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

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A bacterial strain designated M1MS02T was isolated from a surface-sterilized nodule of *Medicago sativa* in Zamora (Spain). The 16S rRNA gene sequence of this strain showed 96.5 and 96.2 % similarity, respectively, with respect to *Gluconacetobacter liquefaciens* IFO 12388T and *Granulibacter bethesdensis* CGDNIH1T from the family *Acetobacteraceae*. The novel isolate was a Gram-stain-negative, non-sporulating, aerobic coccoid to rod-shaped bacterium that was motile by a subpolar flagellum. The major fatty acid was C18:1ω7c and the major ubiquinone was Q-10. The lipid profile consisted of diphosphatidylglycerol, phosphatidylethanolamine, two aminophospholipids, three aminolipids, four glycolipids, two phospholipids and one lipid. Strain M1MS02T was catalase-positive and oxidase- and urease-negative. Acetate and lactate were not oxidized. Acetic acid was produced from ethanol in culture media supplemented with 2 % CaCO3. Ammonium sulphate was assimilated in glucose medium. The strain produced dihydroxyacetone from glycerol. Phylogenetic and phenotypic analyses commonly used to differentiate genera within the family *Acetobacteraceae* showed that strain M1MS02T should be classified as representing a novel species of a new genus within this family, for which the name *Endobacter medicaginis* gen. nov., sp. nov. is proposed. The type strain of the type species is M1MS02T (=LMG 26838T=CECT 8088T). To our knowledge, this is the first report of a member of the *Acetobacteraceae* occurring as a legume nodule endophyte.

The family *Acetobacteraceae* comprises, at the time of writing, 30 recognized genera (http://www.bacterio.cict.fr/) that have been differentiated mainly 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 of sugar cane (Saravanan et al., 2008), and *Gluconacetobacter azotocaptans, Gluconacetobacter johnnae* and *Swaminathania salitolerans*, which are endophytic of coffee, corn and rice, respectively (Dong et al., 1994; Loganathan & Nair, 2004). However, no member of the family *Acetobacteraceae* has previously been reported as endophytes of legume nodules. These structures are induced by rhizobia, but commonly other non-nodulating endophytic bacteria are present inside the nodules, sharing this ecological niche with the bacterial endosymbionts. In the plant genus *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 *Micromonaspora* in nodules of *Medicago sativa* has been reported by Trujillo et al. (2010). However, no data are currently available regarding other alphaproteobacterial endophytes present in *Medicago sativa* nodules, and to our knowledge this is the first report of *Acetobacteraceae* occurring in legume nodules. In this work we isolated an endophytic strain, designated M1MS02T, from a root nodule of *Medicago sativa*, and show that this represents a novel species of a new genus within the family *Acetobacteraceae*.

Strain M1MS02T 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 2.5 % (w/v) HgCl2 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 modified yeast extract mannitol agar (YMA; Vincent, 1970) (per litre: 10 g mannitol, 1 g yeast extract, 0.2 g K2HPO4, 0.2 g MgSO4 · 7H2O, 0.5 g NaCl, 20 g 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 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 48 h 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 24 h incubation at 28 °C. The flagellation type was determined by electron microscopy after 48 h incubation on trypticase soy agar (TSA) at 22 °C as previously described (Rivas et al., 2007). Cells of strain M1MS02T were Gram-negative, rod-shaped, non-sporulating and motile by means of a subpolar flagellum (Fig. S1, available in IJSEM Online).

The 16S rRNA gene sequence of strain M1MS02T was analysed as described by Rivas et al. (2007), the recA gene as described by Gaunt et al. (2001) and the 16S–23S rRNA internal transcribed spacer (ITS) fragment as described by Peix et al. (2005). The sequence obtained was compared with those from the GenBank database using the BLASTN (Altschul et al., 1990) and EzTaxon-e (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012) 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 neighbour-joining and maximum-likelihood models (Saitou & Nei, 1987; Rogers & Swofford, 1998). The package MEGA5 (Tamura et al., 2011) was used for all analyses.

Comparison of the 16S rRNA gene sequence of strain M1MS02T against the EzTaxon database showed that it was phylogenetically equidistant between two genera within the family Acetobacteraceae. Its closest relatives were Gluconacetobacter liquefaciens IFO 12388T, which is the type species of this genus, and Granulibacter bethesdensis CGDNIH1T (96.3 and 96.2 % 16S rRNA gene sequence similarity, respectively). Other recognized species of the genus Gluconacetobacter showed similarities that were similar or lower. The results of the maximum-likelihood phylogenetic analysis (Fig. 1) confirmed the clustering of strain M1MS02T within the family Acetobacteraceae where this strain formed a separate branch. Congruent results were obtained when the phylogenetic tree was constructed using the neighbour-joining method (data not shown). Therefore, strain M1MS02T is considered to represent a member of a new genus within this family, in which several recently described genera show levels of 16S rRNA gene sequence similarity higher than 96 %, as for example between Granulibacter bethesdensis CGDNIH1T and Gluconacetobacter liquefaciens IFO 12388T (96.1 %). Indeed, members of some genera in the family present even higher similarity values, for example between Kozakia and the genera Gluconacetobacter, Asaia and Swaminathania, between Tanticharoenia and Ameyamaea, between Ameyamaea and Neoasaia, between Acetobacter and Neoasaia, and between Asaia and Neoasaia (>97 %), and between Swaminathania and Asaia (98 %). The results of recA gene analysis confirmed that strain M1MS02T is placed in an independent branch phylogenetically very divergent from Granulibacter bethesdensis and from the cluster formed by species of the genus Gluconobacter (Fig. S2) with similarity values lower than 83 % with respect to the remaining analysed species. The levels of recA gene sequence similarity found with respect to its closest relatives (species of the genera Gluconacetobacter and Acidiphilium) were similar to those found, for example, between the type species of the genus Acetobacter, Acetobacter aceti and several species of the genus Gluconobacter. These results support the classification of strain M1MS02T in a new genus within the family Acetobacteraceae.

16S–23S rRNA ITS analysis also showed the phylogenetic divergence of strain M1MS02T with respect to other species within the family Acetobacteraceae (Fig. S3) with similarity values lower than 60 % with respect to the remaining analysed species. These values were much lower than those found among other genera of the family Acetobacteraceae, for example for the type species of Acetobacter and Gluconobacter (84 %).

Therefore, the results of recA gene and ITS fragment analyses of strain M1MS02T agree with those from 16S rRNA gene sequence analysis, supporting the proposal that it represents a member of 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 in trypticase soy broth (Becton Dickinson, BBL) for 24 h at 28 °C and 160 r.p.m. The major fatty acids were C1<sub>18</sub>:<sub>1</sub>ω7c (39.94 %), C<sub>19</sub>:<sub>0</sub> cyclo ω8c (12.15 %) and C<sub>16</sub>:<sub>0</sub> (13.40 %). Other fatty acids identified were C<sub>18</sub>:<sub>1</sub>ω2OH (6.88 %), C<sub>18</sub>:<sub>0</sub> (4.58 %), C<sub>16</sub>:<sub>0</sub> 2OH (2.82 %), C<sub>16</sub>:<sub>0</sub> 3OH (2.73 %), C<sub>17</sub>:<sub>1</sub>ω6c (2.02 %), C<sub>14</sub>:<sub>0</sub> 3OH/iso-C<sub>16</sub>:<sub>1</sub>ωI (summed feature 2, 2.58 %), C<sub>16</sub>:<sub>0</sub> 3OH (1.87 %), C<sub>17</sub>:<sub>0</sub> (1.65 %), iso-C<sub>15</sub>:<sub>0</sub> (1.13 %) and C<sub>16</sub>:<sub>1</sub>ω6c/C<sub>16</sub>:<sub>1</sub>ω7c (summed feature 3, 1.04 %), plus iso-C<sub>13</sub>:<sub>0</sub> anteiso-C<sub>13</sub>:<sub>0</sub> C<sub>13</sub>:<sub>1</sub>, iso-C<sub>14</sub>:<sub>0</sub> anteiso-C<sub>15</sub>:<sub>0</sub> iso-C<sub>16</sub>:<sub>0</sub> iso-C<sub>17</sub>:<sub>0</sub> 11 methyl C<sub>18</sub>:<sub>1</sub>ω7c and C<sub>17</sub>:<sub>0</sub> 2OH in amounts lower than 1 %. These results are in agreement with those obtained for other members of the family Acetobacteraceae that have C<sub>18</sub>:<sub>1</sub>ω7c as the major fatty acid (Sievers & Swings, 2005).

Analysis of respiratory quinones and polar lipids was carried out by the Identification Service of the DSMZ from
freeze-dried cells using the methods described by Tindall (1990a, b). Strain M1MS02 had Q-10 as the major ubiquinone (75%) and Q-9 as a secondary component (25%). This result agrees with those for genera of the family Acetobacteraceae (except Acetobacter, which has Q-9 as the major respiratory quinone; Table 1). Strain M1MS02 displayed a lipid profile (Fig. S4) consisting of diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), two aminophospholipids (PLN1 and PLN2), three aminolipids (AL1–AL3), four glycolipids (GL1–GL4), two phospholipids (PL1–PL4) and one lipid (L1).

DNA for analysis of DNA base composition was prepared according to Chun & Goodfellow (1995). The DNA G+C content was determined using the thermal denaturation method (Mandel & Marmur, 1968). The G+C content of strain M1MS02T was 60.3 mol%. This value is within the range of members of the family Acetobacteraceae (Sievers & Swings, 2005; Greenberg et al., 2006).

Phenotypic characterization was performed using the media and methods commonly used in the family Acetobacteraceae described by Greenberg et al., (2006) and Sievers & Swings (2005). The phenotypic characteristics of strain M1MS02T are reported below in the species description and the differences with respect to its closest phylogenetically related genera are detailed in Table 1. The new genus differed from its closest related genera Granulibacter and Gluconacetobacter in flagellation and in oxidation of acetate and lactate. It also differed from

![Fig. 1. Maximum-likelihood phylogenetic tree based on the nearly complete 16S rRNA gene sequence (1492 nt) of strain M1MS02T and members of other genera of the family Acetobacteraceae. Numbers at nodes are bootstrap values (%) calculated for 1000 subsets; only values greater than 70% are shown. Bar, 2 nt substitutions per 100 nt.](image-url)
**Table 1. Characteristics that differentiate *Endobacter* gen. nov. from phylogenetically related and relevant genera within the family *Acetobacteraceae***

Genera: 1, *Endobacter* gen. nov. (data from this study); 2, *Granulibacter* (Greenberg et al., 2006); 3, *Glucoracetobacter* (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; −, negative; +/−, variable; w, weakly positive; d, delayed; ND, no data.

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*This result was weak in Urakami *et al.* (1989).
†Data from Sievers & Swings (2005) for the type species of the genus.
‡Data from Greenberg *et al.* (2006) for the type strains.
§Data from Lisdiyanti *et al.* (2002).
‖This result is recorded in Loganathan & Nair (2004) and Yukphan *et al.* (2005).
Granulibacter 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 for the family Acetobacteraceae, we suggest that strain M1MS02\textsuperscript{T} represents a novel species of a new genus within this family, for which the name *Endobacter medicaginis* gen. nov., sp. nov. is proposed.

**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 inside of a root nodule of *Medicago sativa*).

Cells are Gram-stain-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. Can grow from 20 to 37 °C with an optimum temperature for growth of 28 °C. Optimum pH for growth is 5.0–7.0 but can grow at pH 3.5. Acetate and lactate are not oxidized. Acetic acid is produced from ethanol in the presence of 2 % CaCO\textsubscript{3} in the medium. The lipid profile consists of diphostatidylglycerol, phosphatidylethanolamine, two aminophospholipids, three amnolipids, four glycolipids, two phospholipids and one lipid. The major fatty acids are C\textsubscript{18:1\textsuperscript{~}ω\textsubscript{7}}, C\textsubscript{19:0} cyclo ω\textsubscript{8}c and C\textsubscript{16:0}. Other fatty acids identified are C\textsubscript{18:1\textsuperscript{~}ω\textsubscript{3}}, C\textsubscript{18:0} C\textsubscript{16:0} 2-OH, C\textsubscript{16:0} 3-OH, C\textsubscript{17:1\textsuperscript{~}ω6c} C\textsubscript{14:0} 3-0H/iso-C\textsubscript{16:1} I (summed feature 2), C\textsubscript{18:0} 3-OH, C\textsubscript{17:0\textsuperscript{~}ω7c} C\textsubscript{15:0} iso-C\textsubscript{15:0} and C\textsubscript{16:1\textsuperscript{~}ω6c} C\textsubscript{16:1\textsuperscript{~}ω7c} (summed feature 3), and in lower amounts iso-C\textsubscript{13:0} anteiso-C\textsubscript{15:0} C\textsubscript{13:1\textsuperscript{~}ω7c} iso-C\textsubscript{14:0} anteiso-C\textsubscript{15:0} iso-C\textsubscript{16:0} iso-C\textsubscript{17:0\textsuperscript{~}ω7c} 11 methyl C\textsubscript{16:1\textsuperscript{~}ω7c} and C\textsubscript{17:0} 2-OH. The major ubiquinone is Q-10. Ammonium sulphate is assimilated on glucose medium. Produces dihydroxyacetone from glycerol. The type species is *Endobacter medicaginis*.

**Description of Endobacter medicaginis** sp. nov.

*Endobacter medicaginis* (me.di.ca’ni.s. N.L. gen. n. medicaginis of Medicago, isolated from *Medicago sativa*).

The species characteristics are the same as those described for the genus with the following additions. Methanol as a sole carbon source is not used. 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.

The type strain is M1MS02\textsuperscript{T} (=LMG 26838\textsuperscript{T} = CECT 8088\textsuperscript{T}), which was isolated from a nodule of *Medicago sativa*. The DNA G+C content of the type strain is 60.3 mol%.

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**References**


