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Sinorhizobium americanum symbiovar mediterranense is a predominant symbiont that nodulates and fixes nitrogen with common bean (*Phaseolus vulgaris* L.) in a Northern Tunisian field

Bacem Mnasri, Sabrine Saïdi, Saif-Allah Chihaoui, Ridha Mhamdi*

Laboratory of Legumes, Centre of Biotechnology of Borj-Cédria, BP 901, Hammam-lif 2050, Tunisia

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ABSTRACT

A total of 40 symbiotic bacterial strains isolated from root nodules of common bean grown in a soil located in the north of Tunisia were characterized by PCR-RFLP of the 16S rRNA genes. Six different ribotypes were revealed. Nine representative isolates were submitted to phylogenetic analyses of *rrs*, *rec*A, *atp*D, *dna*K, *nif*H and *nod*A genes. The strains 23C40 and 23C95 representing the most abundant ribotype were closely related to *Sinorhizobium americanum* CFNEI 156^T. *S. americanum* was isolated from *Acacia* spp. in Mexico, but this is the first time that this species is reported among natural populations of rhizobia nodulating common bean. These isolates nodulated and fixed nitrogen with this crop and harbored the symbiotic genes of the symbiovar mediterranense. The strains 23C2 and 23C55 were close to *Rhizobium gallicum* R602sp^T but formed a well separated clade and may probably constitute a new species. The sequence similarities with *R. gallicum* type strain were 98.7% (*rrs*), 96.6% (*rec*A), 95.8% (*atp*D) and 93.4% (*dna*K). The remaining isolates were, respectively, affiliated to *R. gallicum*, *E. meliloti*, *Rhizobium giardinii* and *Rhizobium radiobacter*. However, some of them failed to re-nodulate their original host but promoted root growth.

Introduction

Common bean (*Phaseolus vulgaris* L.) represents the most important source of proteins for low-income population in Latin America and Africa (Ribeiro et al. 2009). However, this legume is considered as a poor nitrogen-fixer pulse in comparison to other grain legumes (Hardarson 1993). This problem is generally attributed to the ineffectiveness of the native rhizobia (Aouani et al. 1997; Thies et al. 1991) or to adverse abiotic conditions (Mnasri et al. 2007a; Sessitsh et al. 2002). A remarkable attribute of common bean is its ability to nodulate with diverse genera of rhizobia. Most reported phylogenies of rhizobia nodulating *P. vulgaris* have placed them in the genus *Rhizobium* (Aserse et al. 2012; Martinez-Romero 2003; Valverde et al. 2006; Wang et al. 2011). It seems that strains outside this genus formed always ineffective symbiosis with beans (Bromfield and Barran 1990; Mhamdi et al. 2002; Michiels et al. 1998). However, some strains belonging to the genus *Ensifer* (*E. fredii* and *E. meliloti*) formed also effective symbiosis with this crop (Herrera-Cervera et al. 1999; Mnasri et al. 2007b; Zurdo-Piñeiro et al. 2009).

In Tunisia, common bean was shown to be efficiently nodulated by several species including *Rhizobium leguminosarum*, *Rhizobium gallicum*, *Rhizobium giardinii* and *Rhizobium etli* (Mhamdi et al. 2002). Nevertheless, nitrogen-fixing symbioses were also described for some *Ensifer meliloti* and *Ensifer fredii* strains for which the symbiovar mediterranense was attributed (Mhamdi et al. 2002; Mnasri et al. 2007b). It is noteworthy that the distribution of the different species in Tunisia highly varied according to the geographic location and also according to bean cultivation history. Nitrogen-effective isolates were recovered only from sites with a record of bean cultivation. However, in the other sites, common bean was poorly nodulated by opportunistic strains of *E. meliloti* and *Ensifer medicae* (Mhamdi et al. 2002). In Cap Bon and Bizerte regions, the soils are neutral and bean had been extensively cultivated. However, differences in the distribution of the bean nodulating rhizobial species between the two regions were observed, mainly with regard to the relative abundance of the two species, *R. etli* and *R. leguminosarum* (Mhamdi et al. 2002). By contrast, other species, such as *R. gallicum* and *E. fredii*, were recovered from both regions. From the other side, gaps of information still exist about the geographic distribution of bean nodulating species,

* Corresponding author. Tel.: +216 79325948; fax: +216 79325948.
E-mail addresses: ridha.mhamdi@cbbc.rnrt.tn, ridhamhamdi@yahoo.fr (R. Mhamdi).

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mainly with regard to the shape of species diversity in a single site.

The aim of this work is to study the genetic diversity of bean nodulating rhizobia in a single site located in the North of the country in the region of Bizerte.

Materials and methods

Rhizobia isolation

Soil samples were collected from three 50 m-distant-points in a field localized in the north of Tunisia (Azib, 37°12′24″N, 9°56′53″E). The soil was non-saline and slightly alkaline and had been frequently cultivated with common bean. The soil samples were homogenized to one sample and transferred to 5 kg plastic pots. Sterilized common bean seeds (cv. coco) were grown in the soil samples in the greenhouse. Isolation of rhizobia from root nodules was carried out according to standard procedures as previously described (Mhamdi et al. 1999). A total of 40 rhizobial strains were isolated from root nodules of four different plants. One isolate was retained from each nodule. Rhizobial strains were stored in 20% glycerol at −80 °C for further analysis.

PCR-RFLP of chromosomal and symbiotic regions

Bacterial DNAs of the 40 rhizobial strains were extracted as previously described (Mhamdi et al. 2002) and used as templates for 50 μl PCR reactions. The 16S rRNA genes were amplified using primers fD1 and rD1 and digested with *Msp*I and *Nde*II as previously described (Mhamdi et al. 2002).

Sequence analysis

The 16S rRNA genes of the strains 23C2, 23C40, 23C45, 23C55, 23C59, 23C84, 23C88, 23C95 and 23C99 representing all the ribotypes identified by PCR-RFLP were sequenced using primers fD1 and rD1. A 508 bp *rec*A gene and a 507 bp *atp*D gene fragment of these strains were amplified using, respectively, the primers recA-41F and recA-640R, atpD-255F and atpD-782R according to Islam et al. (Islam et al. 2008). A nearly 300 bp *dna*K fragment of the strains 23C2, 23C40, 23C49 and 23C55 was amplified using the primers dnak1466Fd and dnak1777Rd according to Martens et al. (2007). The *nif*H and *nod*A genes of the strains 23C2, 23C40, 23C55, 23C59, 23C84 and 23C95 were amplified using, respectively, $nifH_i$ and $nifH_f$ as previously described (Mhamdi et al. 2002) and nodA1 and nodA2 according to Haukka et al. (1998). PCR-amplified products were purified from agarose gels using the EZ-10 spin column DNA gel extraction kit (Bio Basic Inc.) following the manufacturers' instructions. Purified fragments were sequenced on an ABI310 genetic analyzer (Applied Biosystems). Sequences were assembled by the CAP program (http://pbil.univ-lyon1.fr/cap3.php) and checked manually. The accession numbers are given in Table S1 (supplementary material). The BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search for sequence similarities in the DNA databases. Sequences were aligned using the ClustalW2 software (http://www.ebi.ac.uk/Tools/clustalw2/). Phylogenetic trees were inferred using the phylogenetic inference package MEGA5 with the Neighbor-Joining method. The distances were computed using the Kimura 2-parameter method with the pairwise deletion option. Bootstrap analysis was based on 1000 resamplings.

Plant tests

Nodulation and effectiveness tests of representative strains were conducted on common bean cv. coco. Seeds were sterilized and germinated as previously described (Mhamdi et al. 1999). Seedlings were aseptically transferred to glass tubes containing sterile sand. YEM broth containing flasks were inoculated with a YEM slant pre-culture suspension and incubated on a rotary shaker at 150 rev min^{-1} and 28 °C. At mid-exponential growth phase, the equivalent of 20 μl of broth culture for $OD_{620\,nm}$ = 1 was transferred to 250 ml flasks containing 100 ml of YEM broth and incubated during 48 h. Plants were inoculated with 500 μl of the appropriate strain. Non-inoculated plants were also included. Ten replicates were considered for each treatment. Plants were irrigated with a sterile nitrogen-free solution (Vadez et al. 1996). Plants were checked for nodulation after 60 days. Shoot dry weight and root dry weight were measured and submitted to analysis of variance and mean comparison (HSD test, $p < 0.05$) by the ANOVA program of the STATISTICA software (http://www.statsoft.com).

Table 1
Bean isolates recovered from a single site located in the North of Tunisia and representatives used for sequence analysis.

Ribotype[a]	Species[b]	Number of isolates	Representative isolates used for sequence analysis
1	*S. americanum*	17	23C40, 23C95
2	*R. radiobacter*	12	23C45
3	*R. gallicum*	3	23C84
4	*R. giardinii*	3	23C88
5	*E. meliloti*	3	23C59, 23C99
6	Putative new species	2	23C2, 23C55

Total abundance: 40
Species richness: 6
Evenness: 0.81
Simpson diversity index (1-D): 0.71
Shannon–Wiener diversity index: 1.45

[a] Typing by PCR-RFLP of 16S rRNA genes.
[b] Species assignment on the basis of sequence analysis of 16S rRNA, *atp*D, *rec*A and *dna*K genes.

Results and discussion

Species diversity

The PCR-RFLP ribotyping of the 16S rRNA genes of 40 isolates from the Azib soil was carried out. The isolates were categorized in six ribotypes (data not shown) by combination of the restriction patterns from both enzymes. Representative strains from each genotype were used for sequence analysis (Table 1).

The Neighbor-Joining tree based on nearly full 16S rRNA gene sequences (Fig. 1) showed that the Azib isolates were grouped into six clusters. Isolates 23C40 and 23C95 were closely related to *Sinorhizobium americanum* (not yet formally renamed as *Ensifer* and technically the correct name is still *Sinorhizobium americanum* and not *Ensifer americanus*), *E. fredii* and *Ensifer xinjiangensis* type strains with, respectively, 100%, 100% and 99.9% similarity. Previous reports indicated that the latter two species constitute a single monophyletic lineage and should be reclassified in the same species (Ferreira et al. 2011; Martens et al. 2008); however *S. americanum* should be retained as a separate species (Ferreira et al. 2011). This species was proposed by Toledo et al. (2003) for rhizobia isolated from native *Acacia* spp. in Mexico. The authors claimed that the type strain CFNEI 156^T represents a novel branch of 16S rRNA sequence inside the genus *Sinorhizobium* (renamed as *Ensifer*) and the closest species was *E. fredii* (Toledo et al. 2003). However, when comparing the 16S rRNA gene sequence of strain CFNEI 156^T available in Genbank (accession number AF506513), we found that it is 100% identical to that of *E. fredii* LMG 6217^T (accession number X67231), indicating a discrepancy between the information published by Toledo et al. (2003) and the deposited sequence. Therefore, 16S