Moreira visited laboratories of other institutions: REP-PCR and some 16SrRNA sequences at Center of Microbial Ecology – MSU (2001). MEE and DNA:DNA hybridizations at the Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de Mexico (1991 and 2002). Biolog tests were carried out by Rosa Maria Pitard. Other 16S rRNA sequences were determined by Leonardo Cruz and Fábio Pedrosa at Universidade Federal do Paraná. Sequences determined in this study have been submitted to the public databases with accession numbers AJ003237 and AF391130 (BR5401 = UFLA1-100); AY626222 (UFLA1-510); AY626221 (UFLA1-51B); AY626220 (BR5416).

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seeds. It occurs in Central, Southeast and South Brazil, Argentina, Uruguay and Paraguay [32].

All legume-nodulating bacteria were assigned to the genera *Rhizobium* and *Bradyrhizobium* [17] until 1988, when the new genus *Azorhizobium* was described with a sole species *Azorhizobium caulinodans* [9]. This species was described on the basis of nodule isolates from both stems and roots of *Sesbania rostrata*. Characteristics analyzed included polyacrylamide gel electrophoresis of total cellular proteins (SDS-PAGE), DNA base composition (G + C content) and DNA–rRNA hybridization, among other biochemical assays. Since then, new molecular methods have been developed and isolates from other tropical legume species analyzed, and this has led to a rapid increase in the diversity described among nodule bacteria. Since 1988, five new genera and 43 new species have been described, including some in highly divergent phylogenetic branches of the subclasses α and *β-Proteobacteria* [5,30,37]. However, no new species have been added to the genus *Azorhizobium*, although other stem and root nodulating bacteria had been isolated from *S. rostrata* which were quite distinct from *A. caulinodans* according to the criterion of DNA–DNA hybridization [35].

Studies of nodulating bacteria isolated from native and exotic forest species in Brazil have shown a high diversity, with strains belonging to the genera *Bradyrhizobium, Rhizobium, Azorhizobium, Sinorhizobium, Mesorhizobium* and *Burkholderia* [25,26,28]. Among 171 strains of diverse origins (plant species, ecosystems and soil types) characterized by polyacrylamide gel electrophoresis of their total proteins, two strains isolated from *Sesbania marginata* (syn. *S. virgata*) root nodules (Sm1 = BR5401 and Sm5 = BR5404) [3] occupied a cluster apart from those including type and reference strains of known rhizobium species, as well as *A. caulinodans* [25]. Further studies [6,10] compared a large group of strains from African tropical trees with Brazilian strains [25] by SDS-PAGE protein profile using the database at Gent University (Laboratorium voor Microbiologie). In both studies, BR5401 and BR5404 did not cluster with any other strain and were most similar to each other. Compared to the other 43 strains isolated from 28 forest legume species, strain BR5401 was found to have a unique partial 16S rRNA gene sequence which indicated that it was a potential new species belonging to the genus *Azorhizobium* [26]. The high similarity among strains isolated from *S. virgata* and significant differences between them and ORS 571^T led to the conclusion that they constituted a new species [27]. The name *Azorhizobium johannense* was tentatively suggested, but subsequently we have recognized that *A. doebereinerae* would be a more correct form.

Minimal standards for the description of new genera and species of root- and stem-nodulating bacteria were proposed [14]. The standards included genetic, phenotypic and symbiotic characteristics, besides the analysis of a relatively high number of strains. New strains were isolated from *S. virgata* plants from distinct geographical regions of Minas Gerais and Rio de Janeiro States ([1,13,29], this work). All isolates exhibited the same cultural characteristics as BR5401, which were also similar to those of ORS 571, including the inability to use mannitol from YMA medium. This enables an easy and cheap discrimination of the genus from other nitrogen-fixing legume-nodulating bacteria.

Other plant infection tests were carried out with *S. virgata* and *S. rostrata* growing in Leonard jars with Jensen's modified nutrient solution [13]. *Azorhizobium doebereinerae* (syn. A.johannae) efficiently nodulates *S. virgata* only. While it is capable of nodulating other hosts such as *Macroptilium atropurpureum, Phaseolus vulgaris* and *S. rostrata*, the efficiency, i.e. the amount of N_2 fixation, was substantially reduced compared to *S. virgata*. *A. doebereinerae* was not able to form stem nodules with *S. rostrata*, but induced pseudonodules instead. Conversely, *A. caulinodans* was not able to complete nodule formation in *S. virgata*, although pseudonodules were again observed. Strains belonging to *Bradyrhizobium, Rhizobium, Sinorhizobium* and *Mesorhizobium* do not nodulate *S. virgata*. Recent experiments have shown that NGR 234 nodulates *S. rostrata* but not *S. virgata* (unpublished data).

Ten isolates (including BR5401) from *S. virgata* of various origins exhibited the same tolerance to Zn, Cd and Cu in YMA medium modified by addition of HEPES and MES [39]. The tolerance to heavy metals of sixty strains of different origins and belonging to various rhizobial genera were compared [23] and, two other *S. virgata* isolates had the same tolerance patterns as those described before [39], namely 400 mg L^{-1} Zn, 20 mg L^{-1} Cd and 5 mg L^{-1} Cu. These authors also found that *S. virgata* isolates were less tolerant than isolates of the other genera. However, the tolerance to heavy metal contaminated soil shown by the *S. virgata* symbiosis with three different isolates was intermediate between those of *Enterolobium contortisiliquum* and *Acacia mangium* with *Bradyrhizobium* strains [40].

The objective of this paper was to complete the requirements for the description of a novel species, primarily by the determination of 16S rRNA sequences of five strains and by DNA–DNA hybridization studies.

Materials and methods

Strains

Azorhizobium doebereinerae strains studied in this work are listed in Table 1. This table also includes type

Table 1. Bacterial strains included in this study

Strains and original host	Origin (municipality/state/country)	References
Azorhizobium doebereinerae From *Sesbania virgata*		
BR5401, BR5402, BR5404,BR5405	Seropédica – RJ, BR	[3,13,25,26]
UFLA01-51b, UFLA01-54b	Itutinga-MG	[1,13]
BR5413, BR5414, BR5415	Paracambi – RJ	[13]
BR5416, BR5417, BR5418, BR5430.	Itaguaí – RJ	
BR5419, BR5420, BR5421	Volta Redonda – RJ	
BR5422, BR5423, BR5424, BR5425, BR5426, BR5427, BR5428	CampoGrande – RJ	
UFLA01-601, UFLA01-602	Lavras-MG	[12,13]
UFLA01-605	Ijaci –MG	
UFLA01-606	Ribeirão Vermelho-MG	
UFLA01-603, UFLA01-604	Lavras-MG	
UFLA1-480, UFLA1-483, UFLA1-486, UFLA1-510, UFLA1-515	Tres Marias, MG-Heavy-metal contaminated soil	[23,29,39,40]
Type and reference strains of described species		
A. caulinodans ORS571^T , OR S591 From *Sesbania rostrata*	Senegal	[9]
Bradyrhizobium elkani USDA 76^T from *Glycine max*	USA	[20]
Mesorhizobium loti NZP 2213^T from *Lotus corniculatus*	New Zealand	[16]
Sinorhizobium meliloti NZP 4027^T from *Medicago sativa*	Australia	[6]
Rhizobium tropici CIAT 899^T from *Phaseolus vulgaris*	Colombia	[22]
Reference strains[a]		
INPA54B, BR4406,	*Bradyrhizobium japonicum*	[26]
FL27(= BR 5614), BR5611, BR3617, INPA173A	Bradyrhizobium elkanii	
BR6401, BR809	*Rhizobium tropici*	
INPA95A, BR8802	*Rhizobium leguminosarum*	
BR5001, BR6806, INPA522B	*Sinorhizobium medicae*	
BR4007	*Sinorhizobium* sp.	
BR3804, INPA78B	*Mesorhizobium plurifarium*	[7,26]
BR11040^T	*Azospirillum amazonense*	[21]
BR8801	*Rhizobium leguminosarum*[b]	[25]
LMG 8303	*B.japonicum*	
INPA18A[c]	n.d.	

[a]Strains isolated from Amazonia (prefix INPA) and Atlantic forests (prefix BR).
[b]Probably belonging to this species because SDS-PAGE protein profile is in the same cluster as BR8802.
[c]Almost the same cultural characteristics of *A. doebereinerae*, except for a slightly more EPS production, isolated from *Swartzia* sp.

strains and strains previously isolated from tropical legume species that were used for comparison.

Phenotypic characteristics

Utilization of carbon sources was evaluated with a BIOLOG kit by replacing BUG (Biolog Universal Growth) medium by TY (Tryptone 5 g, yeast extract 3 g, $CaCl_2 \cdot 2H_2O$ 0.9 g/l, agar 15 g, pH 6.8) where cells were allowed to grow for 48 h. Strains tested were: UFLA1-510, UFLA1-51B, BR5416 and BR5401. All strains (Table 1) were also tested for growth in LB medium (Casein peptone 10 g, yeast extract 5 g, NaCl 10 g/l). Growth and nitrogenase activity under free-living conditions were tested in semi-solid N-free LO modified medium [8] with all strains listed in Table 1. Nitrogenase activity was estimated by acetylene reduction, with ethylene production detected by a Varian 3400-X flame ionization gas chromatograph.

Cell characteristics

Cells from log phase cultures grown on semi-solid Yeast extract mannitol medium [11], with mannitol replaced by the same amount of lactate, were observed under phase contrast (Olympus B-max-40-III). A glass capillary tube (with both ends opened, 6 mm diameter, 3 cm length) was put on an isolated colony grown on solid Yeast extract mannitol medium (log phase), then 100 µl of sterile distilled water was inserted within the tube, and left for 10 min to allow cells to migrate to the water. Then, 10 µl were carefully taken out from this

suspension and added to a wax plate for usual preparation of copper grids to be observed by transmission electron microscopy (Carl Zeiss EM 900).

DNA extraction

Genomic DNA was isolated from log-phase cultures grown on solid Yeast extract mannitol medium for 5–6 days. Ultra-clean Soil DNA isolation kits from MOBIO laboratories were used essentially as recommended by the vendor. DNA was quantitated at 260 nm in a diode-array spectrophotometer (Hewlett Packard).

REP-PCR and cluster analysis

For seven isolates, genomic fingerprints were obtained from repetitive extragenic palindromic sequences amplified by polymerase chain reaction (REP-PCR). REP primers [43] were used according to the procedures described by Rademaker et al. [34]. Computer-assisted analysis was performed on normalized gels with GelCompar V. 4–1 (Applied Maths, Kortrijk, Belgium) as described [33]. Similarity matrices derived from REP-PCR profiles were calculated using the pair-wise Pearson's product-moment correlation coefficient (*r*-value). Cluster analysis of *r*-values was performed using the UPGMA method.

Multilocus enzyme electrophoresis (MLEE)

Cultures derived from single colonies (strains BR5401, BR5404, type and reference strains of known rhizobium species) were grown in 30 ml PY medium at 30 °C, centrifuged and re-suspended in 0.3 ml of 10 mM $MgSO_4$. To aid cellular lysis, the extract was incubated with lysozyme (1 mg/ml) at room temperature for 2 min followed by freezing and thawing at −70 °C for two 15 min cycles, and the resulting extracts were maintained at −70 °C. Starch gel electrophoresis was conducted according to Selander et al. [36]. Eight metabolic enzymes were assayed: alcohol dehydrogenase (ADH), malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), glucose-6-phosphate dehydrogenase (G6P), xanthine dehydrogenase (XDH), hexokinase (HEX), phosphoglucomutase (PGM) and esterases (EST). Genetic distance between each pair of strains was estimated as the proportion of loci at which dissimilar alleles occurred. Strains were clustered in a matrix of pairwise genetic distances by Nei's method.

Polyacrylamide gel electrophoresis of total bacterial proteins

Twenty-seven strains were grown at 28 °C for 48 h on TY medium. Whole cell protein extracts were prepared and sodium dodecyl sulphate polyacrylamide gel electrophoresis performed according Moreira et al. [25].

16S rDNA sequencing

Near full-length 16SrRNA genes were amplified with primer pair 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) for strains UFLA1-510, UFLA1-51B and BR5416. Purification of PCR products was performed with Microcon™ filters (Millipore). Single-pass sequencing of PCR amplified rDNAs were performed, using the 27F primer, on an ABI 3700 capillary sequencer at the Michigan State University Sequencing Facility. Near full length 16SrRNA of strains BR5401, BR5414 and BR5426 were sequenced at Universidade Federal do Paraná on an ABI PRISM 377 Genetic analyzer (PE Biosystem) by using primers Y1 and Y3 [31,45] in PCR of boiled cell suspensions. Amplification products were purified with Nucleon QC clean up spin column kit (Amsherham) or QiaKit (Qiagen) as recommended by the vendor.

DNA–DNA hybridization

DNA from *A. caulinodans* ORS 571^T, UFLA01-51B, UFLA01-510, BR5401, *Bradyrhizobium japonicum* USDA 110 and *Bradyrhizobium elkanii* USDA 76^T were purified from 5 ml PY (5 g peptone, 3 g yeast extract and 0.6 g of calcium chloride per liter) cultures using the Amersham Genomic Prep™ Cells and Tissue Isolation kit and digested with *Eco*RI. After electrophoresis and blotting, nylon filters (Hybond-N+, Amersham, Biosciences) were hybridized to total DNA from *A. caulinodans* type strain ORS 571 labeled with ^{32}P by RediPrime (Amersham). Hybridization was performed at 0.1 SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and washing (5 times) was with 2 × SSC and 0.5 × SSC, volume 10 ml. Hybridization temperature was 65 °C for 12 h. After autoradiography, filter lanes were cut and counted with a Beckman scintillation counter, and % hybridization was estimated in reference to the homologous hybridization.

Phylogenetic analysis

Unrooted phylogenetic tree based on aligned 16S rDNA sequences of *A. doebereinerae* strains (BR5401, UFLA01-51B, BR5414 and BR5426) and related sequences from the public DNA database (accession numbers shown), using ClustalW 1.8 program [38], was inferred by the Neighbor-Joining method with Jukes-Cantor correction using the program Mega 2.1 [19].

Strains UFLA1-510 and BR 5416 were not included because the smaller size of their sequences, respectively 845 and 642 bp.

Results and discussion

Utilization of carbon sources

Only nine of the 95 carbon sources supported the growth of all four *Azorhizobium doebereinerae* strains tested: mono-methylsuccinate, formic acid, alpha-, beta- and gamma-hydroxybutyric acids, DL-lactic acid, succinic acid, propionic acid and bromosuccinic acid. Other carbon sources utilized by some strains were: methyl-pyruvate (all except BR 5401), alpha-ketobutyric acid (UFLA1-510), succinamic acid (all except BR 5401), L-aspartic acid (all except BR 5401) and L-pyroglutamic acid (all except BR 5416 and BR 5401). These results show that *A. doebereinerae* can utilize a very narrow range of carbon sources in comparison with the other species of the same genus and with other genera of legume-nodulating bacteria [6,9,16], and this could be related to a low saprophytic competence explaining its very low occurrence in soil in the absence of its host plant. Gris and Moreira [15] found no nodulation of *S. virgata* inoculated with 27 soil samples from diverse sources (Amazon region, central and south Brazil); nodules were found only in the control inoculated with BR5401. Veasey et al. [42] found that *S. virgata* and *S. punicea* were the only ones, among 13 shrub and tree species, that did not nodulate with the native population of a red yellow latosol in south Brazil. Among about 800 nitrogen-fixing legume-nodulating bacteria from forest species, Moreira [24] characterized 598 strains isolated from 49 plant genera from diverse natural ecosystems. Strains with cultural characteristics similar to *Azorhizobium* were only found among those isolated from *S. virgata*, which in turn has been found naturally nodulated only by *A. doebereinerae*.

Neither *A. caulinodans* nor *A. doebereinerae* was able to use mannitol or sucrose, which are carbon sources used by most strains of the genera *Rhizobium, Sinorhizobium, Mesorhizobium* and *Bradyrhizobium*. However, they can use DL- lactic acid. *A. doebereinerae* is different from *A. caulinodans* because it is not able to use either L-leucine or D-glucose.

Growth and nitrogenase activity under free-living conditions and on LB medium

In semisolid LO modified medium all strains of *Azorhizobium doebereinerae* grew, forming a thin pellicle near the surface just like ORS 571 [8], but their pellicles had a slower growth (i.e., they reach the surface later than the pellicle of ORS 571) and they exhibited much less nitrogenase activity than ORS 571. Thus *A. doebereinerae* cannot be confounded with the two distinct unnamed species found nodulating *S. rostrata* by Rinaudo et al. [35]. Unlike *Azorhizobium doebereinerae,* one of those does not fix nitrogen in free-living conditions, and the other does nodulate *S. rostrata* stems. None of these strains, except ORS 571, grew on LB medium.

REP-PCR and cluster analysis

Fig. 1 shows that REP-PCR profiles were quite diverse among *Azorhizobium doebereinerae* isolates and strains belonging to other genera and species. BR5401 was about 70% similar to two strains isolated from heavy metal contaminated sites and 90% similar to strain BR5414 isolated from a nearby place in the same state. Strains UFLA1-51B and UFLA01-605 (98%) were both isolated from municipalities in south Minas Gerais State and they were quite different from the other *A. doebereinerae* isolates as well as from BR5416. The high diversity of REP-PCR profiles corroborates other studies where high diversity was also found among strains belonging to the same species and among species and genera [2,4,7,18,44].

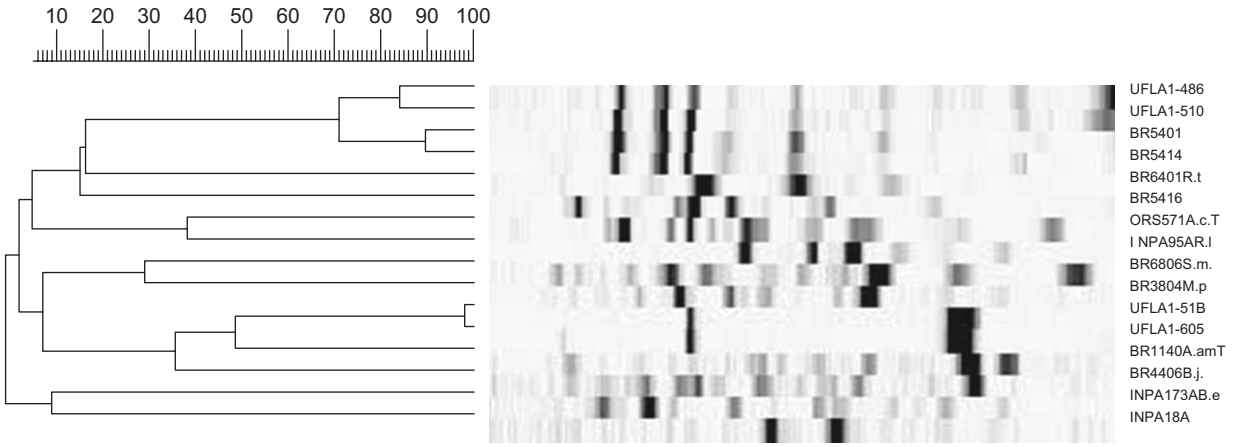

Fig. 1. Cluster analysis of REP-PCR profiles (*r*-values) performed using the UPGMA method.

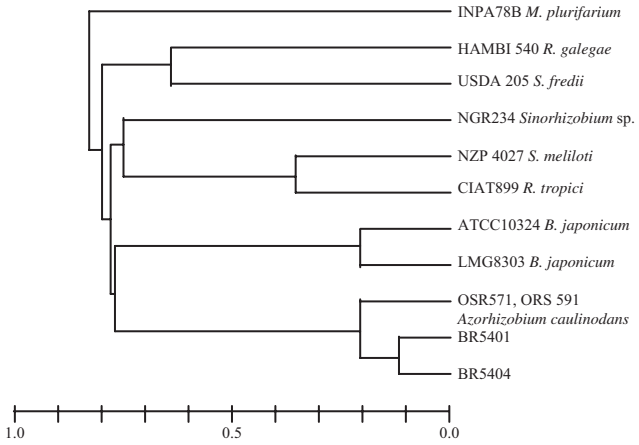

Fig. 2. Dendrogram of relatedness among *Azorhizobium doebereinerae* strains and type and reference strains of other nitrogen-fixing legume-nodulating genera based on the electrophoretic mobility of the following metabolic enzymes analysed for each strain: esterase (EST), glucose-6-phosphate dehydrogenase (G6P), hexokinase (HEX), isocitrate dehydrogenase (IDH), malate dehydrogenase, phosphoglucomutase (PGM), xanthine dehydrogenase (XDH), alcohol dehydrogenase (ADH).

Multilocus enzyme electrophoresis (MLEE)

Considering the allelic variation of eight metabolic enzymes, the type strain ORS 571, the reference strain ORS 591 and the isolates BR5401 and BR5404 constituted an independent cluster separated (at genetic distances above 0.77) from all tested type and reference strains belonging to the genera *Bradyrhizobium, Rhizobium, Sinorhizobium* and *Mesorhizobium* (Fig. 2). This clearly indicates that strains BR5401 and BR5404 belong to the genus *Azorhizobium*. The fact that BR5401 and BR5404 had almost identical electrophoretic type (except for one enzyme) suggests that they belong to the same species. The *A. caulinodans* type and reference strains had an identical electrophoretic type, and they were related to BR5401 and BR5404 at a closer genetic distance than we expected from different species. This might reflect the relatively small number of enzyme loci examined, as also observed with the low genetic distance seen between the genera *Azorhizobium* and *Bradyrhizobium*.

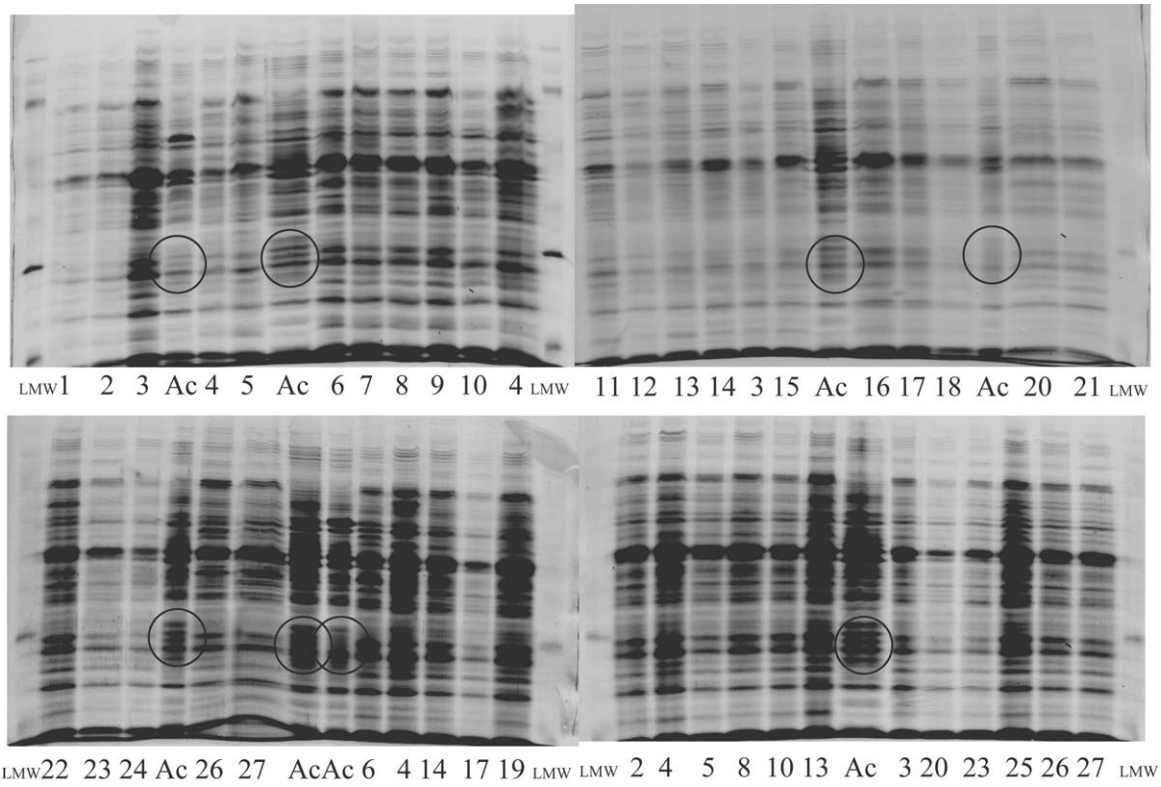

Fig. 3. Protein profiles of 26 *Azorhizobium doebereinerae* strains and of *Azorhizobium caulinodans* ORS571 obtained by sodium dodecyl sulphate polyacrilamide gel electrophoresis. Lines 1 to 27 correspond to *Azorhizobium doebereinerae* strains: BR5419(1), BR5415(2), UFLA01-51B(3), BR5416(4), BR5404(5), UFLA1-606(6), BR5413(7), BR5425(8), UFLA1-602(9), BR5421(10), UFLA1-605(11), BR5401(12), BR5422(13), BR5430(14), UFLA1-604(15), BR5402(16), BR5418(17), BR5405(18), BR5428(19), BR5426(20), UFLA1-603(21), BR5423(22), UFLA1-54B(23), BR5417(24), BR5427(25), UFLA1-601(26), BR5424(27); Ac = *Azorhizobium caulinodans*^{T}ORS571, Circles indicated main differences between profiles of *Azorhizobium caulinodans* and *Azorhizobium doebereinerae.*

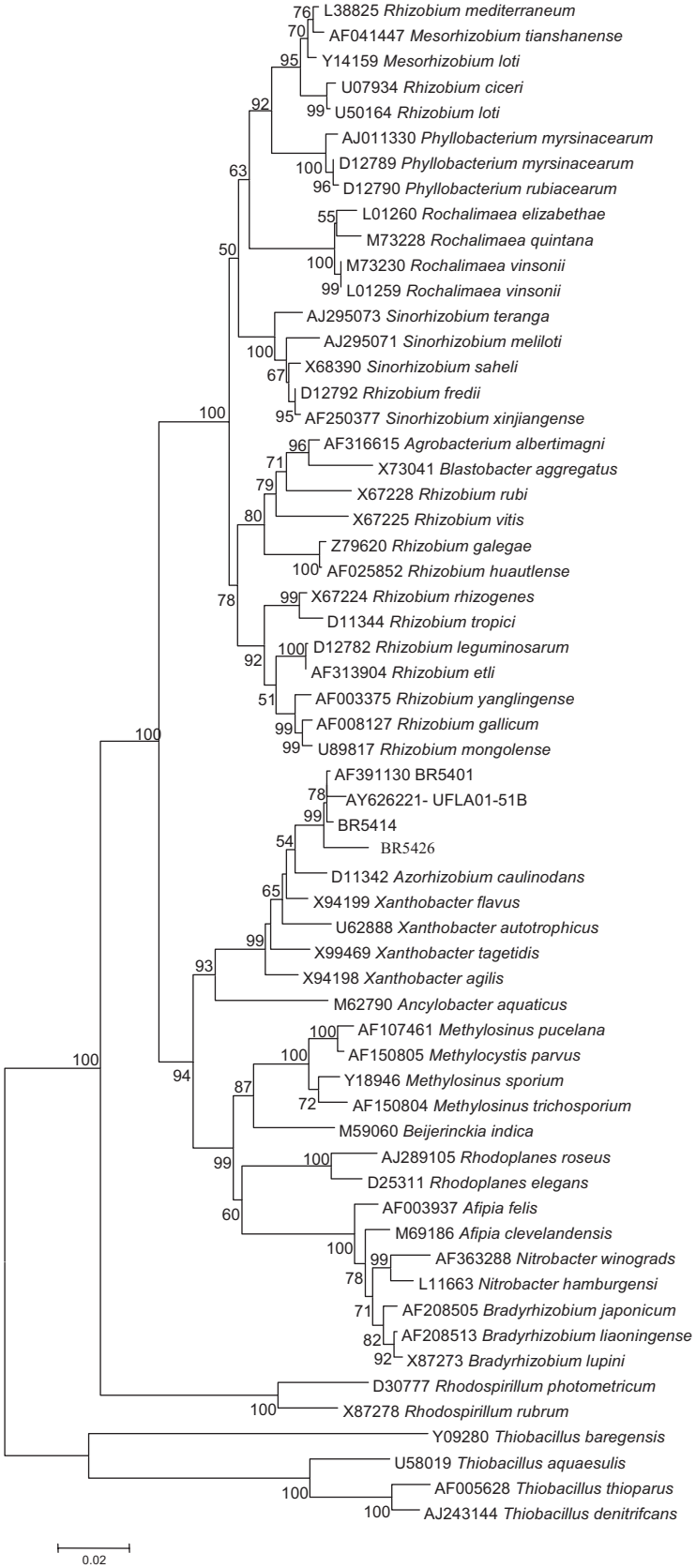

Fig. 4. Phylogenetic tree based on 16S rDNA sequences of *A. doebereinerae* strains and related sequences from the public DNA database (accession numbers shown). The tree was inferred by the Neighbor-Joining method with Jukes-Cantor correction using the program Mega 2.1 [19]. Bootstrap values above 50% are shown (based on 2000 trials); scale bar indicates two substitutions per 100 bases.

Polyacrylamide gel electrophoresis of total bacterial proteins

The protein profiles of the twenty seven strains were quite similar and exhibited small differences in relation to *Azorhizobium caulinodans* ORS 571 protein profile mainly at region corresponding to 30.0 of the low molecular weight marker (Fig. 3).

16S rDNA sequencing

Fig. 4 shows that strains BR5401, UFLA1-51B, BR5414 and BR5426 are quite similar compared to representative species of other branches of the α-*Proteobacteria*, being more similar to *A. caulinodans.* Similarities of UFLA1-51B, BR5414 and BR5426 with BR5401 were 98%, 99.5% and 96%, respectively. UFLA1-510 and BR5416 were also shown to have highest similarity with strain BR5401, respectively 99% (753/755bp) and 93% (598/640bp).

DNA–DNA hybridization

A. caulinodans ORS571 hybridized 25% to UFLA01-51B; 33% to UFLA01-510; 32% to BR5401; 15% to USDA 110; 11.8% to USDA 76. These are averages based on two replicate experiments that did not differ by more than 2%. These results clearly show that the new species is different from *A. caulinodans* but belongs to the genus *Azorhizobium*, as the hybridization values fall within those obtained with rhizobia belonging to the same genus. In fact, the results were expected, due to the high dissimilarity of protein profiles in relation to the other genera and species of nitrogen fixing nodulating bacteria [6,10,25]. It has been demonstrated by many authors that strains with highly similar protein patterns share high DNA hybridization values, so the congruence at the species level between whole-cell-protein pattern similarity and DNA–DNA hybridization is clearly established, and the former technique can be successfully used to identify phenotypically aberrant strains [41].

Cell characteristics

Cells are about 0.6–0.9 μm diameter × 1.5 μm length (Fig. 5), and the number of lateral flagella varies from 2 to 4. These characteristics distinguish the species from *A. caulinodans,* which has cells 0.5–0.6 μm in diameter [9] and only one lateral flagellum in liquid medium but can be peritrichous in solid medium. Cells of the fast-growing genera *Rhizobium, Sinorhizobium* and *Mesorhizobium* also have peritrichous flagella or one polar/sub-polar flagellum. *Bradyrhizobium* has only one sub-polar flagellum and a diameter of 0.5–1.0 μm [46].

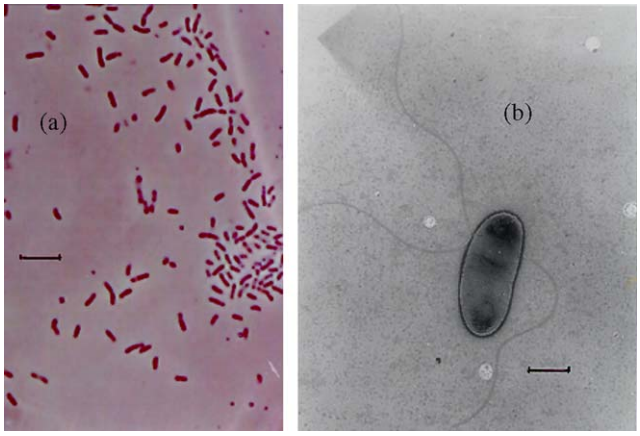

Fig. 5. *Azorhizobium doebereinerae* cells viewed (a) by phase contrast microscopy (scale bar represents 5.4 μm) and (b) by transmission electron microscopy (scale bar represents 0.5 μm).

Description of *Azorhizobium doebereinerae* [doe.ber.ein'erae, N.L. gen. n. doebereinerae, named in honour of Johanna Döbereiner, an outstanding Brazilian microbiologist (1924–2000)]

Aerobic gram-negative, non-spore-forming rods, isolated from *S. virgata* root nodules, strains can establish an effective nitrogen-fixing symbiosis by forming root nodules. They are also able to induce ineffective root nodules in *Sesbania rostrata*, *Phaseolus vulgaris* and *Macroptilium atropurpureum* and pseudo-nodules on *S. rostrata* stems. Cells are approximately 0.6–0.9 μm wide and 1.5 long (Fig. 5) and they are motile by means of two to four lateral flagella. These characteristics distinguish the species from the other species of the genus, *A. caulinodans,* which has cells 0.5–0.6 μm in diameter [9] and only one lateral flagellum in liquid medium but can be peritrichous in solid medium. Members of the species grow in PY, TY and YM but not in LB medium. They are able to grow in N-free semi-solid LO modified medium by fixing nitrogen. Colonies are visible after 3–4 days in YMA, where they reach 1 mm diameter and have a translucent appearance with little exopolysaccharide production, characteristics that are quite similar to *A. caulinodans*. All strains exhibited very similar total protein profiles obtained by polyacrylamide gel electrophoresis (SDS-PAGE). They are not able to use sucrose, mannitol or glucose as sole carbon sources, however they are able to use lactic acid. Strains have a distinct SSU-rRNA gene sequence and low levels of DNA–DNA hybridization with the type strain of its closest phylogenetic relative *A. caulinodans* (between 25% and 33%) and with *Bradyrhizobium japonicum* and *B. elkanii* reference and type strains (15% and 11.8%, respectively).

The type strain is UFLA1-100^T (= BR5401^T, LMG9993^T, SEMIA6401^T) isolated from root nodules

of *S. virgata* (Caz.) Pers. grown in Seropédica, Rio de Janeiro, Brazil.

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