Rhizobium cellulosilyticum sp. nov., isolated from sawdust of *Populus alba*

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During a study of polysaccharide-hydrolysing bacteria present in different plant sources, two strains were isolated from pulverized decaying wood of *Populus alba* and classified in the genus *Rhizobium* on basis of their almost complete 16S rRNA gene sequences. Their closest phylogenetic relatives were *Rhizobium galegae* USDA 4128^T and *Rhizobium huautlense* S02^T, with 98.2 and 98.1 % 16S rRNA gene sequence similarity, respectively. *recA* and *atpD* sequence analysis showed that these species have less than 88 and 92 % similarity, respectively, to the novel strains. In contrast to their closest phylogenetic relatives, the two strains showed strong cellulase activity on plates containing CM-cellulose as a carbon source. They were also distinguishable from these species on the basis of other phenotypic characteristics. The strains were able to induce ineffective nodules on *Medicago sativa* and the sequence of their *nodD* gene was phylogenetically close to that of *Ensifer meliloti* 1021 (99.6 % similarity). DNA–DNA hybridization values ranged from 10 to 22 % with respect to *R. galegae* USDA 4128^T and 14 to 25 % with respect to *R. huautlense* S02^T, showing that the strains from this study belong to a novel species, for which the name *Rhizobium cellulosilyticum* sp. nov. is proposed. The type strain is ALA10B2^T (=LMG 23642^T=DSM 18291^T=CECT 7176^T).

The species of the genus *Rhizobium* are traditionally considered as legume endosymbionts and, although *Rhizobium daejeonense* was isolated from a cyanide-treatment bioreactor (Quan *et al.*, 2005), most of them have been isolated from nodules. Decaying wood is a good source of polysaccharide-hydrolysing micro-organisms, and we have previously isolated several species, mainly actinomycetes, able to hydrolyse xylan and cellulose from similar sources (Rivas *et al.*, 2003, 2004a, b, c, d). In this work, from pulverized decaying wood of *Populus alba*, we isolated two Gram-negative strains, ALA10B2^T and ALA38.2, that

actively hydrolyse CM-cellulose *in vitro*. The 16S rRNA gene sequences of these strains allowed their classification in the genus *Rhizobium*, near to *Rhizobium galegae* and *Rhizobium huautlense*, two species that do not produce cellulases under the conditions used in this study. A polyphasic study of these strains, including phenotypic and molecular taxonomic approaches, showed that they belong to a novel species of the genus *Rhizobium*.

For isolation of strains ALA10B2^T and ALA38.2, a sample of pulverized decaying wood was collected aseptically from a cavity in the trunk of a healthy *Populus alba* tree in Salamanca, Spain. From this sample, 1 g was ground, placed in 9 ml sterile water and stirred for 60 min. Aqueous portions (100 µl of the mixture) were spread on CEA medium containing 0.7 % CM-cellulose as the only carbon source, 0.3 % yeast extract and 2.5 % agar. Colonies were picked from these plates and inoculated on the same medium. Cellulase activity was detected after 5 days incubation at 28 °C after staining with a 1 % aqueous Congo red solution. Two bacterial strains, ALA10B2^T and ALA38.2, showing strong cellulase activity, were isolated (see Supplementary Fig. S1, available in IJSEM Online).

Abbreviation: TP-RAPD, two-primers randomly amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ALA10B2^T is DQ855376, those for the *atpD* sequences of strains ALA10B2^T and ALA38.2 are AM286426 and AM286429, respectively, and those for the *recA* sequences of strains ALA10B2^T and ALA38.2 are AM286427 and AM286428, respectively.

A demonstration of cellulase activity, TP-RAPD profiles, an extended 16S rRNA gene-based neighbour-joining tree, *atpD*-, *recA*- and *nodD*-based neighbour-joining trees, images of nodules and plasmid profiles are available as supplementary material in IJSEM Online.

These strains were subjected to two-primers randomly amplified polymorphic DNA (TP-RAPD) pattern analysis, performed as described previously (Rivas *et al.*, 2002a) using primers 879F (5'-GCCTGGGGAGTACGGCCGCA-3') and 1522R (5'-AAGGAGGTGATCCANCCRCA-3'), which respectively correspond to *Escherichia coli* positions 879–898 and 1509–1522. The patterns obtained contain a band that corresponds to the fragment of the 16S rRNA gene amplified with these primers and several others produced by random amplification of the total DNA (Rivas *et al.*, 2001, 2002a). According to our previous results, bacterial strains showing identical TP-RAPD patterns belong to the same species, and strains with different TP-RAPD patterns belong to different species (Rivas *et al.*, 2001, 2002a; Zurdo-Piñeiro *et al.*, 2004). The TP-RAPD patterns of strains ALA10B2^T and ALA38.2 (Supplementary Fig. S2, lanes 1 and 2) were the same, but were different from those of strains of *R. galegae* (lane 3) and *R. huautilense* (lane 4), suggesting that they belong to the same species, but to a different species from their closest phylogenetic relatives.

Nearly complete 16S rRNA gene sequences of the strains isolated in this study were obtained according to a previously described method (Rivas *et al.*, 2002b). The sequences of strains ALA10B2^T and ALA38.2 exhibit 100 % similarity and therefore only the sequence of the strain ALA10B2^T is included in the phylogenetic tree. The sequence was compared with those held in GenBank using the BLASTN program (Altschul *et al.*, 1990). 16S rRNA gene sequences were aligned using the CLUSTAL X software (Thompson *et al.*, 1997) and distances were calculated according to the model of Kimura (1980) and the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis was based on 1000 resamplings. The MEGA2 package (Kumar *et al.*, 2001) was used for all analyses. The resulting neighbour-joining tree is shown in Fig. 1 (a phylogenetic tree including a wider selection of reference strains is available as Supplementary Fig. S3). The results indicate that the strains from this study are phylogenetically related to members of the genus *Rhizobium* within the family *Rhizobiaceae*. According to 16S rRNA gene sequence comparisons, the closest relatives of strain

ALA10B2^T are *R. galegae* USDA 4128^T, showing 98.2 % similarity, followed by *R. huautilense* S02^T, showing 98.1 % similarity.

We also determined partial *recA* (550 bp) and *atpD* (492 bp) sequences for the two new isolates using methods and primers reported previously (Gaunt *et al.*, 2001; Willems *et al.*, 2003). Phylogenetic sequence analysis was performed with the Bionumerics software (Applied Maths), including sequences of close relatives available from EMBL. Distances were calculated and clustering was performed with the neighbour-joining algorithm as described above. Bootstrap analysis was performed using 500 replications. The two strains had the same sequence for each of these genes. *recA* gene sequence analysis supports the separate phylogenetic position of the strains from this study within the genus *Rhizobium*, with *Rhizobium mongolense* USDA 1844^T (87.9 %), *R. galegae* LMG 6214^T (87.8 %) and *R. huautilense* LMG 18254^T and *Rhizobium etli* USDA 9032^T (both 87.2 %) as the most closely related strains (Supplementary Fig. S4). *atpD* sequence analysis revealed similarly low sequence similarities between strain ALA10B2^T and several species from the genera *Rhizobium* and *Ensifer*. The most similar strains were '*Sinorhizobium morelense*' LMG 21331 (91.3 %), *Ensifer adhaerens* STM 2072^T (91.1 %), *R. galegae* USDA 4128^T (90.1 %) and *R. huautilense* S02^T (88.9 %) (Supplementary Fig. S5). The low similarities found between the *recA* and *atpD* sequences of the strains from this study and those of *R. galegae* and *R. huautilense*, their closest neighbours from the 16S rRNA gene sequence analysis, indicate that these strains may represent a separate *Rhizobium* species.

The white poplar tree from which the strains from this study were isolated was located in a soil in which indigenous plants of *Medicago sativa* constituted the main herbaceous legume plant. Therefore we included this plant in nodulation tests, performed as described previously (Velázquez *et al.*, 2005), together with other plants of wide host range such as *Phaseolus vulgaris* or *Macroptilium atropurpureum*. The results obtained showed that the strains were able to nodulate *Medicago sativa* only, forming ineffective nodules (Supplementary Fig. S6).

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of strain ALA10B2^T (*Rhizobium cellulosilyticum* sp. nov.) and related organisms of the genus *Rhizobium*. The significance of each branch is indicated by a bootstrap percentage calculated for 1000 subsets. Bar, 1 substitution per 100 nucleotide positions. An extended version of this tree is available as Supplementary Fig. S3 in IJSEM Online.

The plasmid content was determined by the method of Plazinski *et al.* (1985), with the modifications described by Rivas *et al.* (2002b), using the strain *Ensifer meliloti* GR4 as a reference (Toro & Olivares, 1986). The symbiotic plasmid was identified by Southern hybridization analysis. Plasmid DNA was transferred to a nylon membrane and immobilized by baking at 80 °C for 2 h. Oligonucleotide primers were designed to amplify fragments of the *nodD* and *nifH* genes conserved among members of the family *Rhizobiaceae* as described previously (Rivas *et al.*, 2002b). PCR-amplified fragments of *nodD* were labelled with the DIG DNA Labelling kit (Roche Diagnostics) following the manufacturer's instructions and used as a probe. Hybridization was detected with the DIG nucleic acid detection kit (Boehringer Mannheim), using BCIP and NBT as substrates for alkaline phosphatase, according to the manufacturer's instructions. The two strains from this study showed identical plasmid profiles (represented in Supplementary Fig. S7, lane 2), with two plasmids of about 40 and 250 kb. The plasmid of 250 kb was identified as the pSym after hybridization with the *nodD* probe (Supplementary Fig. S7, lane 4).

A partial *nodD* gene fragment (512 bp) from strain ALA10B2^T was amplified and sequenced as described previously (Rivas *et al.*, 2002b). Comparison against sequences held in GenBank showed that the closest relative to the *nodD* gene from strain ALA10B2^T was that of *Ensifer meliloti* 1021, with 99.6 % similarity (Supplementary Fig. S8). The *nifH* gene could not be amplified using the conditions reported by Rivas *et al.* (2002b). The results of nodulation tests in alfalfa showed the formation of ineffective nodules in this legume by the strains from this study (Supplementary Fig. S6).

For base composition analysis, DNA was prepared according to Chun & Goodfellow (1995). The G + C content of DNA from strain ALA10B2^T was determined as 57 mol% using the thermal denaturation method (Mandel & Marmur, 1968). DNA–DNA hybridization was carried out by using the method of Ezaki *et al.* (1989), as described by Willems *et al.* (2001). The two novel strains showed 10–22 % relatedness to *R. galegae* USDA 4128^T and 14–25 % relatedness to *R. huautilense* S02^T (Supplementary Table S1). These results indicate that strains ALA10B2^T and ALA38.2 do not belong to either of these species when the recommendation of a threshold value of 70 % DNA–DNA relatedness for species definition is considered (Wayne *et al.*, 1987).

Phenotypic characterization of the strains from this study was based on growth with different carbon sources and resistance to different antibiotics, as described by Wang *et al.* (1998), and by using API 20NE according to the manufacturer's instructions (bioMérieux). The temperature range for growth was determined by incubating cultures in YMA medium at 4 to 40 °C. The pH range was determined in the same medium with a final pH between 4.0 and 10.0. Salt tolerance was studied in YMA medium containing 0–5 % (w/v) NaCl. Cellulase activity was detected after 5 days incubation at 28 °C on plates containing CEA medium as

explained above. The type strains of *R. galegae* and *R. huautilense* were used as references.

Slight phenotypic differences were observed between the two strains from this study, as shown in Table 1, which also includes results for *R. galegae* and *R. huautilense*. The strains from this study differ from *R. galegae* in cellulase production, growth in the presence of 3 % NaCl, use of L-methionine as a carbon source and resistance to erythromycin and ampicillin. They differ from *R. huautilense* in cellulase production, growth at 4 °C and pH 9, growth in 3 % NaCl and resistance to kanamycin and erythromycin. Therefore, the new group can be differentiated genotypically and phenotypically from previously described species and we therefore propose to name it *Rhizobium cellulosilyticum* sp. nov.

Description of *Rhizobium cellulosilyticum* sp. nov.

Rhizobium cellulosilyticum (cel.lu.lo.si.ly'ti.cum. N.L. n. *cellulosum* cellulose; N.L. neut. adj. *lyticum* dissolving from Gr. adj. *lytikos* able to loose, able to dissolve; N.L. neut. adj. *cellulosilyticum* cellulose-dissolving).

Gram-negative rods, as for other species of the genus. Colonies are small and pearl white in YMA at 28 °C, the optimal growth temperature. The optimum pH is 7–7.5. Grows at 4–37 °C, pH 6–8 and 0–4 % (w/v) NaCl. Strains of this species actively produce cellulases on media containing

Table 1. Differential phenotypic characteristics between the strains from this study and the type strains of the phylogenetically closest species

Strains: 1, *R. galegae* USDA 4128^T (unless indicated, data from Lindström, 1989); 2, *R. huautilense* S02^T (unless indicated, data from Wang *et al.*, 1998); 3, strain ALA10B2^T (*R. cellulosilyticum* sp. nov.); 4, strain ALA38.2 (*R. cellulosilyticum* sp. nov.).

Characteristic	1	2	3	4
Growth at/in:				
4 °C	+	–	+	+
40 °C	–	+	–	–
pH 9	–	+	–	–
3 % NaCl	–	–	+	+
Hydrolysis of CM-cellulose	–	–	+	+
Urease	+	+	+	w
Assimilation of:				
Maltose	+	+	+	w
Methionine	–	+	+	+
Resistance to (µg ml ^{–1}):				
Ampicillin (5)	–	+	+	+
Kanamycin (5)	–	+	–	–
Erythromycin (5)	+	+	–	–

*Data from the type strains obtained in this study.
†After 5 days incubation, the strains formed 1 cm lysis ‘haloes’.

CM-cellulose as substrate. Nitrate reduction is negative. Produces β -galactosidase and hydrolyses aesculin in API 20NE. Production of urease is positive in strain ALA10B2^T but weak in strain ALA38.2. Does not produce indole, arginine dihydrolase or gelatinase in the same system. Utilizes glucose, L-arabinose, mannose, mannitol, N-acetylglucosamine and malate as carbon sources. Maltose is used as a carbon source, but growth of strain ALA38.2 is weak. Grows on glutamate and methionine as carbon and nitrogen sources. Does not grow on gentiobiose, caprate, adipate, citrate or phenylacetate. Known strains are resistant to 5 μ g ampicillin ml⁻¹ and 50 μ g chloramphenicol ml⁻¹ and sensitive to 5 μ g erythromycin and kanamycin ml⁻¹. The G + C content of the type strain is 57 mol%.

The type strain ALA10B2^T (=LMG 23642^T=DSM 18291^T=CECT 7176^T) and reference strain ALA38.2 (=LMG 23643) were isolated from sawdust of *Populus alba* in Spain.

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