Reclassification of *Beijerinckia fluminensis* CIP 106281^T and *Beijerinckia fluminensis* UQM 1685^T as *Rhizobium radiobacter* strains, and proposal of *Beijerinckia fluminensis* LMG 2819 as *Beijerinckia doebereinerae* sp. nov.

Monike Oggerin[1], David R. Arahal[2], Víctor Rubio[3], Irma Marín[1]*

[1]Centro de Biología Molecular Severo Ochoa, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

[2]Spanish Type Culture Collection (CECT) and Department of Microbiology and Ecology, University of Valencia, Campus de Burjassot, 46100 Valencia, Spain

[3]Instituto de Ciencias Agrarias, Centro de Ciencias Medio Ambientales, CSIC, 28006 Madrid, Spain

Running title: *Beijerinckia doebereinerae* sp. nov.

Correspondence: Irma Marín

e-mail: imarin@cbm.uam.es. Phone: +34 914978078. Fax: +34 914978300

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Footnote: The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LMG 2819^T, UQM 1685 and CIP 106281 are EU401905, EU401907 and EU401908 respectively.

Transmission electron micrographs of strains LMG 2819^T and UQM 1685 are available as a supplementary figure in IJSEM Online.

During the course of a research project with free-living nitrogen fixing bacteria we determined the 16S rRNA gene sequence of strains *Beijerinckia fluminensis* UQM 1685^T and *B. fluminensis* CIP 106281^T and discovered that they were only 90.6-91.2 % similar to the sequences of other *Beijerinckia* species and subspecies. Moreover, the highest similarity to these sequences (99.7 %) corresponded to *Rhizobium radiobacter* (including *Agrobacterium tumefaciens*). Other diagnostic features confirmed that both strains have the same origin but do not descend from the nomenclatural type. At the same time, *Beijerinckia fluminensis* LMG 2819 was characterized and it was found that it does 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 reclassification of *Beijerinckia fluminensis* CIP 106281^T and *Beijerinckia fluminensis* UQM 1685^T as *Rhizobium radiobacter* strains, and ii) the designation of strain LMG 2819^T (= CECT 7311^T) as *Beijerinckia doebereinerae* sp. nov.

The relationship between plants and rhizosphere microorganisms and how they can affect the plant growth and development has excited great interest nowadays and in past times. Kloepper & Schrott (1978) introduced the term Plant Growth Promoting Rhizobacteria (PGPR) to describe a group of soil bacteria able to colonize plant roots and rhizosphere affecting positively their growth and development due to microbial activity.

The literature that we find about it is very wide doing reference to genera such as *Rhizobium*, *Frankia*, *Azotobacter* and *Azospirillum* just as other similar microorganisms capable of Biological Nitrogen Fixation. This can be performed by prokaryotes called diazothophs, including free living bacteria and symbiotic bacteria associated to plant roots and it is a direct very important mechanism in the promotion of plant growth, because although nitrogen is a very abundant element in the atmosphere very few organisms are able to use it directly being necessary the fixation of nitrogen in ammonium and nitrate form.

The genus *Beijerinckia* was described by Derx (1950) consisting of two species, *Beijerinckia indica* (type species) and *Beijerinckia mobilis*. Only a few taxonomic studies leading to

descriptions of new taxa within the genus have been conducted thereafter: *Beijerinckia derxii* (Tchan, 1957), *Beijerinckia fluminensis* (Döbereiner & Ruschel, 1958), and the two subspecies *Beijerinckia derxii* subsp. *venezuelae* and *Beijerinckia indica* subsp. *lacticogenes* (Thompson & Skerman, 1981). Currently, the genus is classified together with the genera *Chelatococcus*, *Methylocapsa*, and *Methylocella* within the family *Beijerinckiaceae* in the order *Rhizobiales* (Garrity *et al*., 2006).

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

B. fluminensis was first isolated from soil samples collected in different Brazilian locations (Döbereiner & Ruschel, 1958) and later it has been reported to occur in other countries from South America, 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 alphabetic 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 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 in the row (CIP). We also noticed that DSM 2327^T is no longer in catalogue (but the last record for that number was *Rhizobium radiobacter*).

In addition to the type strain, we also obtained the rest of strains of *B. fluminensis* listed in StrainInfo.net, namely: *B. fluminensis* LMG 2819, *B. fluminensis* NCIMB 9881, *B. fluminensis* NCIMB 9882, *B. fluminensis* NCIMB 11068, and *B. fluminensis* NCIMB 11069. Finally, the following strains were also included for comparative purposes: *Beijerinckia indica* subsp. *indica* LMG 2817^T, *Beijerinckia indica* subsp. *lacticogenes* LMG 2818^T, *Beijerinckia derxii* subsp.

derxii LMG 3899^T, *Beijerinckia derxii* subsp. *venezuelae* DSM 2329^T, *Beijerinckia mobilis* LMG 3912^T, *Derxia gummosa* LMG 3977^T, and *Chelatococcus assacharovorans* DSM 6462^T. For maintenance and further analysis, cells were grown in a Nitrogen free mineral medium (10 g glucose l^{-1}, 0.8 g K_2HPO_4 l^{-1}, 0.2 g KH_2PO_4 l^{-1}, 0.1 g $MgSO_4 \cdot 7H_2O$ l^{-1}, 20 mg $FeSO_4 \cdot 7H_2O$ l^{-1}, 1.3 mg $MnSO_4 \cdot H_2O$ l^{-1}, 5 mg $ZnSO_4 \cdot 7H_2O$ l^{-1}, 4 mg $CuSO_4 \cdot 5H_2O$ l^{-1}, 5 mg $Na_2MoO_4 \cdot 2H_2O$ l^{-1}, 15 g Agar l^{-1} (just for solid media) pH 6.5 at 30ºC. However, *B. fluminensis* UQM 1685^T and *B. fluminensis* CIP 106281^T could not be sub-cultured in this medium and were incubated in Tryptone Soy Agar (TSA) or Tryptone Soy Broth (TSB) (Pronadisa) at 30 ºC as recommended by the suppliers.

Genomic bacterial DNA was isolated with UltraClean Microbial DNA Isolation Kit (MO BIO). As first inspection 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 (Roche) as specified by the manufacturer. Two enzymes were used: *Alu*I and *Msp*I. Restricted DNA was analyzed by horizontal electrophoresis in 3 % (w/v) agarose MetaPhor (FMC). Electrophoreses were carried out at 100 V for 4 hours, and gels were stained with ethidium bromide (2 µg ml^{-1}) and photographed under UV illumination. Sequence divergences between the 16S rDNA 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 a statistical analysis by bootstrap (Felsenstein, 1985) based on 1000 resamplings.

ARDRA patterns derived from both enzymes (Supplementary Fig. S1) clearly allow recognizing that those of *B. fluminensis* CIP 106281^T and *B. fluminensis* UQM 1685^T are identical to each other but quite different from the rest of strains of the genus *Beijerinckia*. This observation was confirmed on the dendrogram that was generated with the data of a similarity matrix (Supplementary Fig. S2). In this dendrogram the genus *Beijerinckia* forms two clusters very well resolved, one that includes the types of all the species and subspecies of the genus (except *B. fluminensis*) and another one that contains all *B. fluminensis* strains except the type

strain that appears as deeper branch among the *Alphaproteobacteria* tested (*D. gummosa* LMG 3977^T is a *Betaproteobacteria*). Once proven that strains *B. fluminensis* CIP 106281^T and *B. fluminensis* UQM 1685^T can not be considered members of the genus *Beijerinckia*, our efforts concentrated on performing a better characterization of strain LMG 2819^T as 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 ultra structure 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, Spain). No flagellation was detected at strain LMG 2819^T, not even in fresh cultures, although monotrichous polar flagellation was observed in strains CIP 106281 and UQM 1685. According to the original description of *B. fluminensis* 'motility is slow and occurs only in fresh cultures' [translated from Portuguese] (Döbereiner & Ruschel, 1958).

The optimal temperature for growth of strain LMG 2819^T was 30 ºC, and no growth occurred at 37 ºC. Growth was enabled at pH values within the range 3-10 and optimally at pH 6.5. Different rich media formulations were tested (such as LB medium, nutrient agar and Tryptone Soy Agar) and no growth was observed in them. Only in Nitrogen free mineral medium the growth was optimal.

Strain LMG 2819^T was tested by standard procedures (Gerhard *et al*., 1994) and also by using different biochemical tests, namely bioMérieux API 20NE (for biochemical reactions), API ZYM (for extracellular enzyme reactions) and API 50 CH in conjunction to API 50 CHB/E medium according to the manufacturer's instructions.

The 16S rRNA gene was amplified by PCR with the primers 27f and 1492r (Lane, 1991) as described by Orphan *et al.* (2001). The products were sequenced directly as described 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 sequences (about 1453 nucleotides) was obtained and compared with the sequences placed in the GenBank database (National Center for

Biotechnology Information) by using the BLAST (Basic Local Alignment Search Tool, National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/BLAST/). Related sequences were further analyzed using the program package ARB (Ludwig *et al*., 2004) (http://www.mikro.biologie.tu-muenchen.de). Sequence alignments 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^T was more related to other type strains of the genus *Beijerinckia* (97.0 to 97.5 %) than to strains CIP 106281^T and UQM 1685^T (only 91.2 %). Indeed, these two shared 99.7 % sequence similarity with *Rhizobium radiobacter* (including *Agrobacterium tumefaciens*). Although strain LMG 2819^T formed the deepest branch of the true *Beijerinckia* cluster, its position was supported both by high bootstrap values and identical local branching order with alternative treeing methods (Fig. 2).

The DNA G+C content (mol%) 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^T was 57.1 mol%, which is closer to that of *B. indica* LMG 2817^T (58.8 mol%), the type species of the genus, than to strain CIP 106281 (60.7 mol%).

The cellular fatty acid composition of strain LMG 2819^T, *B. indica* subsp. *indica* LMG 2817^T, *B. mobilis* LMG 3912^T, and strains CIP 106281 and UQM 1685 were analyzed by GLC at the DSMZ using a method described by Kämpfer & Kroppenstedt (1996) and resulting profiles are shown in Table 1. Strain LMG 2819^T, *B. indica* subsp. *indica* LMG 2817^T and *B. mobilis* LMG 3912^T were grown in Nitrogen free mineral medium for 50 hours, whereas strains CIP 106281 and UQM 1685 grew in 24 hours in TSB. Again, strains CIP 106281 and UQM 1685 were almost indistinguishable from each other and differed markedly from strain LMG 2819^T which in turn presents more resemblance with the type species of the genus *Beijerinckia*.

All the data collected provide sufficient evidence for the reclassification of *B. fluminensis* CIP 106281^T and *B. fluminensis* UQM 1685^T as *Rhizobium radiobacter* strains. Regarding *B. fluminensis* LMG 2819^T, our data support its classification within the genus *Beijerinckia*, but we have found important differences between this strain and the original description of the species provided by Döbereiner & Ruschel (1958). Strain LMG 2819^T has slightly curved cells that are non motile, it grows abundantly in five days and forms, on Nitrogen free solid medium, creamy colonies that are very viscous. Liquid media become highly viscous after five days and acquire also a creamy taint. Cells possess two or more large lipid bodies of PHB and zoogleas or cysts have never been observed. In contrast, *B. fluminensis* (Döbereiner & Ruschel, 1958) was described as straight motile rods that grow very slowly. After 15-20 days colonies on Nitrogen free solid medium are brown, 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 zoogleas (groups of cells surrounded by a common capsule) are frequent. Further differences include wider temperature and pH ranges for strain LMG 2819^T, formation of acid from glucose (negative for strain LMG 2819^T) and several nutritional tests. Our study provides evidence that strain LMG 2819^T 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 as a novel species named *Beijerinckia doebereinerae* sp. nov. (type strain LMG 2819^T = CECT 7311^T).

Description of *Beijerinckia doebereinerae* (Döbereiner & Ruschel 1958)

Beijerinckia doebereinerae (doe.be.rei'ne.rae N.L. fem. n. doebereinerae, 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 measuring about 3.25 x 1 μm that do not form spores or zoogloas. A high number of fimbriae are present around the cell but no flagella could be observed. Two polar lipoid bodies of PHB or more are present per cell. Colonies on Nitrogen free mineral agar medium are irregular, convex, creamy colour, opaque

and highly viscous. After 5 days growing 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 growth range is 10-35ºC and no growth is obtained at 37ºC. It grows at pH values between 3 and 10. Optimal growth occurs at 30ºC and pH 6.5. No growth on peptone media. Cytochrome oxidase and catalase tests are positive. Reduces nitrate to nitrite.

The following API 20NE test gives positive result: reduction of nitrate to nitrite. The remaining tests are negative: indole production, acids from glucose, arginine dihydrolase, urease, hydrolysis of esculin and gelatin, β-galactosidase activity, and assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, D-maltose, gluconate, caproate, adipate, malate, citrate and phenylacetate. The following enzymatic activities (API ZYM) were recorded as positive: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, naphtol-AS-BI-phosphohydrolase and α-glucosidase. The remaining enzymatic activities in the API ZYM system were 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, D-maltose, sucrose, D-turanose, D-lyxose and D-fucose. It 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, amigdalin, arbutin, aesculin, salicin, D-cellobiose, D-lactose, D-melibiose, D-trehalose, inulin, D-melezitose, D-raffinose, starch, glycogen, xylitol, gentiobiose, D-tagatose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate, potassium 5-ketogluconate.

Fatty acids detected are in order of abundance: 18:1 w7c (86.03 %), summed features 16:1 w7c and/or 15:0 iso 2-OH (7.03 %) and 16:0 (6.94 %).

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 LMG before 1966 according to LMG on-line catalog (http://bccm.belspo.be/).

Acknowledgments

We are most grateful to Professor Dr Hans G. Trüper for his assistance with the Latin nomenclature. This work was supported by a grant from Soil Biogenics Ldt. M. Oggerin was funded by a fellowship from Soil Biogenics Ldt. D. R. Arahal has a contract with the Universitat de València under the "Ramón y Cajal" program (Ministerio de Educación y Ciencia, Spain).

REFERENCES

Amor Asunción, J. M., Frontera, G., Dellepiane, E. (1980). Species of the genus *Beijerinckia* Derx isolated from a soil of Corrientes (Argentina) *Rev Argent Microbiol* **12,** 23–28. [Article in Spanish]

Becking, J. H. (1961). Studies on nitrogen-fixing bacteria of the genus *Beijerinckia*. I. Geographical and ecological distribution in soils. *Plant Soil* **14,** 49–81.

Derx, H. G. (1950). *Beijerinckia*, a new genus of nitrogen-fixing bacteria occurring in tropical soils. *Proceed Konink Nederl Akad Wetensch Series C* **53,** 140–147.

Döbereiner, J. & Ruschel, A. P. (1985). Uma nova espécie de Beijerinckia. *Rev Biol* **1,** 261–272. [Article in Portuguese]

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39,** 783–791.

Garrity, G. M., Bell, J. A. & Lilburn, T. (2006). Family VI. *Beijerinckiaceae* fam. nov. In *Validation of the Publication of New Names and New Combinations Previously Effectively Published Outside the IJSEM,* List no. 107. *Int J Syst Evol Microbiol* **56,** 1–6.

Gerhardt, P., Murray, R. G. E., Wood, W. A. & Krieg, N. R. (1994). *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology.

Kämpfer, P. & Kroppenstedt, R. M. (1996). Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Can J Microbiol* **42,** 989–1005.

Kloepper, J. W., & Schroth, M. N. (1978). Plant growth-promoting rhizobacteria on radishes. II. In *Proceedings of the 4th International Conference on Plant Pathogenic Bacteria*, pp. 879–882. Angers, France: Station de Pathologie Vegetale et Phytobacteriologie, INRA.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.

Ludwig, W., Strunk, O., Klugbauer, S., Klugbauer, N., Weizenegger, M., Neumaier, J., Bachleitner, M. & Schleifer, K.-H. (1998). Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* **19,** 554–568.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G. & other authors (2004). ARB: a software environment for sequence data. *Nucl Acids Res* **32,** 1363–1371.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39,** 159–167.

Moore, E. R. B., Arnscheidt, A., Krüger, A., Strömpl, C. & Mau, M. (1999). Simplified protocols for the preparation of genomic DNA from bacterial cultures. In *Molecular Microbial Ecology Manual*, pp. 1.6.1.1–1.6.1.15. edited by A. D. L. Akkermans, J. D van Elsas & F. J. de Bruijn. Dordrecht: Kluwer Academic.

Nei, M. & Li, W. H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc Nat Acad Sci* **76,** 5269–5273.

Orphan, V. J., Hinrichs, K. U., Ussler, W., III, Paull, C. K., Taylor, L. T., Sylva, S. P., Hayes, J. M. & DeLong, E. F. (2001). Comparative analysis of methane-oxidizing archaea and sulphate-reducing bacteria in anoxic marine sediments. *Appl Environ Microbiol* **67,** 1922–1934.

Sokal, R. R. & Michener, C. D. (1958). A statistical method for evaluating systematic relationships. *Kansas Univ Sci Bull* **38,** 1409–1438.

Tchan, Y. T. (1957). Studies of nitrogen-fixing bacteria. VI. A new species of nitrogen-fixing bacteria. *Proc Linn Soc New South Wales* **82,** 314–316.

Thompson, J. P. & Skerman, V. B. D. (1980). *Azotobacteraceae: the taxonomy and ecology of the aerobic nitrogen-fixing bacteria*. London: Academic Press.

Table 1. Fatty acid methyl ester profiles. All data from this study.

Strains: 1, *Beijerinckia indica* subsp. *indica* LMG 2817^T; 2, *Beijerinckia doebereinerae* LMG 2819^T; 3, *Beijerinckia mobilis* LMG 3912^T; 4, *Beijerinckia fluminensis* CIP 106281^T; 5, *Beijerinckia fluminensis* UQM 1685^T. Values are mean percentages of total fatty acid methyl esters. tr, trace amount (≤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
17:0 ISO	2.0	nd	nd	nd	nd
18:0	4.2	nd	2.1	tr	tr
18:0 3-OH	nd	nd	nd	tr	tr
19:0 10-methyl	nd	nd	nd	tr	tr
Unsaturated fatty acids					
13:1	tr	nd	nd	tr	tr
18:1 w7c	90.5	86.0	92.4	75.7	76.2
Cyclopropane acids					
19:0 cyclo w8c	nd	nd	nd	3.1	1.4
Summed features					
12: ALDE?	nd	nd	nd	tr	tr
14:0 3OH and/or 16:1 iso I	nd	nd	nd	5.8	6.6
16:1 w7c and/or 15:0 iso 2-OH	nd	7.0	nd	1.6	1.9
17:1 iso I/ante I B	nd	nd	1.5	nd	nd

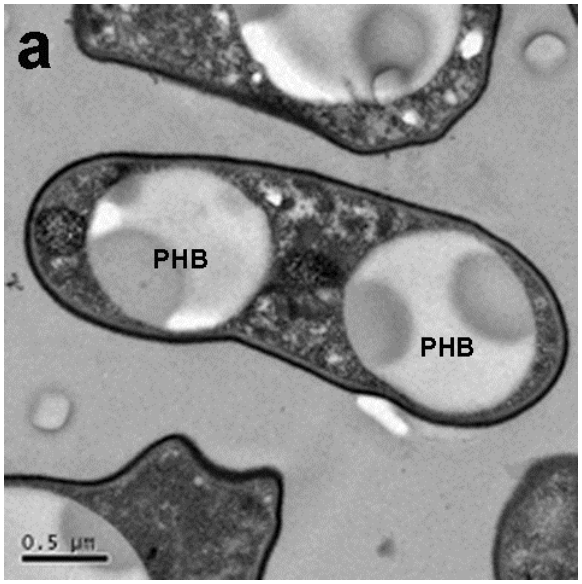
Table 2. Differentiating characteristics among the strains included in this study

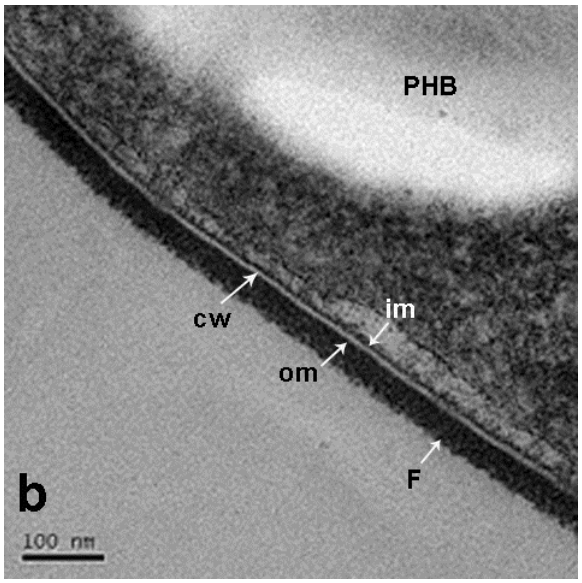
Strains: 1, *Beijerinckia indica* subsp. *indica* LMG 2817^T; 2, *Beijerinckia doebereinerae* LMG 2819^T; 3, *Beijerinckia fluminensis* CIP 106281^T; 4, *Beijerinckia fluminensis* UQM 1685^T. +, positive; -, negative; nd, not determined; w, weak reaction.

Characteristic	1	2	3	4
Colony pigmentation	white	creamy	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
D-Maltose	-	+	+	+
Lactose	w	-	+	+
D-Turanose	-	+	+	+
DNA G+C content (mol%)	58.8	57.1	60.7	nd

Figure 1. Electron micrographs. Bars, 0.5 μm (a); 100 μm (b); 1 μm (c).

(a). *Beijerinckia doebereinerae* LMG 2819^{T} shape: light curved-rods with two big polar lipoid bodies of poliyhydroxibutirate (PHB) about 3.25 x 1 μm. (b). *Beijerinckia doebereinerae* LMG 2819^{T}. cw, cell wall; im, inner membrane; om, outer membrane; F, fimbriae; PHB, poliyhydroxibutirate. (c). Flagellum observed in *Beijerinckia fluminensis* UQM 1685^{T}.





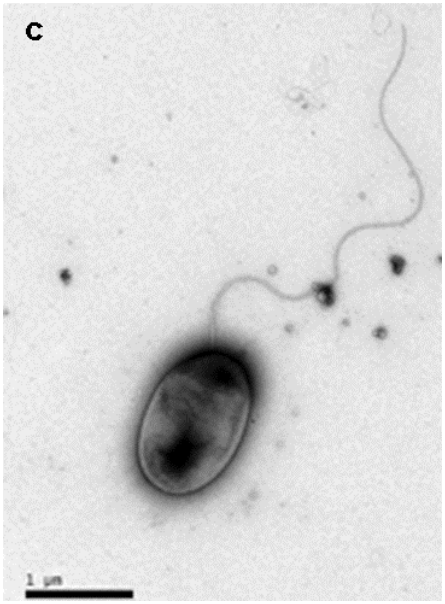
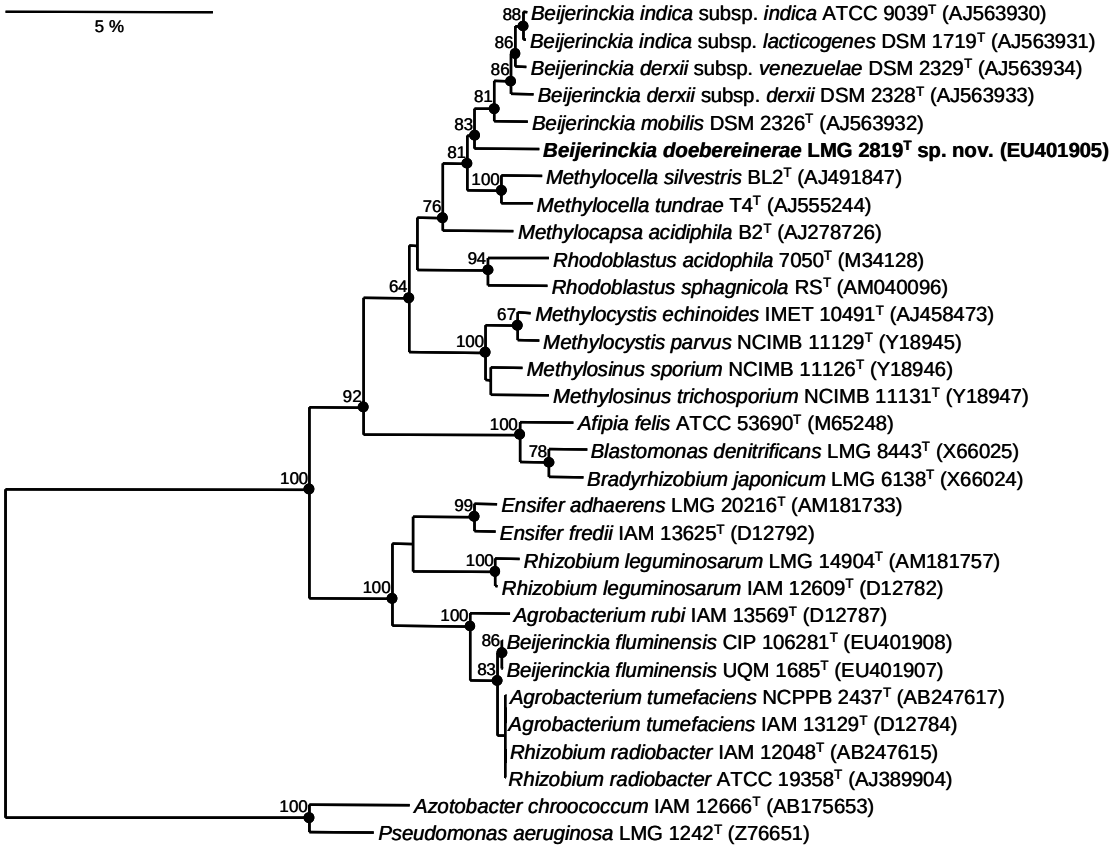
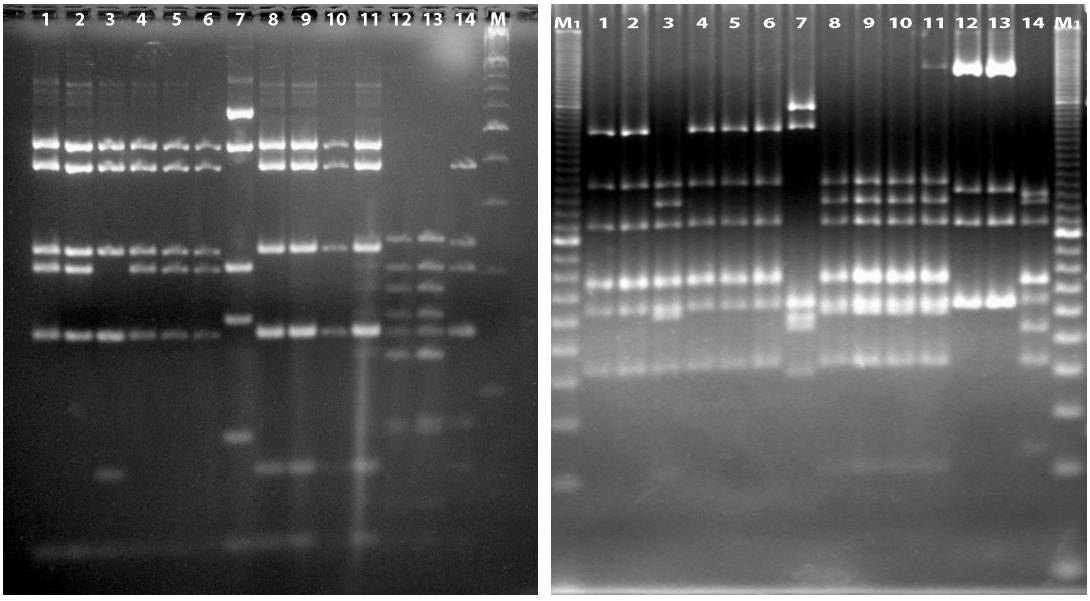


Figure 2. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of *Beijerinckia doebereinerae* LMG 2819^T, *Beijerinckia fluminensis* CIP 106281^T, *Beijerinckia fluminensis* UQM 1685^T and other related species. Bootstrap values (percentages of 1000 resamplings) greater than 60% are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



Supplementary Figure 1. ARDRA profiles. (a) *MspI* (b) *AluI*.

Lanes: 1, *Beijerinckia indica* subsp. *indica* LMG 2817^{T}; 2, *Beijerinckia indica* subsp. *lacticogenes* LMG 2818^{T}; 3, *Beijerinckia doebereinerae* LMG 2819^{T}; 4, *Beijerinckia derxii* subsp. *venezuelae* DSM 2329^{T}; 5, *Beijerinckia derxii* subsp. *derxii* LMG 3899^{T}; 6, *Beijerinckia mobilis* LMG 3912^{T}; 7, *Derxia gummosa* LMG 3977^{T}; 8, *Beijerinckia doebereinerae* NCIMB 9881; 9, *Beijerinckia doebereinerae* NCIMB 9882; 10, *Beijerinckia doebereinerae* NCIMB 11068; 11, *Beijerinckia doebereinerae* NCIMB 11069; 12, *Beijerinckia fluminensis* CIP 106281^{T}; 13, *Beijerinckia fluminensis* ACM 1685^{T}; 14, *Chelatococcus assacharovorans* DSM 6462^{T}; M, DNA Molecular Weight Marker XVII (Roche); M1, 20 bp DNA Ladder (Takara).



Supplementary Figure 2. ARDRA dendrogram showing the phylogenetic relationship between some members of the family *Beijerinckiaceae*. Bootstrap values (percentages of 1000 resamplings) greater than 50% are shown at branching points. Bar, estimated substitution per 100 base positions.