***Endobacter medicaginis* gen. nov. sp. nov. isolated from alfalfa nodules in an acidic soil in Spain**

Martha Helena Ramírez-Bahena^{1,2}, Carmen Tejedor³, Isidro Martín³, Encarna Velázquez^{2,3}, Alvaro Peix^{1,2*}

1: Instituto de Recursos Naturales y Agrobiología de Salamanca. Consejo Superior de Investigaciones Científicas. (IRNASA-CSIC). Salamanca. Spain.

2: Unidad Asociada Grupo de Interacciones planta-microorganismo Universidad de Salamanca-IRNASA (CSIC). Salamanca. Spain

3: Departamento de Microbiología y Genética. Universidad de Salamanca. Salamanca. Spain

Running title: *Endobacter medicaginis* gen. nov. sp. nov.

Abstract

A bacterial strain designed M1MS02^T was isolated from a surface sterilised nodule of *Medicago sativa* in Zamora (Spain). The 16S rRNA gene sequence of this strain showed 96.5 and 96.2% identities, respectively, with respect to *Gluconacetobacter liquefaciens* IFO 12388^T and *Granulibacter bethesdensis* CGDNIH1^T from the Family *Acetobacteraceae*. The isolate was Gram negative, non-sporulated aerobic motile by a subpolar flagellum coccoid to rod-shaped bacterium. Major fatty acid is C_{18:1} ω7c (39.94%) and major ubiquinone is Q-10. The lipid profile consisted of diphosphatidylglycerol, phosphatidylethanolamine, two aminophospholipids, three aminolipids, four glycolipids, two phospholipids and a lipid. Catalase positive and oxidase and urease negative. Acetate and lactate are not oxydized. Acetic acid is produced from ethanol in culture media supplemented with 2% CaCO₃. Ammonium sulphate is assimilated in glucose medium. It produces dihydroxyacetone from glycerol. The phylogenetic and phenotypic analyses commonly used to differentiate the family *Acetobacteraceae* genera showed that the strain M1MS02^T should be classified into a new genus within this family for which the name *Endobacter medicaginis* gen nov., sp. nov. is proposed (type strain M1MS02^T, LMG 26838^T, CECT 8088^T). To our knowledge, this is the first report of *Acetobacteraceae* bacteria occurrence as legume nodules endophytes.

Keywords: endophytes, *Medicago*, nodules, *Acetobacteraceae*, *Endobacter*

* Corresponding author:

Alvaro Peix. Instituto de Recursos Naturales y Agrobiología, IRNASA-CSIC, c/Cordel de Merinas 40-52, 37008 Salamanca, Spain. Tel. +34923219606; Fax. +34923219609; E-mail: alvaro.peix@csic.es

Genebank Accession numbers for strain M1MS02 gene sequences are: JQ436923 for 16S rRNA, JX424585 for recA and JX424586 for 16S-23S rRNA ITS

The family *Acetobacteraceae* currently includes 30 validly described genera recorded in the List of Prokaryotic Names with Standing in Nomenclature by Dr. Euzéby (<http://www.bacterio.cict.fr>) mainly differentiated on the basis of *rrs* gene sequences (Sievers & Swings, 2005). Some of these genera contain species that are plant endophytes such as *Gluconacetobacter diazotrophicus* which is endophytic in sugar cane (Saravanan *et al.*, 2008) but other species such as *Gluconacetobacter azotocaptans*, *Gluconacetobacter johannae* and *Swaminathania salitolerans* are endophytic of coffee, corn and rice, respectively (Dong *et al.*, 1994, Loganathan & Nair, 2004). Nevertheless no member of family *Acetobacteraceae* have been reported up to date as endophytes of legume nodules. These structures are induced by rhizobia, but commonly other non-nodulating endophytic bacteria are present inside the nodules sharing niche with the bacterial endosymbionts. In the case of *Medicago*, several strains of the former genus *Agrobacterium* have been isolated from nodules of different species of this legume (Kan *et al.*, 2007, Djedidi *et al.*, 2011) and the presence of the actinobacteria *Micromonospora* in nodules of *Medicago sativa* has been reported by Trujillo *et al.* (2010). However no data are available up to date about other endophytes from alpha-Proteobacteria present in *Medicago sativa* nodules, and to our knowledge this is the first report of *Acetobacteriaceae* occurring in legume nodules. In this work we isolated an endophytic strain named M1MS02^T from a root nodule of this host that belongs to a new genus and species within family *Acetobacteraceae* for which the name *Endobacter medicaginis* gen. nov. sp. nov. is proposed.

The strain M1MS02^T was isolated from a nodule of *Medicago sativa* growing in Matilla la Seca (Zamora, Spain) during a study of rhizobia and nodular endophytes present in different legumes. To sterilize the root nodules they were washed several times with sterile distilled water and were then surface sterilized in HgCl₂ 2,5% (w/v) for 2 min. The nodules were rinsed five times with sterile distilled water and then crushed using a sterile pestle. The homogenized nodule tissue was inoculated on yeast extract mannitol agar (Vincent, 1970) modified (10 g l⁻¹ mannitol, 1 g l⁻¹ yeast extract, 0.2 g l⁻¹ K₂HPO₄, 0.2 g l⁻¹ MgSO₄·7H₂O, 0.5 g l⁻¹ NaCl, 20 g l⁻¹ agar) and the plates were incubated at 28 °C for 4 days. In parallel, some of the disinfected entire nodules were incubated in the same medium in order to ensure their complete external disinfection and no growth was observed around these nodules. The cultures used in further phenotypic and molecular

studies were purified from a single colony after 2 days incubation at 28 °C on YMA. The colonies were white, mucoid, translucent and convex on this medium. The strain was grown in nutrient broth (Difco, Becton Dickinson, BBL) for 48h at 22°C to check for motility by phase-contrast microscopy using the hanging drop method. Gram staining was carried out by the procedure described by Doetsch (1981) after 24h incubation at 28°C. The flagellation type was determined by electron microscopy after 48h incubation in TSA at 22°C as was previously described (Rivas *et al.*, 2007). Cells of strain M1MS02^T were Gram-negative, rod-shaped, non-sporulating, and motile by means of a subpolar flagellum (figure S1). The 16S rRNA gene of the strain M1MS02^T was analyzed as described by Rivas *et al.*, 2007), the *recA* gene as described by Gaunt *et al.* (2001) and the ITS fragment as described by Peix *et al.* (2005). The sequence obtained was compared with those from the GenBank using the BLASTN (Altschul *et al.*, 1990) and EzTaxon (Chun *et al.*, 2007) programs. Sequences were aligned using the Clustal_X software (Thompson *et al.*, 1997) and distances were calculated according to Kimura's two-parameter model (Kimura, 1980). The phylogenetic tree was inferred using the neighbor joining and maximum likelihood models (Saitou & Nei, 1987, Rogers & Swofford, 1998). MEGA5 package (Tamura *et al.*, 2011) was used for all analyses. The comparison of the 16S rRNA gene sequence of strain M1MS02^T against those of EzTaxon database showed that it is phylogenetically equidistant to two genera of family Acetobacteraceae. The closest related species are *Gluconacetobacter liquefaciens* IFO 12388^T, which is the type species of this genus, and *Granulibacter bethesdensis* CGDNIH1^T with 96.3 and 96.2% identities, respectively. The remaining species of genus *Gluconacetobacter* showed identities similar or lower according to the results of the mentioned database. The results of the maximum likelihood phylogenetic analysis (figure 1) confirmed the clustering of M1MS02^T within family *Acetobacteraceae* where this strain forms a separated branch. Congruent results were obtained when the phylogenetic tree was constructed using the neighbor joining method (data not shown). Therefore M1MS02^T represents a new genus within this family in which several recently described genera have identity values in their 16S rRNA genes higher than 96% as occurs in the case of *Granulibacter bethesdensis* CGDNIH1^T that presented 96.1% identity with *Gluconacetobacter liquefaciens* IFO 12388^T. There are some genera that presented even higher identity values as occurs between *Kozakia* and the genera *Gluconacetobacter*, *Asaia* and *Swaminathania*, between *Tanticharoenia* and

Ameyamaea, between *Ameyamaea* and *Neoasaia*, between *Acetobacter* and *Neoasaia*, between *Asaia* and *Neoasaia* with identities higher than 97% and even some genera have more than 98% identity such as *Swaminathanian* and *Asaia*.

The results of the *recA* gene analysis confirmed that strain M1MS02^T is placed in an independent branch phylogenetically very divergent to *G. bethesdensis* and to the cluster formed by species of genus *Gluconobacter* (figure S2) with identity values lower than 83% with respect to the remaining analysed species. The distances found in the *recA* gene with respect to their closest relatives (species from genus *Gluconacetobacter* and *Acidiphilum*) were similar to those found for example between the type species of genus *Acetobacter*, *A. aceti*, and several species of genus *Gluconobacter*. These results support the classification of strain M1MS02^T in a new genus within family *Acetobacteraceae*.

The 16S-23S rRNA internal transcribed spacer (ITS) analysis also showed the phylogenetic divergence of the strain M1MS02^T with respect to other species of family *Acetobacteraceae* (figure S3) with identity values lower than 60% with respect to the remaining analyzed species. These values were much lower than those found among other genera of family *Acetobacteraceae*, as occurs for example between the type species of *Acetobacter* and *Gluconobacter* that showed 84% identity.

Therefore, the results of *recA* gene and ITS fragment of strain M1MS02^T agree with those of the 16S rRNA gene analysis supporting the proposal that it represents a new genus within the family *Acetobacteraceae*.

The cellular fatty acids were analysed by using the Microbial Identification System (MIDI Inc Sherlock MIS software 6.1) using an Agilent 6890N gas chromatograph at DSMZ. The strains were grown on TSB (Becton Dickinson, BBL) for 24h at 28°C and 160 rpm. The major fatty acids are C_{18:1} ω7c (39.94%), C_{19:0} cyclo ω8c (12.15%) and C_{16:0} (13.40%). Other fatty acids identified were C_{18:1} 2OH (6.88%), C_{18:0} (4.58%), C_{16:0} 2OH (2.82%), C_{16:0} 3OH (2.73%), C_{17:1} ω6c (2.02%), C_{14:0} 3OH/ C_{16:1} ISO I in summed feature 2 (2.58%), C_{18:0} 3OH (1.87%) C_{17:0} (1.65%), C_{14:0} (1.46%), C_{15:0} ISO (1.31%), C_{16:1} ω6c/C_{16:1} ω7c in summed feature 3 (1.04%), and C_{13:0} ISO, C_{13:0} anteiso, C_{13:1}, C_{14:0} ISO, C_{15:0} anteiso, C_{16:0} ISO, C_{17:0} ISO, 11methyl C_{18:1} ω7c, C_{17:0} 2OH in amounts lower than 1%. These results are in agreement with those obtained for other members of family *Acetobacteraceae* that have C_{18:1} ω7c as major fatty acid (Sievers & Swings, 2005).

Analysis of respiratory quinones and polar lipids was carried out by Dr. Brian Tindall and the Identification Service and at DSMZ, from freeze dried cells using the methods described by Tindall (1990a; 1990b). The strain M1MS02 has Q-10 as major ubiquinone (75%) and Q-9 as secondary ubiquinone (25%). This result agrees with those of the remaining genera of family *Acetobacteraceae* except in the case of genus *Acetobacter* that has Q-9 as major respiratory quinone (Table 1). The strain M1MS02^T displayed a lipid profile (figure S4) consisting of diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), two aminophospholipids (PLN1-PLN2), three aminolipids (AL1-AL3), four glycolipids (GL1-GL4), two phospholipids (PL1-PL4) and a lipid (L1).

DNA for analysis of DNA base composition was prepared according to Chun & Goodfellow (1995). The mol % G+C content of DNA was determined using the thermal denaturation method (Mandel & Marmur, 1968). The G+C content of strain M1MS02^T was 60.3%. This value is within the range of family *Acetobacteraceae* members (Sievers & Swings, 2005; Greenberg *et al.*, 2006).

The phenotypic characterization was performed using the media and methods commonly used in family *Acetobacteraceae* described in Greenberg *et al.*, (2006) and Sievers & Swings (2005). Phenotypic characteristics of the new species are reported below in the species description and the differences with respect to the closest phylogenetically related genera are recorded in Table 1. The new genus differed from its closest relative genera *Granulibacter* and *Gluconacetobacter* in the flagellation and in the oxidation of acetate and lactate. From *Granulibacter* it also differs in the absence of pigmentation, in the production of dihydroxyacetone from glycerol, in the assimilation of methanol and in the production of acid from glycerol.

Therefore on the basis of phylogenetic and phenotypic tests usually performed in the family *Acetobacteraceae* we propose the strain M1MS02^T to be classified into a new genus within this family for which the name *Endobacter medicaginis* gen. nov. sp. nov. is proposed (type strain M1MS02^T, LMG 26838^T, CECT 8088^T).

Description of *Endobacter* gen. nov.

Endobacter (En.do.bac'ter. Gr. pref. endo, within; N.L. masc. n. bacter, a rod; N.L. masc. n. Endobacter, a rod isolated from the inner of a root nodule of *Medicago sativa*).

Cells are Gram-negative, motile by a subpolar flagellum and coccoid to rod-shaped. Strictly aerobic. Catalase positive. Oxidase negative. Urease negative. Colonies are white and mucoid on YMA medium. It can grow from 20°C to 37°C with an optimum temperature for growth of 28°C. Optimum pH for growth is 5.0–7 but it can grow to pH 3.5. Acetate and lactate are not oxydized. Acetic acid is produced from ethanol in presence of 2% CaCO₃ in the medium. The lipid profile consists of diphosphatidylglycerol, phosphatidylethanolamine, two aminophospholipids, three aminolipids, four glycolipids, two phospholipids and a lipid. The major fatty acids are C_{18:1} ω7c, C_{19:0} cyclo ω8c and C_{16:0}. Other fatty acids identified are C_{18:1} 2OH, C_{18:0}, C_{16:0} 2OH, C_{16:0} 3OH, C_{17:1} ω6c, C_{14:0} 3OH/ C_{16:1} ISO I in summed feature 2, C_{18:0} 3OH, C_{17:0}, C_{14:0}, C_{15:0} ISO, C_{16:1} ω6c/C_{16:1} ω7c in summed feature 3, and in lower amounts C_{13:0} ISO, C_{13:0} anteiso, C_{13:1}, C_{14:0} ISO, C_{15:0} anteiso, C_{16:0} ISO, C_{17:0} ISO, 11methyl C_{18:1} ω7c, C_{17:0} 2OH. Major ubiquinone is Q-10. Ammonium sulphate is assimilated on glucose medium. It produces dihydroxyacetone from glycerol. The type species is *Endobacter medicaginis*.

Description of *Endobacter medicaginis* sp. nov.

Endobacter medicaginis (me.di.ca'gi.nis. N.L. gen. n. medicaginis, of *Medicago*, isolated from *Medicago sativa*).

Characteristics are the same as those described for the genus. Characteristics at species level are the following: Methanol as a sole carbon source is not used. It grows on glutamate agar and mannitol agar. Acid is produced from glucose, xylose, glycerol and ethanol, but not from mannitol, sorbitol, dulcitol, lactose, sucrose or maltose. DNA base composition is 60.3% mol% G+C. The type strain is M1MS02^T (LMG 26838^T, CECT 8088^T), which was isolated from a nodule of *Medicago sativa*.

Acknowledgments

This research was funded by Junta de Castilla y León (Regional Government, Spain) under research project CSI02A09 and by MICINN (Central Government, Spain) under research project AGL2010-17380. MHRB is recipient of a JAE-Doc researcher contract from CSIC. We thank Dr. J. Euzeby for his valuable help in providing the correct etymology for the name of the new taxon, and M. Ortiz for technical assistance in electron microscopy.

References

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D.J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403-410.
- Chun, J. & Goodfellow, M. (1995). A phylogenetic analysis of the genus *Nocardia* with 16S rRNA sequences. *Int J Syst Bacteriol* **45**, 240-245.
- Chun, J., Lee, J.H., Jung, Y., Kim, M., Kim, S., Kim, B.K. & Lim, Y. W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int J Syst Evol Microbiol* **57**, 2259-2261.
- Djedidi, S., Yokoyama, T., Ohkama-Ohtsu, N., Risal, C. P., Abdelly, C. & Sekimoto, H. (2011). Stress tolerance and symbiotic and phylogenic features of root nodule bacteria associated with *Medicago* species in different bioclimatic regions of Tunisia. *Microbes Environ* **26**, 36-45.
- Doetsch, R.N. (1981). Determinative Methods of Light Microscopy. In: *Manual of Methods for General Bacteriology*, pp. 21-33. Edited by P. Gerhardt, R. G. E. Murray, R.N. Costilow, E.W. Nester, W. A. Wood, N.R. Krieg, & G. B. Phillips. Washington, American Society for Microbiology Press.
- Dong, Z., Canny, M.J., McCully, M.E., Roboredo, M.R., Fernández-Cabadilla, C., Ortega, E. & Rodés, R. (1994). A nitrogen-fixing endophyte of sugarcane-A new role for the apoplast. *Plant Physiol* **105**, 1139-1147.
- Gaunt, M.W., Turner, S.L., Rigottier-Gois, L., Lloyd-Macgilp, S.A. & Young, J.W.P. (2001). Phylogenies of *atpD* and *recA* support the small subunit rRNA-based classification of rhizobia. *Int J Syst Evol Microbiol* **51**, 2037 - 2048.
- Greenberg, D.E., Porcella, S.F., Stock, F., Wong, A., Conville, P.S., Murray, P.R., Holland, S.M. & Zelazny, A. Z. (2006). *Granulibacter bethesdensis* gen. nov., sp.

nov., a distinctive pathogenic acetic acid bacterium in the family *Acetobacteraceae*. *Int J Syst Evol Microbiol* **56**, 2609-2616.

Kan, F.L., Chen, Z.Y., Wang, E.T., Tian, C.F., Sui, X.H. & Chen, W.X. (2007). Characterization of symbiotic and endophytic bacteria isolated from root nodules of herbaceous legumes grown in Qinghai-Tibet plateau and in other zones of China. *Arch Microbiol* **188**, 103-15.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111-120.

Mandel, M. & Mamur, J. (1968). Use of ultraviolet absorbance temperature profile for determining the guanine plus cytosine content of DNA. *Methods Enzymol* **12B**, 195-206.

Lisdiyanti, P., Kawasaki, H., Widyastuti, Y., Saono, S., Seki, T., Yamada, Y., Uchimura, T. & Komagata, K. (2002). *Kozakia baliensis* gen. nov., sp. nov., a novel acetic acid bacterium in the α -Proteobacteria. *Int J Syst Evol Microbiol* **52**, 813–818.

Loganathan, P. & Nair, S. (2004). *Swaminathania salitolerans* gen. nov., sp. nov., a salt-tolerant, nitrogen-fixing and phosphate solubilizing bacterium from wild rice (*Porteresia coarctata* Tateoka). *Int J Syst Evol Microbiol* **54**, 1185–1190.

Peix, A., Rivas, R., Velázquez, E., Mateos, P.F., Martínez-Molina, E., Muñoz-Herrera, A., Gómez-Alonso, A. & Velázquez, E. (2005). Application of horizontal staircase electrophoresis in agarose minigels to the random intergenic spacer analysis of clinical samples. *Electrophoresis* **26**, 4402-4410.

Rivas, R., García-Fraile, P., Mateos, P.F., Martínez-Molina, E. & Velázquez, E. (2007). Characterization of xylanolytic bacteria present in the bract phyllosphere of the date palm *Phoenix dactylifera*. *Lett Appl Microbiol* **44**, 181-187.

- Rogers, J.S. & Swofford, D.L. (1998).** A fast method for approximating maximum likelihoods of phylogenetic trees from nucleotide sequences. *Syst Biol* **47**, 77-89.
- Saitou, N. & Nei, M. (1987).** A neighbour-joining method: a new method for reconstructing phylogenetics trees. *Mol Biol Evol* **4**, 406-425.
- Saravanan, V.S., Madhaiyan, M., Osborne, J., Thangaraju, M. & Sa, T.M. (2008).** Ecological occurrence of *Gluconacetobacter diazotrophicus* and nitrogen-fixing *Acetobacteraceae* members: their possible role in plant growth promotion. *Microb Ecol* **55**, 130-140.
- Sievers, M. & Swings, J. (2005).** Family II: Acetobacteraceae Gillis and de Ley 1980, 23^{VP}. In: *Bergey's Manual of Systematic Bacteriology*, Vol. 2, part C. pp. 41-95. Edited by D. J. Brenner, N. R. Krieg & J. T. Stanley. New York, Springer Press.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011).** MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony. *Mol Biol Evol* **28**, 2731-2739.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. & Higgins, D.G. (1997).** The clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acid Res* **24**, 4876-4882.
- Tindall, B.J. (1990a).** A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst. Appl. Microbiol.* **13**, 128-130
- Tindall, B.J. (1990b).** Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol. Letts.* **66**, 199-202
- Trujillo, M.E., Alonso-Vega, P., Rodríguez, R., Carro, L., Cerda, E., Alonso, P. & Martínez-Molina, E. (2010).** The genus *Micromonospora* is widespread in legume root nodules: the example of *Lupinus angustifolius*. *ISME J* **4**: 1265-1281.

Urakami, T., Tamaoka, J., Suzuki, K.I. & Komagata, K. (1989). *Acidomonas* gen. nov., incorporating *Acetobacter methanolicus* as *Acidomonas methanolica* comb. nov. *Int. J. Syst. Bacteriol.* **39**, 50-55.

Vincent J.M. (1970). The cultivation, isolation and maintenance of rhizobia. In: *A Manual for the Practical Study of Root-Nodule*, pp. 1-13. Edited by J. M. Vincent. Oxford. Blackwell Scientific Publications Press.

Yamada, Y., Katsura, K., Kawasaki, H., Widyastuti, Y., Saono, S., Seki, T., Uchimura, T. & Komagata, K. (2000). *Asaia bogorensis* gen. nov., sp. nov., an unusual acetic acid bacterium in the α -Proteobacteria. *Int J Syst Evol Microbiol* **50**, 823–829.

Yamashita, S., Uchimura, T. & Komagata, K. (2004). Emendation of the genus *Acidomonas* Urakami, Tamaoka, Suzuki and Komagata 1989. *Int J Syst Evol Microbiol* **54**: 865-870.

Yukphan, P., Malimas, T., Potacharoen, W., Tanasupawat, S., Tanticharoen, M. & Yamada, Y. (2005). *Neoasaia chiangmaiensis* gen. nov., sp. nov., a novel osmotolerant acetic acid bacterium in the alpha-Proteobacteria. *J Gen Appl Microbiol* **51**, 301–311.

Figure legend:

Figure 1. Maximum likelihood phylogenetic tree based on nearly complete 16S rRNA gene sequence (1492 nucleotides) of *Endobacter medicaginis* M1MS02^T and the remaining genera of family *Acetobacteraceae*. The significance of each branch is indicated by a bootstrap value (%) calculated for 1000 subsets. Bar, 2 nt substitutions per 100 nt.

0,02

Table 1. Characteristics that differentiate *Endobacter* gen. nov. from phylogenetically related and relevant genera within family *Acetobacteraceae*

Genera: 1, *Endobacter*; 2, *Granulibacter* (Greenberg *et al.*, 2006); 3, *Gluconacetobacter* (Sievers & Swings, 2005); 4, *Acetobacter* (Sievers & Swings, 2005); 5, *Gluconobacter* (Sievers & Swings, 2005); 6, *Kozakia* (Lisdiyanti *et al.*, 2002); 7, *Acidomonas* (Yamashita *et al.*, 2004; Sievers & Swings, 2005); 8, *Asaia* (Yamada *et al.*, 2000); 9, *Neoasaia* (Yukphan *et al.*, 2005); 10, *Swaminathania* (Loganathan & Nair, 2004). +, positive; 2, negative; W, weakly positive; d, delayed; nd, no data. [§]Data recorded by Yamada *et al.* (2000). For the type strains. [‡]This result was weak in Urakami *et al.* (1989). [¶]This result was weak according to Lisdiyanti *et al.* (2002). [†]This result is recorded in Loganathan & Nair (2004) and Yukphan *et al.* (2005). [¶]This result is recorded by Sievers & Swings (2005) for the type species of the genus. [£]These results are from Lisdiyanti *et al.* (2002). [‡]These results are recorded in Lisdiyanti *et al.* (2002). [§]These results are from Yupkhan *et al.* (2009).

Characteristic	1	2	3	4	5	6	7	8	9	10
Flagellation	Subpolar	Nonmotile	Peritrichous or non-motile	Peritrichous or non-motile	Polar or non-motile	Nonmotile	Polar or non-motile	Peritrichous or non-motile	Nonmotile	Peritrichous
Pigmentation	-	+	+/-	+/-	+/-	-	+/-	+/-	+	+
Oxidation of:										
Acetate	-	w	+	+	-	w	+ [‡]	+	-	w
Lactate	-	+	+ [¶]	+	-	w	-	+	-	w
Assimilation of ammonium sulfate on glucose medium*	+	w [§]	w [§]	w [§]	- [§]	w/- [§]	+	+	w	nd
Growth on:										
Glutamate agar	+	+	+ [£]	+ [£]	- [£]	-	- [£]	+	+	+
Mannitol agar	+	w	+/-	+/-	+	+	w	+	+	+
Dihydroxyacetone from glycerol	+	-	+/-	+/-	+	+	w	+/-	w	+
Utilization of methanol	-	+	-	-/W	-	-	+	-	-	-
Acetic acid from ethanol- 2.0% CaCO ₃ agar	+	w/-	+	+	+	+	+	w/-	+	+
Acid production from:										
Mannitol	-	-	+/-	+/-	+	-	-	+/-	w	-
Sorbitol	-	-	- [£]	- [£]	+	-	-	+/-	+(d)	+
Dulcitol	-	-	- [£]	- [£]	- [£]	-	-	+	w	+/-
Glycerol	+	w/-	+	-	+	+	-	+	+	+
D-Xylose	+	-	+/- [†]	+ [†]	+ [†]	+	-	+	+	+/-
Sucrose	-	-	- [†]	w [†]	+ [†]	+/-	-	+ [†]	+	nd
Major isoprenoid quinone	Q-10	Q-10	Q-10	Q-9	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10
DNA G+C content (mol%)	60.3	59	55–67	50.5–60.3	52–64	56.8–57.2	63–66	59.3–61	63.1	57.6–59.9

Figure S1. Electron micrograph of strain M1MS02^T showing the subpolar flagellum.
Bar (1cm), 1 μ m

Figure S2. Neighbour joining phylogenetic tree based on partial *recA* gene sequences (527 nucleotides) of *Endobacter medicaginis* M1MS02^T and other genera of family *Acetobacteraceae*. The significance of each branch is indicated by a bootstrap value (%) calculated for 1000 subsets. Bar, 5 nt substitutions per 100 nt.

Figure S3. Neighbour joining phylogenetic tree based on complete ITS fragment sequences (797 nucleotides) of *Endobacter medicaginis* M1MS02^T and other genera of family Acetobacteraceae. The significance of each branch is indicated by a bootstrap value (%) calculated for 1000 subsets. Bar, 5 nt substitutions per 100 nt.

Figure S4. Polar lipids profile of strain *Endobacter medicaginis* M1MS02. DPG: Diphosphatidylglycerol; PN1-PN2: Aminophospholipids; PE: Phosphatidylethanolamine; AL1-AL3: Aminolipids; GL1-GL4: Glycolipids; L1: Lipids; PL1-PL2: Phospholipid (PL1 overlaps with the amino lipids AL1 and AL2, for which its possible identity as phosphatidylglycerol could not be confirmed).