Identification of *Beijerinckia fluminensis* strains CIP 106281^T and UQM 1685^T as *Rhizobium radiobacter* strains, and proposal of *Beijerinckia doebereineriae* sp. nov. to accommodate *Beijerinckia fluminensis* LMG 2819

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During the course of a research project with free-living, nitrogen-fixing bacteria, we determined the 16S rRNA gene sequence of *Beijerinckia fluminensis* strains UQM 1685^T and CIP 106281^T and discovered that they were only 90.6–91.2 % similar to the sequences of strains of other *Beijerinckia* species and subspecies. Moreover, the highest similarity to these sequences (99.7 %) corresponded to strains of *Rhizobium radiobacter* (including *Agrobacterium tumefaciens*). Other diagnostic features confirmed that the two strains have the same origin but do not descend from the nomenclatural type. At the same time, *B. fluminensis* LMG 2819 was characterized and it was found that its properties also do not agree with the original description of the species, although it can be considered a member of the genus. Further characterization, including chemotaxonomic and other phenotypic traits, allows us to propose (i) the identification of *B. fluminensis* strains CIP 106281^T and UQM 1685^T as strains of *Rhizobium radiobacter* and (ii) the designation of strain LMG 2819^T (=CECT 7311^T) as the type strain of a novel species, *Beijerinckia doebereineriae* sp. nov.

The relationship between plants and rhizosphere micro-organisms and how they can affect plant growth and development continues to arouse great interest. Kloepper & Schroth (1978) introduced the term 'plant-growth-promoting rhizobacteria' to describe a group of soil bacteria able to colonize plant roots and the rhizosphere, positively affecting their growth and development through microbial activity. Members of genera such as *Rhizobium*, *Frankia*, *Azotobacter* and *Azospirillum* and other similar micro-organisms are capable of biological nitrogen fixation. These prokaryotes are referred to as diazotrophs, and they include free-living bacteria and also symbiotic bacteria associated with plant roots. Biological nitrogen fixation is an important mechanism in the promotion of plant growth because, although

nitrogen is abundant in the atmosphere, very few organisms are able to use it directly, instead requiring fixed nitrogen in the form of ammonium or nitrate.

The genus *Beijerinckia* was described by Derx (1950) as consisting of two species, *Beijerinckia indica* (the type species) and *Beijerinckia mobilis*. Only a few taxonomic studies leading to descriptions of new taxa within the genus have been conducted since: *Beijerinckia derxii* (Tchan, 1957), *Beijerinckia fluminensis* (Döbereiner & Ruschel, 1958) and the subspecies *B. derxii* subsp. *venezuelae* and *B. indica* subsp. *lacticogenes* (Thompson & Skerman, 1979) have been described. Currently, the genus is classified together with the genera *Chelatococcus*, *Methylocapsa* and *Methylocella* within the family *Beijerinckiaceae* in the order *Rhizobiales* (Garrity *et al.*, 2005).

As part of a study with fertilizers, we realized that strain CIP 106281^T, the type strain of *B. fluminensis*, differed significantly from other members of the genus *Beijerinckia*. Therefore, we decided to explore further and elucidate the implications of this observation for the taxonomic status of this species.

Abbreviation: PHB, polyhydroxybutyrate.

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene sequences of strains LMG 2819^T, UQM 1685 and CIP 106281 are respectively EU401905, EU401907 and EU401908.

ARDRA profiles with two restriction enzymes and a dendrogram based on the profiles are available as supplementary material with the online version of this paper.

B. fluminensis was first isolated from soil samples collected in different Brazilian locations (Döbereiner & Ruschel, 1958) and it was later reported from elsewhere in South America and in Africa and Asia (Becking, 1961; Amor Asunción *et al.*, 1980). In spite of this, only a few strains have been deposited in public culture collections. By using the StrainInfo.net bioportal (<http://www.straininfo.net>), we could only find six entries, including the type strain. The equivalences of the type strain were (in alphabetical order): CCUG 53676^T, CIP 106281^T, DSM 2327^T, NCAIM B.01797^T, UQM 1685^T. By following the hyperlinks, we found out that, for CCUG 53676^T, the following text is displayed: 'original DSM ampoules, not yet processed'. For the rest, the history of the strain can be reconstructed as follows: UQM → DSMZ → Varga, Sz. → NCAIM → CIP. Therefore, we obtained the type strain of *B. fluminensis* from the first collection (UQM) in this sequence of exchanges, since we already had the last one (CIP 106281^T). We also noticed that DSM 2327^T is no longer listed in the DSMZ catalogue (but the last record for that number was *Rhizobium radiobacter*).

In addition to the type strain, we also obtained the remaining strains of *B. fluminensis* listed in StrainInfo.net, namely LMG 2819, NCIMB 9881, NCIMB 9882, NCIMB 11068 and NCIMB 11069. Finally, the following strains were included for comparative purposes: *B. indica* subsp. *indica* LMG 2817^T, *B. indica* subsp. *lacticogenes* LMG 2818^T, *B. derxii* subsp. *derxii* LMG 3899^T, *B. derxii* subsp. *venezuelae* DSM 2329^T, *B. mobilis* LMG 3912^T, *Derxia gummosa* LMG 3977^T and *Chelatococcus asaccharovorans* DSM 6462^T.

For maintenance and further analysis, strains were grown at 30 °C in a nitrogen-free mineral medium [l^{-1} : 10 g glucose, 0.8 g K₂HPO₄, 0.2 g KH₂PO₄, 0.1 g MgSO₄·7H₂O, 20 mg FeSO₄·7H₂O, 1.3 mg MnSO₄·H₂O, 5 mg ZnSO₄·7H₂O, 4 mg CuSO₄·5H₂O, 5 mg Na₂MoO₄·2H₂O and 15 g agar (for solid media); pH 6.5]. However, *B. fluminensis* UQM 1685^T and CIP 106281^T could not be subcultured in this medium, and were instead incubated in tryptone soy agar (TSA) or broth (TSB) (Pronadisa) at 30 °C as recommended by the suppliers.

Genomic bacterial DNA was isolated with an UltraClean microbial DNA isolation kit (MO BIO). For a preliminary analysis, ARDRA profiles were generated using purified [GFX PCR, DNA and gel band purification kit (GE Healthcare)] aliquots of PCR products that were digested with restriction endonucleases (*AluI* and *MspI*; Roche) as specified by the manufacturer. Restricted DNA was analysed by horizontal electrophoresis in 3 % (w/v) agarose MetaPhor (FMC). Electrophoresis was carried out at 100 V for 4 h, and gels were stained with ethidium bromide (2 µg ml⁻¹) and photographed under UV illumination. Sequence divergences between the 16S rRNA gene regions of pairs of strains were estimated from the proportion of shared restriction fragments by the Nei-Li method (Nei & Li, 1979). A dendrogram was constructed from the distance

matrix by using the unweighted pair group method using arithmetic averages (UPGMA) (Sokal & Michener, 1958), with statistical analysis by bootstrap (Felsenstein, 1985) based on 1000 resamplings.

ARDRA patterns derived from the two enzymes (Supplementary Fig. S1, available in IJSEM Online) showed clearly that the profiles of *B. fluminensis* CIP 106281^T and UQM 1685^T are identical but quite different from those of the remaining *Beijerinckia* strains examined. This observation was confirmed by the dendrogram generated from the data of a similarity matrix (Supplementary Fig. S2). In this dendrogram, the genus *Beijerinckia* forms two well-resolved clusters, one that includes the types of all the species and subspecies of the genus except *B. fluminensis* and another that contains all *B. fluminensis* strains except the type strain, which appears as a deeper branch among the other alphaproteobacteria tested (*D. gummosa* LMG 3977^T is a betaproteobacterium). Once it was clear that *B. fluminensis* strains CIP 106281^T and UQM 1685^T can not be considered members of the genus *Beijerinckia*, our efforts concentrated on performing a better characterization of strain LMG 2819 as a representative of the other *B. fluminensis* strains.

Cell morphology and motility of cultures was observed under a Nikon Phase-contrast-2 microscope. The size and ultrastructure of the cells were determined by transmission electron microscopy (Fig. 1). Cells were negatively stained with uranyl acetate (0.5 % w/v, pH 4.5) and were observed with a JEOL microscope (Centro de Biología Molecular, Universidad Autónoma de Madrid, Madrid). No flagellation was detected for strain LMG 2819, not even in fresh cultures, although monotrichous polar flagellation was observed in strains CIP 106281^T and UQM 1685^T. According to the original description of *B. fluminensis* in which the type strain is specified, 'motility is slow and occurs only in fresh cultures' (translated from Portuguese; Döbereiner & Ruschel, 1958).

The optimal temperature for growth of strain LMG 2819 was 30 °C, and no growth occurred at 37 °C. Growth was observed at pH 3–10, optimally at pH 6.5. Different rich media formulations were tested (such as LB medium, nutrient agar and TSA), and no growth was observed on them. Growth was optimal in nitrogen-free mineral medium only.

Strain LMG 2819 was tested by standard procedures (Gerhardt *et al.*, 1994) and also by using the bioMérieux API 20NE (for biochemical reactions), API ZYM (for extracellular enzyme reactions) and API 50 CH kits in conjunction with API 50 CHB/E medium according to the manufacturer's instructions. Characteristics useful for differentiating the strains examined in this study are shown in Table 1.

The 16S rRNA gene was amplified by PCR with primers 27f and 1492r (Lane, 1991) as described by Orphan *et al.* (2001). The products were sequenced directly as described

Fig. 1. (a) Electron micrograph showing the cell shape of strain LMG 2819: slightly curved rods, about $3.25 \times 1 \mu\text{m}$, with two large polar lipid bodies of PHB. (b) Electron micrograph of a cell of strain LMG 2819. cw, Cell wall; im, inner membrane; om, outer membrane; F, fimbriae; PHB, polyhydroxybutyrate body. (c) Electron micrograph showing flagellum observed in [*B. fluminensis*] UQM 1685^T. Bars, 0.5 μm (a), 100 nm (b) and 1 μm (c).

by Moore *et al.* (1999) using the Taq dideoxy terminator cycle sequencing kit (Perkin Elmer Applied Biosystems), carrying out the reactions in an Applied Biosystems 373S DNA sequencer.

A nearly complete 16S rRNA gene sequence (about 1453 nt) was obtained and compared with the sequences placed in the GenBank database (National Center for Biotechnology Information) by using BLAST (<http://www.ncbi.nlm.nih.gov/blast/>). Related sequences were further analysed using the program package ARB (Ludwig *et al.*, 2004) (<http://www.arb-home.de>). Sequence align-

ments were corrected manually using the sequence editor ARB_EDIT. Phylogenetic analysis using various treeing methods (neighbour-joining, maximum-parsimony and maximum-likelihood) and data subsets were performed using the appropriate ARB tools (Ludwig *et al.*, 1998). Fig. 2 shows the tree derived from the neighbour-joining method using Jukes–Cantor evolutionary corrections. Phylogenetic analysis revealed that strain LMG 2819 was more related to other type strains of the genus *Beijerinckia* (97.0–97.5 %) than to strains CIP 106281^T and UQM 1685^T (only 91.2 %). The latter strains shared 99.7 % sequence similarity with strains of *Rhizobium radiobacter* (including

Table 1. Differentiating characteristics among strains included in this study

Strains: 1, *B. indica* subsp. *indica* LMG 2817^T; 2, *B. fluminensis* LMG 2819; 3, *B. fluminensis* CIP 106281^T; 4, *B. fluminensis* UQM 1685^T. +, Positive; –, negative; ND, not determined; w, weak reaction.

Characteristic	1	2	3	4
Colony pigmentation	White	Cream	Light brown	Light brown
Motility	–	–	+	+
Nitrate to nitrite reduction	ND	+	–	–
Urease	ND	–	+	+
β -Galactosidase	ND	–	+	+
Assimilation of:				
D-Arabinose	+	–	+	+
L-Arabinose	–	+	+	+
Methyl β -D-xylopyranoside	+	–	+	+
Methyl α -D-glucopyranoside	–	+	w	w
Maltose	–	+	+	+
Lactose	w	–	+	+
Turanose	–	+	+	+
DNA G+C content (mol%)	58.8	57.1	60.7	ND

Fig. 2. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of strain LMG 2819, [*B. fluminensis*] strains CIP 106281^T and UQM 1685^T and other related species. Bootstrap values (percentages of 1000 resamplings) greater than 60 % are shown at branching points. Bar, 5 estimated substitutions per 100 base positions. Sequence accession numbers are given in parentheses. Dots at nodes indicate that the same clusters were retrieved in the maximum-parsimony and maximum-likelihood trees.

Agrobacterium tumefaciens strains). Although strain LMG 2819 formed the deepest branch of the true *Beijerinckia* cluster, its position was supported by both high bootstrap values and identical local branching order with alternative treeing methods (Fig. 2).

The DNA G+C content was determined by HPLC at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) according to Mesbah *et al.* (1989). The G+C content of strain LMG 2819 was 57.1 mol%, which is closer to that of *B. indica* LMG 2817^T (58.8 mol%), the type strain of the type species of the genus, than to that of [*B. fluminensis*] CIP 106281^T (60.7 mol%).

The cellular fatty acid composition of strain LMG 2819, *B. indica* subsp. *indica* LMG 2817^T, *B. mobilis* LMG 3912^T and [*B. fluminensis*] strains CIP 106281^T and UQM 1685^T were analysed by GLC at the DSMZ using the method described by Kämpfer & Kroppenstedt (1996); the resulting profiles are shown in Table 2. Strain LMG 2819, *B. indica* subsp. *indica* LMG 2817^T and *B. mobilis* LMG 3912^T were grown in nitrogen-free mineral medium for 50 h, whereas [*B.*

fluminensis] strains CIP 106281^T and UQM 1685^T were grown in TSB for 24 h. Again, [*B. fluminensis*] strains CIP 106281^T and UQM 1685^T were almost indistinguishable from each other and differed markedly from strain LMG 2819 which, in turn, resembled the type species of the genus *Beijerinckia*.

The data collected provide sufficient evidence for the identification of [*B. fluminensis*] strains CIP 106281^T and UQM 1685^T as strains of *Rhizobium radiobacter*. The data also support the classification of *B. fluminensis* LMG 2819 within the genus *Beijerinckia*, but we have found important differences between the properties of this strain and the descriptions of the species provided by Döbereiner & Ruschel (1958) and Thompson & Skerman (1979). Strain LMG 2819 has slightly curved cells that are non-motile, it grows abundantly in 5 days and forms creamy colonies on nitrogen-free solid medium that are very viscous. Liquid cultures become highly viscous after 5 days and also acquire a creamy taint. Cells possess two or more large lipid bodies of polyhydroxybutyrate (PHB), and zoogloas or cysts have never been observed. In contrast, *B.*

Table 2. Fatty acid methyl ester profiles

Strains: 1, *B. indica* subsp. *indica* LMG 2817^T; 2, *B. fluminensis* LMG 2819; 3, *B. mobilis* LMG 3912^T; 4, *B. fluminensis* CIP 106281^T; 5, *B. fluminensis* UQM 1685^T. All data are from this study. Values are mean percentages of total fatty acid methyl esters. tr, Trace amount ($\leq 1.0\%$); ND, not detected.

Fatty acid	1	2	3	4	5
Saturated fatty acids					
16:0	2.8	6.9	4.0	7.5	7.5
16:0 3-OH	ND	ND	ND	3.6	3.8
iso-17:0	2.0	ND	ND	ND	ND
18:0	4.2	ND	2.1	tr	tr
18:0 3-OH	ND	ND	ND	tr	tr
10-Methyl 19:0	ND	ND	ND	tr	tr
Unsaturated fatty acids					
13:1	tr	ND	ND	tr	tr
18:1 ω 7c	90.5	86.0	92.4	75.7	76.2
Cyclopropane acids					
19:0 cyclo ω 8c	ND	ND	ND	3.1	1.4
Summed features					
12:0 ALDE?	ND	ND	ND	tr	tr
14:0 3-OH and/or iso-16:1 I	ND	ND	ND	5.8	6.6
16:1 ω 7c and/or iso-15:0 2-OH	ND	7.0	ND	1.6	1.9
iso-17:1 I and/or anteiso-17:1 B	ND	ND	1.5	ND	ND

fluminensis was described as follows (Döbereiner & Ruschel, 1958). Cells are straight, motile rods that grow very slowly. After 15–20 days, colonies on nitrogen-free solid medium are brown and small, with a stiff and rough surface. Liquid media acquire a blue–white turbidity but no viscosity. Cells contain only two small lipid bodies (one at each end) and zoogloas (groups of cells surrounded by a common capsule) are frequent. Further differences include wider temperature and pH ranges for strain LMG 2819, formation of acid from glucose (negative for strain LMG 2819) and several nutritional tests. Our study provides evidence that strain LMG 2819 can not be considered a member of the species *B. fluminensis* nor of any other established species of the genus *Beijerinckia*. Therefore, we propose it be classified within a novel species, named *Beijerinckia doebereineriae* sp. nov. The implications of this study for the status of the name *Beijerinckia fluminensis* will be the subject of a subsequent paper.

Description of *Beijerinckia doebereineriae* sp. nov.

Beijerinckia doebereineriae (doe.be.rei'ne.rae. N.L. fem. n. *doebereineriae* to honour the Brazilian microbiologist Johanna Doebereiner, in recognition of her contribution to the study of nitrogen-fixing bacteria).

Cells are Gram-negative, regular, non-motile and slightly curved rods, about $3.25 \times 1 \mu\text{m}$, that do not form spores or zoogloas. A large number of fimbriae are present around the cell, but no flagella are observed. Two or more polar lipid bodies of PHB are present per cell. Colonies on nitrogen-free

mineral agar are irregular, convex, cream in colour, opaque and highly viscous. After 5 days of growth in nitrogen-free mineral broth, the medium becomes opaque, creamy and highly viscous. Strictly aerobic. Molecular nitrogen is fixed under aerobic conditions and requires molybdenum. Temperature range for growth is 10–35 °C; no growth at 37 °C. Grows at pH 3–10. Optimal growth at 30 °C and pH 6.5. No growth on peptone media. Cytochrome oxidase and catalase tests are positive. Reduces nitrate to nitrite. Positive for reduction of nitrate to nitrite (API 20NE). The remaining tests of the API 20NE kit are negative: indole production, acids from glucose, hydrolysis of aesculin and gelatin, arginine dihydrolase, urease and β -galactosidase activities and assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, maltose, gluconate, caproate, adipate, malate, citrate and phenylacetate. The following enzyme activities are recorded as positive (API ZYM): alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α -glucosidase. The remaining activities in the API ZYM system are negative: α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. Utilizes the following substrates (API 50 CH): L-arabinose, L-xylose, D-galactose, D-glucose, D-fructose, D-mannose, methyl α -D-glucopyranoside, maltose, sucrose, turanose, D-lyxose and D-fucose. Does not utilize glycerol, erythritol, D-arabinose, D-ribose, D-xylose, D-adonitol, methyl β -D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α -mannopyranoside, N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, lactose, melibiose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, D-tagatose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. Fatty acids detected are, in decreasing order of abundance, 18:1 ω 7c, summed feature 3 (16:1 ω 7c and/or iso-15:0 2-OH) and 16:0. The DNA G+C content of the type strain is 57.1 mol%.

The type strain is LMG 2819^T (=CECT 7311^T), which was deposited by F. Hilger at the LMG before 1966 according to the online catalogue (<http://bccm.belspo.be/>).

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