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Bradyrhizobium algeriense sp. nov., a novel species isolated from effective nodules of *Retama sphaerocarpa* from Northeastern Algeria

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ABSTRACT

We have characterized genetic, phenotypic and symbiotic properties of bacterial strains previously isolated from nitrogen-fixing nodules of *Retama sphaerocarpa* from Northern Algeria. Phylogenetic analyses of 16S rRNA genes and three concatenated housekeeping genes, *recA*, *atpD* and *glnII*, placed them in a new divergent group that is proposed to form a new *Bradyrhizobium* species, *Bradyrhizobium algeriense* sp. nov. (type strain RST89^T, LMG 27618 and CECT 8363). Based on these phylogenetic markers and on genomic identity data derived from draft genomic sequences, *Bradyrhizobium valentinum* LmjM3^T, *Bradyrhizobium lablabi* CCBAU 23086^T, *Bradyrhizobium retamae* Ro19^T, and *Bradyrhizobium jicamae* PAC68^T are the closest relatives of *B. algeriense* RST89^T, with sequence identities of 92–94% and Average Nucleotide Identities (ANIm) under 90%, well below the 95–96% species circumscription threshold. Likewise, a comparison of whole-cell proteomic patterns, estimated by Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight (MALDI-TOF) mass spectrometric analysis, yielded almost identical spectra between *B. algeriense* strains but significant differences with *B. valentinum*, *Bradyrhizobium paxllaeri*, *Bradyrhizobium icense*, *B. lablabi*, *B. jicamae* and *B. retamae*. A phylogenetic tree based on symbiotic gene *nodC* revealed that the *B. algeriense* sequences cluster with sequences from the *Bradyrhizobium* symbiovar retamae, previously defined with *B. retamae* strains isolated from *Retama monosperma*. *B. algeriense* strains were able to establish effective symbioses with *Retama raetam*, *Lupinus micranthus*, *Lupinus albus* and *Genista numidica*, but not with *Lupinus angustifolius* or *Glycine max*.

Introduction

Retama spp. (tribe *Genisteae*, family *Fabaceae*) are shrubs native to the Mediterranean basin and adapted to grow under arid conditions. They are able to produce N_2-fixing root nodules, which makes them useful for restoration of arid or semiarid degraded ecosystems [30]. All endosymbiotic bacteria isolated from *Retama* nodules belong to the genus *Bradyrhizobium* [2]. Isolates from *Retama sphaerocarpa* growing in central Spain have been classified as *Bradyrhizobium canariense* [31]. Isolates from *R. sphaerocarpa* growing in Bouarfa (Morocco) and Ciudad Real (Spain), and isolates from *Retama monosperma* growing in Ras el Ma (Morocco) have been recently included in the *Bradyrhizobium retamae* species [14]. One hundred twenty-five isolates from root nodules of *Retama raetam* and *R. sphaerocarpa*, native of Northeastern Algeria, were described as *Bradyrhizobium* sp. [2]. Some of the *R. sphaerocarpa* isolates appeared to differ from the above-mentioned species, and have been characterized in this work by molecular, phenotypic and phylogenetic methods. These *R. spaherocarpa* strains are proposed to define the novel *Bradyrhizobium algeriense* sp. nov.

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Materials and methods

Bacteria and culture conditions

The isolates were obtained in a previous work [2] from surface-sterilized, effective nodules from *R. sphaerocarpa* plants growing in Algerian soils, and grown on Yeast Mannitol Agar (YMA [35]) at 28 °C. All pure cultures were tested for their ability to establish effective symbiosis with *R. sphaerocarpa* under controlled conditions (see below).

Phenotypic characterization

Cultural characteristics were assessed on YMA plates or YM Broth. Mean generation times were determined by spectrophotometric (600 nm) sampling of aerated (200 rpm orbital shaking) cultures. Growth temperature and pH range were determined by incubating cultures in YMA at 28 and 37 °C, and at pH of 4.0 or 10.0, respectively. Basal medium pH was adjusted with 1 M HCl to pH 7, and 0.05 g L^{-1} of bromothymol blue was added before sterilization. Salt tolerance was assayed by adding 1% (w/v) NaCl to the medium. GEN III MicroPlates (Biolog Inc.) were used to study assimilation of several compounds as carbon or nitrogen sources, using YNB (Becton Dickinson USA) as basal medium. Intrinsic antibiotic resistance was tested on YMA plates containing the following antibiotics: ampicillin (50 and 100 μg mL^{-1}), erythromycin (50 μg mL^{-1}), gentamycin (30 μg mL^{-1}), tetracycline (5 μg mL^{-1}), spectinomycin (50 μg mL^{-1}), kanamycin (50 μg mL^{-1}), streptomycin (10, 60 and 100 μg mL^{-1}), chloramphenicol (20 μg mL^{-1}) or rifampicin (5 μg mL^{-1}).

Protein composition was assessed by whole-cell matrix-assisted laser-desorption time-of-flight mass spectrometry (WC MALDI-TOF MS), using freshly-grown colonies analyzed as previously described [3,26].

Cellular fatty acid composition analyses were carried out at the Spanish Type Culture Collection (Colección Española de Cultivos Tipo, CECT, Paterna, Valencia). Cultures were grown aerobically on YM broth at 28 °C, and cells were collected at the late log phase of growth. Fatty acid methyl esters were prepared and resolved using the methods described by Sasser [34], and identified with the MIDI Sherlock Microbial Identification System (version 6.1), using the TSBA6 database.

Genotypic characterization

DNA fragment amplification and sequencing were performed with primers: 41F and 1488R for 16S rRNA [17]; atpD255F and atpD782R, recA41F and recA640R for partial sequences of *atpD* and *recA* respectively [13]; GSII-1 and GSII-4 for *glnII* [41]; nodCfor540 and nodCrev1160 for *nodC* [21]. Sequences were compared with those from GenBank using the BLASTN algorithm [1]. Sequences from *R. sphaerocarpa* isolates and from all the available *Bradyrhizobium* type species were aligned using the SINA alignment service from the SILVA database (http://www.arb-silva.de/aligner/) [27] for 16S rRNA genes, and ClustalX software [38] for housekeeping genes and symbiotic gene *nodC*. Most phylogenetic analyses were carried out with the software package MEGA 6.06 [37]. Kimura's two-parameter model was used to calculate distances [20], and phylogenetic trees were inferred using either the neighbor-joining (NJ [32]), maximum parsimony (MP [12]) and maximum likelihood (ML [11]) methods. Bootstrap analyses were based on 1000 subsets. For maximum likelihood trees, the PhyML 3.0 package [15,16] was used, and the best nucleotide substitution model for ML trees was determined by means of jModelTest 2.1.1 [7,16]. Robustness of ML tree topologies was inferred by nonparametric bootstrap tests based on 100 pseudoreplicates.

Table 1
Percentage Average Nucleotide Identity (ANIm) between *B. algeriense* strains RST89^T and RST91, and related *Bradyrhizobium* strains.

Strain	RST89^T	RST91
B. algeriense RST89^T	–	99.95%
B. algeriense RST91	99.74%	–
B. retamae Ro19^T	89.26%	89.27%
B. valentinum LmjM3^T	88.73%	88.70%
B. lablabi CCBAU 23086^T	88.48%	88.46%
B. jicamae PAC68^T	88.26%	88.28%
B. elkanii USDA 76^T	85.39%	85.42%
B. japonicum USDA 6^T	84.68%	84.66%
B. diazoefficiens USDA 110^T	84.63%	84.60%
B. sp. ORS278	84.32%	84.37%
B. sp. BTAi1	84.31%	84.40%

Draft genome sequences (Illumina HiSeq 2000, 500 bp paired-end libraries, 100 bp reads, 7 million reads), were obtained for *R. sphaerocarpa* strains and for type strains of closely-related *Bradyrhizobium* species by BGI (Hong Kong, China). These partial genomic sequences were assembled with SOAPdenovo2 [23] and used, together with available genome sequences from databanks, to calculate Average Nucleotide Identities (ANI). Genome sequences obtained in this work were deposited in Genbank as the following Bioprojects: *Bradyrhizobium lablabi* CCBAU 23086^T, PRJNA241376; *Bradyrhizobium jicamae* PAC68^T, PRJNA241374; *B. retamae* Ro19^T, PRJNA241375; *B. algeriense* RST89, PRJNA438070; and *B. algeriense* RST91, PRJNA438074. Pairwise genome comparisons were carried out with the JSpecies software package and the MUMMER option (ANIm) [29].

Plant tests

The plant symbiotic behavior of *B. algeriense* strains was tested in sterile Leonard jars filled with vermiculite containing Jensen's solution [35,42]. *B. algeriense* culture suspensions (2 mL, 10^8–10^9 cells mL^{-1}) were added onto seedlings of the legume to be tested (2–5-day-old) in the Leonard jars, and plants were grown in the greenhouse (25 °C) for a minimum of 3 and a maximum of 8 weeks, after which plants were examined for the presence of nodules, their number, size and appearance, as well as for appearance and weight of the aerial part. Tests were run in triplicate and compared with negative (uninoculated plants) and positive (plants inoculated with cognate symbionts) controls.

Results and discussion

R. sphaerocarpa root-nodule isolates were Gram-negative, non spore-forming rods, and extremely slow-growing bacteria with mean generation times >20 h in YMB. Non-mucous colonies (<2 mm) took at least 10 days to appear on YMA plates incubated at 28 °C and produced an alkaline reaction on YMA medium supplied with bromthymol blue, suggesting that isolates belong to the genus *Bradyrhizobium* [18]. Three related isolates from the Toudja maquis, RST88bis, RST89, and RST91, out of four belonging to IGS type 9 [2], were chosen for further work. RAPD-PCR analysis showed two band patterns for RST88bis and RST89/RST91, respectively (Supplementary Fig. S1). Further DNA sequence analyses (see Table 1 and Figs. 1 and 2, below) showed, however, that RST89 and RST91 are not clones.

Fig. 1 shows the tree derived from 16S rRNA alignments by the neighbor-joining method, but similar results were obtained with the MP and ML methods (data not shown). All *R. sphaerocarpa* strains isolated in our previous work had similar 16S rRNA gene sequences [2], and the three included in the phylogenetic tree (RST89^T, RST88bis and RST91) showed >99.6% identity. The *R. sphaerocarpa* strains cluster with other *Bradyrhizobium* species,

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