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Phaseolus vulgaris seed-borne endophytic community with novel bacterial species such as *Rhizobium endophyticum sp. nov.*

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ABSTRACT

The bacterial endophytic community present in different *Phaseolus vulgaris* (bean) cultivars was analyzed by 16S ribosomal RNA gene sequences of cultured isolates derived from surface disinfected roots and immature seeds. Isolated endophytes from tissue-macerates belonged to over 50 species in 24 different genera and some isolates from *Acinetobacter*, *Bacillus*, *Enterococcus*, *Nocardioides*, *Paracoccus*, *Phyllobacterium*, and *Sphingomonas* seem to correspond to new lineages. Phytate solubilizing bacteria were identified among *Acinetobacter*, *Bacillus* and *Streptomyces* bean isolates, phytate is the most abundant reserve of phosphorus in bean and in other seeds. Endophytic rhizobia were not capable of forming nodules. A novel rhizobial species *Rhizobium endophyticum* was recognized on the basis of DNA–DNA hybridization, sequence of 16S rRNA, *recA*, *rpoB*, *atpD*, *dnaK* genes, plasmid profiles, and phenotypic characteristics. *R. endophyticum* is capable of solubilizing phytate, the type strain is CCGE2052 (ATCC BAA-2116; HAMBI 3153) that became fully symbiotic by acquiring the *R. tropici* CFN299 symbiotic plasmid.

Introduction

Microbial communities seem to be determined by the niches [13,28], thus inside a particular plant species there must be a selection of bacteria that are adapted to the conditions in the plant. By definition, endophytic bacteria and fungi reside inside plant tissues without causing evident harm. Endophytes may be – and often are – beneficial to their host plants [8,9,34]. Different bacterial species have been found as endophytes and some promote plant growth by supplying hormones, by stimulating plant defense responses or exerting antagonism towards plant pathogens [29,30,32] specially xylem borne pathogens [27]. Many endophytes have antifungal properties [28].

In legumes few studies on bacterial endophytes have been conducted, although soybean associated bacteria, clover and pea endophytes have been reported [4,14,18,19,20,36]. Seed-borne endophytes may be transmitted vertically from generation to generation and plants inheriting beneficial symbionts may have an ecological advantage. Plant "hosts may reduce the transmission of more pathogenic symbionts to seeds" [33]. Seed endophytes may spread as seeds disperse. "Not much information is available about plant beneficial endophyte bacteria isolated from seeds" [24]. In *Phaseolus vulgaris* seeds abundant bacilli were obtained from spermosphere [42] and ovules but a molecular characterization was not performed [26]. While bean nodule rhizobial diversity has been largely studied [22], little is known of the endophytic community in *P. vulgaris*. This study was performed in one of the sites of origin of bean and using different bean cultivars and seed isolates. *P. vulgaris* is a very convenient species to study seed-borne endophytes as surface disinfected pods may be easily dissected to detach seeds, and immature seeds are easily macerated.

Materials and methods

Isolation of bean endophytes

Phaseolus vulgaris (bean) cultivar BAT 477 has low phosphate requirements and is efficient for nitrogen fixation. DOR 364 is phosphate inefficient [1,25]. Seeds from Mesoamerican bean cultivars BAT 477 and DOR 364 were obtained from CIAT, Centro Internacional de Agricultura Tropical in Cali, Colombia [1]. Seeds from Mesoamerican cultivar Negro Xamapa were from Pronase, Productora Nacional de Semilla, Mexico. Bean seeds were surface sterilized with serial washes of 70% ethanol for 5 min, 1.5% sodium hypochlorite for 15 min and several rinses with distilled water over a 20 min period.

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After surface disinfection, germinated bean seeds were transferred into 250 ml Erlenmeyer flasks (covered with sterile foam) containing 220 ml of Fahraeus solution [5] solidified with 0.75% agar or into pots with sterilized vermiculite watered with Fahraeus solution. Roots were maintained in sterile conditions. Plants were kept in a plant growth chamber at 28 °C with a photoperiod of 12 h light/12 h dark.

Roots from 3-day-old and 12-day-old bean seedlings as well as immature bean seeds from developing pods were sampled. Roots of 3-day-old plants were surface sterilized with serial washes of 70% ethanol for 1 min, 1.5% sodium hypochlorite for 3 min, two times with sterile distilled water for 3 min, 2% sodium thiosulphate for 30 s and then rinsed several times with distilled water. 1.5% sodium hypochlorite for 6 min was used instead for roots of 12-day-old plants. Green bean pods of 4–7 cm size were subjected to indirect sonication for 15 min in sterile water to release any attached microbes, then rinsed in 70% ethanol before surface disinfection in hypochlorite. The disinfection process for bean pods was as described above for roots, but using 1.5% sodium hypochlorite for 15 min. Tests for bacterial contamination of plant samples were made after the surface disinfection protocol by plating water from the final rinsings or placing the pods or seeds on LB [37] and yeast-mannitol (YM) [40] media plates. These tests for contamination were consistently negative, indicating an adequate surface disinfection of pods and seeds.

Roots or immature seeds were homogenized with a sterile mortar and pestle. Serial dilutions were plated onto LB and YM agar containing 10 μg ml^{-1} cycloheximide. LB and YM medium allowed the recovery of a large number of morphologically distinct bacteria. Plates were incubated at 28 °C for up to 20 days to recover endophytes that were selected based on different morphologies.

PCR amplification, ARDRA, sequencing, analysis of bacterial genes and G+C content

Bacteria were grown on LB or YM plates for DNA extraction. Bacterial DNA was obtained according to the procedure for the Genomic Prep cell and tissue DNA isolation Kit (Amersham) with one modification: in addition to RNase, 5 μl of protease (Sigma, 50 mg ml^{-1}), 5 μl of lysozyme (10 mg ml^{-1}) and 5 μl of 10% SDS were added and the samples were incubated at 37 °C for 45 min.

Primers fD1 and rD1 [43] were used for PCR amplification of the 16S rRNA gene as described [21]. DNA restriction (ARDRA) was performed with *Msp*I, *Hinf*I, *Hha*I, *Sau*3AI and *Dde*I and patterns were observed in agarose gels by electrophoresis as described [41]. The percent identity of almost complete 16S rRNA gene sequences to the closest type strain in GenBank database was determined with BLASTN. New phylotypes were identified by having 16S rRNA gene identities equal or lower than 97% [17] to the type strain from the closest reported species (Supplementary Table S1 and Fig. S1).

recA primers were from Gaunt et al. [6], *rpoB* primers were from Khamis et al. [16]. Primers for *dnaK* and *atpD* were dnaK-RS-F (5′-CACSACGATCCCGACSAA-3′) and *dnaK*-RS-R (5′-TCGTAGTCGGCRTCGACSAC-3′); and *atpD*-F (5′-TCGGCGCCGTCGTSGAC-3′) and *atpD*-R (5′-RGCTTCCGGCAGRTK RTCGTA-3′) that amplify fragments corresponding to positions 1230–1880 of the *dnaK* gene and 47–1362 of the *atpD* gene from *R. etli* CFN42. Primers for *nodB* and *nifH*, and amplification conditions were as described [21].

For phylogenetic analysis, sequences were aligned with ClustalW, and trees were constructed with the neighbor-joining (NJ) method using Tamura-Nei distances with the MEGA 4 software, or by the maximum-likelihood (ML) method using the PhyML program. Bootstrap analysis was done by using 1000 (NJ) or 100 (ML) pseudoreplications.

nod, *nif* and other genes were searched in the genome of CCGE 2052 obtained by the Illumina sequencing technology using the Illumina Genome Analyzer II at the UNAM Cuernavaca sequencing facility. The G+C content was calculated from the assembled genome with BioEdit. The analysis of the genome sequence will be published elsewhere.

DNA–DNA hybridization

Hybridization was performed as we have described before [21,23,37,41]: DNA was quantified with the Nanodrop 2000 spectrophotometer and 2 μg digested with *Eco* RI restriction enzyme, after gel electrophoresis the DNA was blotted to nylon filters that were prehybridized with Rapid-hyb buffer (GE, UK). DNA probes were labeled with ^{32}P dCTP using the Rediprime II Amersham kit (GE, UK). Radioactivity was counted and around 1 million dcp were used per lane. Southern blot hybridization was performed at 65 °C for 16 h, washings were at 65 °C with SSC 2X and SSC 1X twice each. Filters were counted in a liquid scintillation counter and the homologous hybridization was referred as 100%.

Solubilization of phytate and other phenotypic assays

Halo forming strains were considered as positive in qualitative assays for phytase production. Phytase-screening media contained (per liter): 2.1 g MOPS pH 7, 3.34 g NH_4Cl, 0.5 g $MgSO_4{\cdot}7H_2O$, 10 mg $FeSO_4{\cdot}7H_2O$, and 10 mg $4MnSO_4{\cdot}H_2O$. After autoclaving, 100 ml 20% glucose, 100 ml 4% phytate (inositol hexaphosphoric acid sodium salt from corn), and 10 ml 20% $CaCl_2$ were added [38]. Phytate was tested as a carbon source as described [39]. Plates without phytate were used as controls. Plates were incubated at 29 °C for up to 5 days and triplicates were tested for each isolate. Assays to evaluate resistance to antibiotics, acidity, high temperatures and growth in different media were as described [23,41].

Plasmid transfer and inoculation assays

R. tropici CFN 299 Tn*5mob*6 was used as donor to transfer the symbiotic plasmid to *R. endophyticum* CCGE 2052 as described [31], transconjugants were selected in PY medium with Cb (50 mg l^{-1}) and Nm (60 mg l^{-1}). Plasmid patterns were determined by a modified Eckhardt procedure [11]. Bean, *Macroptilium atropurpureum*, *Vigna* sp., *Leucaena leucocephala* and *Mimosa affinis* were inoculated with CCGE 2052 and the transconjugant as described in Rogel et al. [31].

GenBank accession numbers

The 16S rRNA gene sequences determined in this study have been deposited in the GenBank database under accession numbers (EU867297–EU867387, EU938359). The 3 *atpD*, 4 *dnaK*, 1 *recA* and 1 *rpoB* gene sequences from CCGE 2052 and other rhizobial type strains have been assigned accession numbers HM142760 to HM142768.

Results and discussion

Diverse Phaseolus vulgaris endophytes

Bacteria were recovered from 32 surface disinfected roots of plants grown under gnotobiotic conditions and from 13 immature seeds from 6 different surface disinfected developing pods. From 318 isolates having different morphologies, 99 isolates were selected based on differences in restriction enzyme patterns of *rrs* genes by ARDRA (not shown). The 99 isolates were identified by sequencing 16S rRNA gene PCR products and classified in 3 phyla,

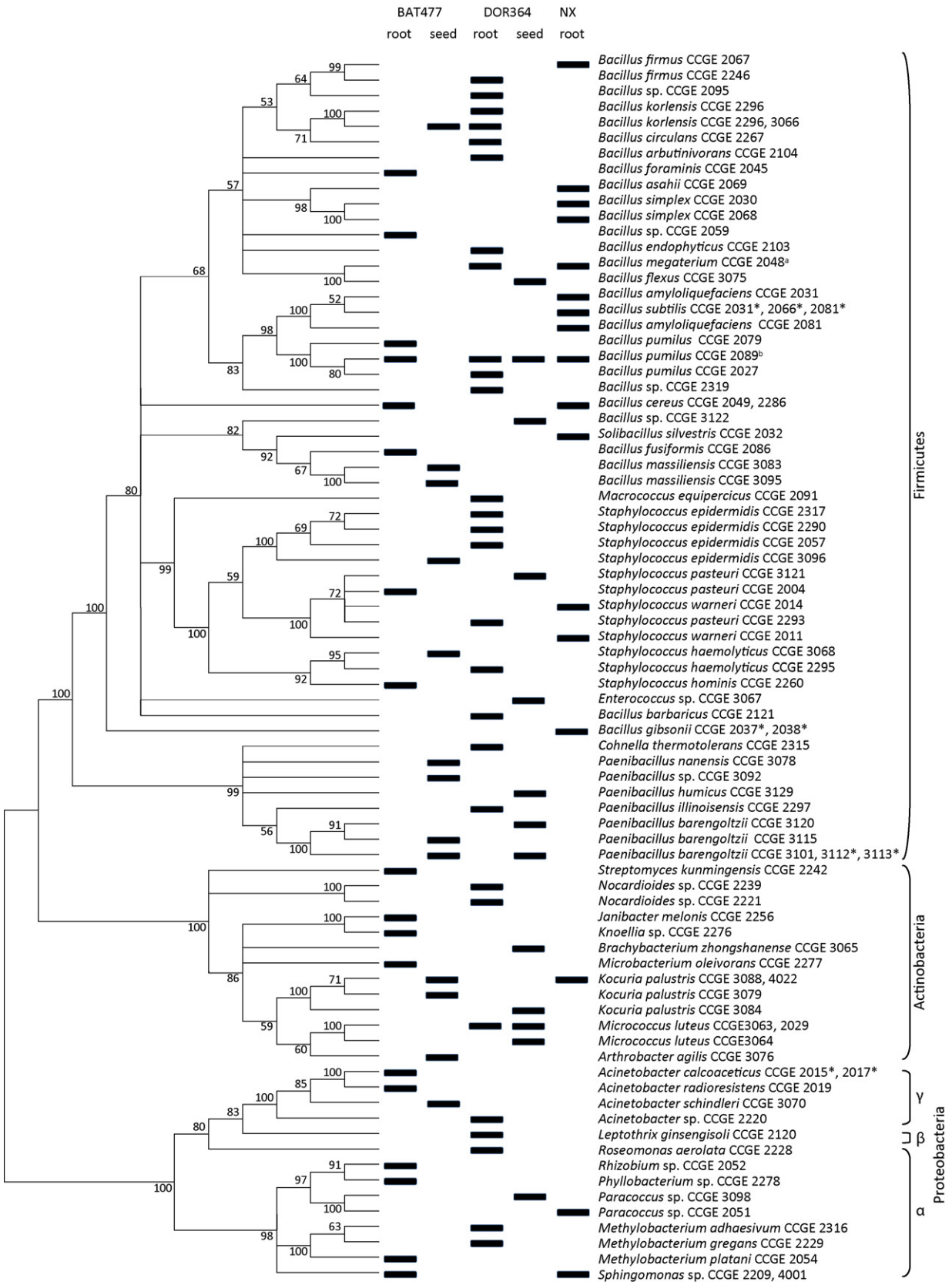

Fig. 1. Neighbor-joining phylogenetic tree of 16S rRNA gene sequences from the bacteria obtained as bean endophytes. Bars indicate presence of endophytes in different bean cultivars. *Indicates that isolates were from the same cultivar and stage. Isolates whose numbers start with 3 are from seeds. [a]Isolates corresponding to *Bacillus megaterium* were CCGE 2048 and CCGE 2050 from Negro Xamapa roots, CCGE 2105 and CCGE 2187 from DOR364 roots. [b]Isolates corresponding to *Bacillus pumilus* were CCGE 2005, CCGE 2028, and CCGE 2077 from DOR364 roots, CCGE 2080 from Negro Xamapa roots, CCGE 2083 and CCGE 2089 from BAT477 roots, and CCGE 3123 from DOR364 seed.

24 genera and over 50 species (Fig. 1, Supplementary Table S1 and Fig. S1, repeated identical sequences are not shown). Sequences corresponding to Firmicutes, Actinobacteria, and α-, γ- and β-Proteobacteria were obtained from inside root tissues and seeds.

Bacillus and the related *Staphylococcus* as well as *Paenibacillus*, *Micrococcus* and *Methylobacterium* were isolated from many of the bean samples tested (Fig. 1). Bacteria belonging to the genus *Bacillus* were recovered from the three bean cultivars analyzed. *Bacillus* isolates were the most diverse bean endophytes in number of species (Fig. 1, Supplementary Table S1 and Fig. S1).

Some isolates such as those from *Enterococcus*, ***Nocardioides***, *Roseomonas*, ***Leptothrix, Cohnella, Rhizobium***, ***Phyllobacterium***, ***Microbacterium***, ***Janibacter***, ***Knoellia***, ***Macrococcus***, *Brachybacterium* and ***Streptomyces*** were obtained from only certain bean cultivars (Fig. 1) and not in all plants sampled. All these were found in low numbers and those in bold were not found in seeds, but the seed sample was smaller than the root sample. Endophytic species were found unequally distributed in bean individual plants even from the same cultivar and age (not shown). *Streptomyces* (known for producing antibiotics, vitamins and other metabolites) were found only in bean cultivar BAT 477, but as differences among individual plants or seeds from the same cultivar were as large as among cultivars, no conclusions can be drawn on cultivar preferences for endophytic species.

Many of the bacterial genera encountered in bean have been previously reported as endophytes in different plants and some have shown clear beneficial effects [3,32], but many of the species are not the same. Bean is closely related to soybean, however, the bean endophytic community is distinct as the only bacteria in common in both legumes belonged to the genera *Lysobacter*, *Acinetobacter*, *Methylobacterium*, and rhizobia. In contrast, similar *Bacillus*, *Staphylococcus*, *Paenibacillus* but other *Methylobacterium* spp. strains were found in *Medicago truncatula* as seed endophytes (our unpublished results).

The isolation of bacteria from immature seeds obtained from fresh, surface sterilized bean pods that had not opened (Supplementary Fig. S2) strongly supported the notion that the isolates are *bona fide* endophytes and seed-borne. Surface disinfection control tests (showing no contaminating surface bacteria) support this notion. There was not a single bean seed without bacteria. Root isolates from plantlets grown in sterile conditions from surface disinfected seeds are presumably seed-borne. Except for *Arthrobacter*, *Brachybacterium* and *Enterococcus*, all genera obtained as seed isolates (from pods) were recovered as well from bean roots suggesting that seed-borne bacteria were able to multiply and persist in germinated plants.

Phytate assimilation

In plates with phytate from all 99 isolates tested, 13 *Bacillus* and 3 *Acinetobacter* isolates, *Streptomyces kunmingensis* CCGE 2242 and *Rhizobium endophyticum* CCGE 2052 formed a characteristic halo observed in phytate using bacteria being able to grow using phytate as phosphorus (P) source. Most bacteria with positive phytase activity (indicated by asterix in Supplementary Table 1) were isolated from 12-day roots of Negro Xamapa and BAT477 cultivars. Strains of *Bacillus*, *Stenotrophomonas*, *Agrobacterium rhizogenes*, *Klebsiella* and *Burkholderia* are capable of solubilizing phytate [15,38,39] while *Acinetobacter*, *Rhizobium* and *Streptomyces* were not known to solubilize phytate [35]. The role of phytate catabolism in plant–endophyte interactions has not been explored. During germination, seed endophytes must be able to proliferate with seed nutrients such as phytate, which is the main storage form of P in many seeds especially in cereals and legumes such as bean with up to 72% of P as phytate [2]. Seed endophytes may help in seed germination and supposedly germination declines as bacteria die [10]. The extracellular phytase activity of *Bacillus amyloliquefaciens* FZB45 contributes to its plant growth-promoting effect [12].

New species

Some isolates from *Acinetobacter*, *Bacillus*, *Enterococcus*, *Nocardioides*, *Paracoccus*, *Phyllobacterium*, and *Sphingomonas* seem to correspond to new lineages (Supplementary Fig. S1 and bold in Supplementary Table S1). It seems reasonable to encounter new species of endophytes in seeds from plants not previously analyzed as different plant species represent unique niches and there should be a selection of seed bacteria to be transmitted to new plant generations. We analyzed the bacterial endophytic community present in the most widely consumed grain legume for human nutrition

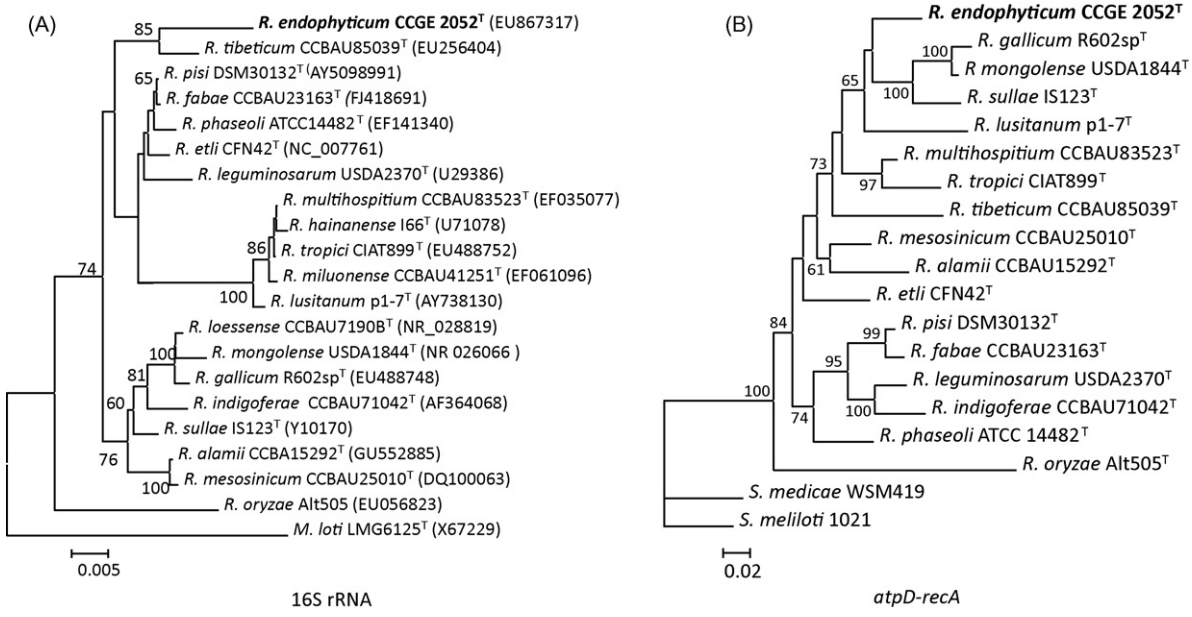

Fig. 2. Maximum-likelihood phylogenetic trees of *Rhizobium* type and reference strains related to *Rhizobium endophyticum* based on the sequences of (A) 16S rRNA and (B) *recA* and *atpD* concatenated genes.

Table 1
Distinctive phenotypic characteristics of *Rhizobium endophyticum* and related type strains.

Characteristic	*R. endophyticum* CCGE 2052	*R. gallicum* R602sp	*R. tibeticum* CCBAU 85039	*R. etli* CFN42	*R. tropici* CIAT 899
Growth in/at					
LB	+	–	–	–	+
PY no Ca[a]	–	–	–	–	+
PY 37°	–	+	–	+	+
PY pH 4.5	+	–	–	–	+
PY NaCl 0.25M (1.5%)	+	w	–	–	+
PY NaCl 0.5M (3.0%)	+	–	–	–	+
PY Cb[b]	+	+	–	–	–
PY Cm[b]	+	–	–	–	+
Phytate degradation	+	–	–	–	+
Swimming in agar (0.3%)	–	–	+	+	+
Utilization of carbon source					
Arginine	+	+	–	+	+
Asparagine	+	+	–	w	+
Citrate	+	–	–	–	+
Proline	+	+	–	+	+
Phytate	+	–	+	–	+
Utilization of nitrogen source:					
Asparagine	+	+	+	–	+
Arginine	+	w	–	+	+
Phaseolus vulgaris nodulation	–	+	+	+	+

+, positive; –, negative; w, weakly positive result.
[a] Calcium.
[b] Cb = carbenicillin 50 μg ml^{-1}; Cm = chloramphenicol 25 μg ml^{-1}.

and identified novel bacteria supposedly well adapted for bean that could be evaluated for their use as bean inoculants (not considering the human pathogens or opportunistic pathogens such as *Staphylococcus haemolyticus* and *Acinetobacter calcoaceticus*) together with rhizobia.

The rhizobial strain endophyte CCGE 2052, isolated from 12-day-old *Phaseolus vulgaris* roots was further characterized because its 16S ribosomal RNA gene sequence did not correspond to any described rhizobial species (Fig. 2A). CCGE 2052 represented a distinct branch in individual *dnaK*, *recA*, *atpD*, and *rpoB* gene phylogenies (Supplementary Fig. S3) and in the concatenated *recA* + *atpD* phylogeny (Fig. 2B). Phenotypic characteristics distinguishing CCGE 2052 from related species are shown in Table 1 and DNA–DNA hybridization results are shown in Table 2. *Rhizobium* strain CCGE 2052 did not form nodules in beans, neither in the highly promiscuous hosts *Macroptilium atropurpureum* (siratro) and *Vigna* sp. nor in *Leucaena leucocephala* or *Mimosa affinis* plants, additionally *nodB* and *nifH* genes were not detected by PCR. Furthermore *nifH*, *nifD* and *nodDABC* genes were not found in the genome sequence of CCGE 2052 but *recA*, *atpD*, *dnaK*, *rpoB* and 16S rRNA genes were found in the genome with identical sequences as those reported here. CCGE 2052 transconjugant that acquired the *R. tropici* symbiotic plasmid (Supplementary Fig. S4) forms nitrogen-fixing nodules on beans. The other Rhizobiales bacteria isolated as bean endophyte, *Phyllobacterium* sp. CCGE 2267 (probably corresponding to a new species of *Phyllobacterium*, Supplementary Table S1) did not form nodules on bean in plant nodulation assays.

Table 2
Average DNA–DNA hybridization of *Rhizobium endophyticum* CCGE 2052 and related type strains.

	Strain	% and standard error
Rhizobium endophyticum	CCGE 2052^T	100
R. mongolense	USDA1844^T	13.6 ± 2
R. gallicum	R602sp^T	16.4 ± 3
R. multihospitium	CCBAU 83523^T	16.1 ± 3
R. tropici	CIAT 899^T	12.6 ± 6
R. tibeticum	CCBAU 85039^T	16.9 ± 5
R. mesosinicum	CCBAU 25010^T	16.9 ± 2
R. etli	CFN42^T	17.0 ± 0

Average of three hybridizations per strain.

Description of Rhizobium endophyticum sp. nov.

Rhizobium endophyticum (en.do.phy'ti.cum. Gr. pref. endo, within; Gr. n. phyton, plant; L. neut. suff. -icum, adjectival suffix used with the sense of belonging to; N.L. neut. adj. endophyticum, endophytic, within plants, pertaining to the original isolation from plant tissues). It was isolated from surface disinfected roots of *Phaseolus vulgaris* beans grown in the laboratory. It grows in LB medium, YM, PY and minimal medium [7] and uses arginine, asparagine, citrate, proline and gluconate as carbon sources and asparagine and arginine as N sources. In PY medium it forms pearly regular colonies typical of rhizobia, in YM colonies are semi-translucent and gummy. Duplication time is 2.25 h in PY medium. It grows in NaCl 3%, producing a biofilm in liquid medium and a yellow substance. CCGE 2052 is resistant to nalidixic acid (20 mg l^{-1}), carbenicillin (50 mg l^{-1}), and chloramphenicol (25 mg l^{-1}). It does not grow at 37 °C in PY medium nor in PY without calcium. It has the capacity to solubilize phytate and use it as a P and C source; it is not motile in soft agar (0.3%). *R. endophyticum* is distinguished by the nucleotide sequence of 16S rRNA, *recA*, *rpoB*, *atpD* and *dnaK* genes (Fig. 2 and Supplementary Fig. S2). DNA–DNA hybridization was low to all related species (Table 2). Strain CCGE 2052 (ATCC BAA-2116; HAMBI 3153) is the type strain isolated in Cuernavaca, Mexico from roots of the phosphate efficient cultivar BAT 477 of *Phaseolus vulgaris* bean. The DNA G+C content of the strain is 61.23%.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.syapm.2010.07.005.

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