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Cicer canariense, an endemic legume to the Canary Islands, is nodulated in mainland Spain by fast-growing strains from symbiovar trifolii phylogenetically related to *Rhizobium leguminosarum*

Pilar Martínez-Hidalgo[a,1], José-David Flores-Félix[a], Esther Menéndez[a], Raúl Rivas[a], Lorena Carro[a], Pedro F. Mateos[a], Eustoquio Martínez-Molina[a], Milagros León-Barrios[b], Encarna Velázquez[a,*]

[a] Departamento de Microbiología y Genética, Universidad de Salamanca, 37007 Salamanca, Spain
[b] Departamento de Bioquímica, Microbiología, Biología Celular y Genética, Universidad de La Laguna, 38071 La Laguna, Tenerife, Canary Islands, Spain

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ABSTRACT

Cicer canariense is a threatened endemic legume from the Canary Islands where it can be nodulated by mesorhizobial strains from the symbiovar ciceri, which is the common worldwide endosymbiont of *Cicer arietinum* linked to the genus *Mesorhizobium*. However, when *C. canariense* was cultivated in a soil from mainland Spain, where the symbiovar ciceri is present, only fast-growing rhizobial strains were unexpectedly isolated from its nodules. These strains were classified into the genus *Rhizobium* by analysis of the *recA* and *atpD* genes, and they were phylogenetically related to *Rhizobium leguminosarum*. The analysis of the *nodC* gene showed that the isolated strains belonged to the symbiovar trifolii that harbored a *nodC* allele (β allele) different to that harbored by other strains from this symbiovar. Nodulation experiments carried out with the lacZ-labeled strain RCCHU01, representative of the β *nodC* allele, showed that it induced curling of root hairs, infected them through infection threads, and formed typical indeterminate nodules where nitrogen fixation took place. This represents a case of exceptional performance between the symbiovar trifolii and a legume from the tribe *Cicereae* that opens up new possibilities and provides new insights into the study of rhizobia–legume symbiosis.

Introduction

Legumes establish symbiosis with soil bacteria called rhizobia that carry out nitrogen fixation in nodules formed on their roots or stems [26]. They can be promiscuous or restrictive depending on the diversity of rhizobial symbiovars able to nodulate them [26]. For instance, members of the tribe *Phaseoleae* are promiscuous hosts, whereas those from tribes *Cicereae*, *Trifolieae* and *Fabeae* (*Vicieae*) are considered restrictive hosts [7].

The tribe *Cicereae* only comprises the genus *Cicer* that contains several species, of which *Cicer arietinum* (chickpea) is widely cultivated worldwide [33]. This legume is nodulated by several species of the genus *Mesorhizobium*, with *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum* as the major endosymbionts [13,24,25]. It is also nodulated by *Mesorhizobium amorphae* and *Mesorhizobium tianshanense* in mainland Spain and Portugal [1,30], *Mesorhizobium huakuii* and *Mesorhizobium opportunistum* in Portugal [1,22], *Mesorhizobium muleiense* in China [42], and by other *Mesorhizobium* genospecies in Portugal [22] and India [17].

Therefore, *C. arietinum* establishes endosymbiosis with several *Mesorhizobium* species; nevertheless, it is a restrictive legume because all the strains nodulating chickpea belong to the symbiovar ciceri [21,30,42]. Moreover, all strains nodulating chickpea isolated from different continents have been shown to harbor closely related *nodC* genes, including those isolated in Syria and Turkey, the distribution centers of chickpea [33], which suggests a dispersion of chickpea endosymbionts together with its seeds, as already reported for other legumes such as *Phaseolus* [8,10,27,37] or *Vicia* [3]. Chickpea seeds may have dispersed strains from their distribution centers that harbored the typical nodulation genes of symbiovar ciceri, acquired through horizontal transfer from several *Mesorhizobium* species in Africa, Asia and Europe, where the *nodC*

* Corresponding author at: Departamento de Microbiología y Genética, Lab. 209, Edificio Departamental de Biología, Campus Miguel de Unamuno, 37007 Salamanca, Spain. Tel.: +34 923 294532; fax: +34 923 224876.
E-mail address: evp@usal.es (E. Velázquez).
[1] Current address: Department of Molecular, Cell and Developmental Biology, University of California, Los Angeles 621, Charles E. Young Drive South, Los Angeles, CA 90095-1606, United States.

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genes are highly conserved across *Mesorhizobium* strains nodulating chickpea [21].

The species *C. canariense*, also from the tribe *Cicereae*, is a threatened, wild perennial species endemic to the Canary Islands [38], where it has coevolved with its endosymbionts that belong to different species of the genus *Mesorhizobium*, and to several symbiotic lineages according to *nodC* gene analysis [4]. One of them has been shown to have 100% identity in the *nodC* gene sequence with respect to the *M. ciceri* type strain, and forms a number of nodules per plant similar to those formed by the remaining symbiotic lineages in reinfection experiments [4].

Considering that *C. canariense* is an endemism never cultivated outside the Canary Islands, it is interesting to study the rhizobia able to establish symbiosis with this species in other geographical locations. In a continental soil from North Spain, we have previously shown the presence of the symbiovar ciceri linked to *Mesorhizobium* species in *C. arietinum* nodules [30]. Therefore, the aim of this study was to analyze the rhizobial species nodulating *C. canariense* in this soil identified at the species level on the basis of *recA* and *atpD* gene analyses, and at the symbiovar level through analysis of the *nodC* gene.

Materials and methods

Isolation of strains

C. canariense was used as a trap plant in a soil from Riego de la Vega (León) in North Spain where the symbiovar ciceri strains were shown to nodulate *C. arietinum* [30]. The experiment was carried out using 10 plants in two different experiments performed in soil samples collected in 2011 and 2012. The rhizobial strains were isolated from the *C. canariense* nodules on YMA medium, according to Vincent [39].

RAPD fingerprinting

RAPD patterns were obtained as previously described [31] using the primer M13 (5′-GAGGGTGGCGGTTCT-3′) and the Dream-Taq™ DNA Green PCR Master Mix (Fisher Scientific, USA). PCR conditions were as follows: preheating at 95 °C for 9 min; 35 cycles of denaturing at 95 °C for 1 min; annealing at 45 °C for 1 min and extension at 75 °C for 2 min, and a final extension at 72 °C for 7 min. A total of 17 μL of each PCR product were electrophoresed on 1.5% (w/v) agarose gel in TBE buffer (100 mM Tris, 83 mM boric acid, 1 mM EDTA, pH 8.5) at 6 V/cm, stained in a solution containing 0.5 g mL^{-1} ethidium bromide, and photographed under UV light. Standard VI (Roche, USA) was used as a size marker.

Analysis of atpD, recA *and* nodC *genes*

The *recA* and *atpD* genes were amplified and sequenced as described by Gaunt et al. [11], and the *nodC* gene as described by Laguerre et al. [20]. PCR amplifications were performed with a REDExtract-N-Amp™ PCR Kit (Sigma Co., USA) following the manufacturer's instructions. Bands corresponding to the different genes were purified directly from the gel by room temperature centrifugation using a DNA gel extraction device (Millipore Co., USA) for 10 min at 5000 × *g*, according to the manufacturer's instructions. Sequencing reactions were performed on an ABI PRISM® 3100 sequencer using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc., USA), as supplied by the manufacturer. The sequences obtained were compared to those held in GenBank by using the BLASTN program [2]. The sequences were aligned by using Clustal W software [36]. Distances calculated according to Kimura's two-parameter model [18] were used to infer phylogenetic trees with the neighbor-joining method [32] and MEGA5 software [34]. Confidence values for nodes in the trees were generated by bootstrap analysis using 1000 permutations of the data sets.

Nodulation experiments

Nodulation assays were performed with *Trifolium repens*, *C. arietinum* and *C. canariense*. The seeds of the first two hosts were surface disinfected with 70% ethanol for 1 min and later sterilized with 5% sodium hypochlorite for 25 min. The seeds of *C. canariense* were scarified with concentrated sulfuric acid for 2 h and then surface sterilized with 5% sodium hypochlorite for 15 min. From each host, 15 seeds were germinated in sterile vermiculite and then inoculated with 1 mL per plant of a suspension containing approximately 1×10^8 CFU mL^{-1} of strains RCCHU01 and *Rhizobium leguminosarum* sv. trifolii ATCC 14480 in the case of *Trifolium repens*, and strains RCCHU01 and *M. ciceri* USDA 3383^T in the case of *C. canariense* and *C. arietinum*. Four weeks after inoculation, the plants were harvested and the number of nodules, weight and nitrogen content of aerial parts of plants were measured for *C. canariense*. Data were analyzed by one-way analysis of variance, and mean values were compared by Fisher's Protected LSD test (least significant differences) ($P \leq 0.05$).

Metagenomic studies

In order to exclude that the nodulation of *C. canariense* was due to contamination with *Mesorhizobium*, nodulation experiments were repeated in the same conditions, and the nodules were harvested and watered fivefold with sterile water. The DNA of nodules was extracted and PCR amplifications were performed with the REDExtract-N-Amp™ PCR kit (Sigma Co., USA) following the manufacturer's instructions. The *nodC* gene was used as a marker for metagenomic studies using the primers described for *Rhizobium* by Laguerre et al. [20] for amplification and those described for *Mesorhizobium* by Rivas et al. [29]. The bands obtained were sequenced using the same reagents and conditions described above.

Lac-Z labeling of strain RCCHU01

Plasmid pXLGD4 expressing hemA::lacZ [23] was transferred from *Escherichia coli* DH5α cells to strain RCCHU01 by triparental mating using the helper plasmid pRK2013 [9]. Recombinant strains were routinely grown at 28 °C on TY medium [5] supplemented with tetracycline (10 mg mL^{-1}).

Microscopy studies

Four days-old seedlings of *C. canariense* were inoculated with 1 mL of 0.5 OD_{600} strain RCCHU01 carrying plasmid pXLGD4 (hemA::lacZ). Roots were collected 21 and 30 days post infection, fixed with 2.5% glutaraldehyde for 1.5 h at room temperature, rinsed three times in Z buffer [35], and stained overnight at 28 °C in Z buffer containing 0.02 M 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-GAL), 10 mM $K_3Fe(CN)_6$, and 10 mM $K_4Fe(CN)_6$ in dark conditions. Then, two washes of Z buffer were performed, the first one lasting a few seconds while the second one involved immersion for one hour in the buffer before removal. Stained nodules and root hairs were observed by light microscopy using a NIKON Eclipse 8Oi fluorescence microscope.

Moreover, nodules from nodulation experiments carried out with unstained RCCHU01 were harvested at 30 dpi and fixed with 4% formaldehyde for 24 h, dehydrated in an increasing ethanol series, and embedded in paraffin. The sections (2 μm) of embedded nodules were stained with 0.01% toluidine blue for 1 min and examined by light microscopy.

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