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Characterization of Rhizobial Bacteria Nodulating *Astragalus corrugatus* and *Hippocrepis areolata* in Tunisian Arid Soils

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Abstract

Fifty seven bacterial isolates from root nodules of two spontaneous legumes (*Astragalus corrugatus* and *Hippocrepis areolata*) growing in the arid areas of Tunisia were characterized by phenotypic features, 16S rDNA PCR-RFLP and 16S rRNA gene sequencing. Phenotypically, our results indicate that *A. corrugatus* and *H. areolata* isolates showed heterogenic responses to the different phenotypic features. All isolates were acid producers, fast growers and all of them used different compounds as sole carbon and nitrogen source. The majority of isolate grew at pHs between 6 and 9, at temperatures up to 40°C and tolerated 3% NaCl concentrations. Phylogenetically, the new isolates were affiliated to four genera *Sinorhizobium*, *Rhizobium, Mesorhizobium* and *Agrobacterium*. About 73% of the isolates were species within the genera *Sinorhizobium* and *Rhizobium*. The isolates which failed to nodulate their host plants of origin were associated to *Agrobacterium* genus (three isolates).

K e y w o r d s: 16S rDNA sequencing, arid areas, PCR-RFLP, phenotypic properties, rhizobial bacteria

Introduction

Rhizobia or "legume nodulating bacteria (LNB)" or "root nodule bacteria' (RNB)" are defined as nitrogen-fixing bacteria that form nodules on legume plants. In the last few years, a large diversity of LNB has been revealed, which has caused deep changes in the taxonomy of these bacteria. Rhizobia currently consist of 98 species belonging to 13 different genera. The predominant symbionts for most legume species in habitats throughout the world are found in the *a*-class of *Proteobacteria*: *Rhizobium*, *Azorhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Mesorhizobium*, *Bradyrhizobium*, *Methylobacterium* (Jaftha *et al*., 2002; Jourand *et al*., 2004), *Devosia* (Rivas *et al*., 2003), *Shinella* (Lin *et al*., 2008), *Ochrobactrum* (Trujillo *et al*., 2005; Zurdo-Pineiro *et al*., 2007), *Phyllobacterium* (Valverde *et al*., 2005; Mantelin *et al*., 2006) and *Microvirga* (Ardley *et al*., 2012). Moreover, about eight species within two genera of *β*-class of *Proteobacteria – Burkholderia* and *Cupriavidus* have been reported (Moulin *et al*., 2001; Chen *et al*., 2001; 2006; 2008; Klonowska *et al*., 2012). In addition, bacteria from *γ*-class of *Proteobacteria* have also been reported (Benhizia *et al*., 2004; Muresu *et al*., 2008; Mahdhi *et al*., 2012). On the other hand, many *Agrobacterium*-like strains have been isolated from root nodules of different legumes species (Gurtler *et al*., 1991; Liu *et al*., 2005; Mahdhi *et al*., 2008), but all of them failed to nodulate their original plant hosts and until now no definitive explanation of the presence of these bacteria inside nodules could be demonstrated.

Legumes belonging to the genera *Astragalus* and *Hippocrepis* are distributed in northern Africa, southern Europe and East Asia. Several *Astragalus* species are used as herbal medicine and *Hippocrepis* species have a wide range of uses as minor crops including consumption, fodder, forage and land stabilization. Despite the high number of these legume species (3000 species for *Astragalus* and 20 for *Hippocrepis*), only few of them have been considered for their nitrogen symbiotic fixation. Previous studies reported that microsymbionts associated to root nodules of some *Astragalus* species belonged to *Sinorhizobium*, *Rhizobium*, *Agrobacterium, Bradyrhizobium* and *Mesorhizobium* (Zhang *et al*., 2000; Gao *et al*., 2001; 2004; Wei *et al*., 2003; Zhao *et al*., 2012;

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Guerrouj *et al*., 2013; Gnat *et al*., 2014). Surprisingly, Muresu *et al.* (2008) reported that strains isolated from *Hippocrepis* unisiliquosa are identified as members of the genus *Bacillus* or as uncultured bacteria.

At Tunisia, *Astragalus* and *Hippocrepis* nitrogen-fixing symbiotic associations are poorly documented (Zakhia *et al*., 2004; Mantelin *et al*., 2006). Previous research's reported that rhizobia associated to *Astragalus glombiformis*, *Astragalus armatus*, *Astragalus corrugatus* and *Astragalus algerianus* were assigned to the genus of *Rhizobium* and *Phyllobacterium*, and only one isolate from nodules *Hippocrepis areolata* was affiliated to the genus *Sinorhizobium* (Zakhia *et al*., 2004).

Considering the potential value of the *Astragalus* and *Hippocrepis* species in the arid regions of Tunisia and the little information available about the diversity of their root nodulating bacteria, the present paper aim to determine the taxonomic diversity of 57 bacterial collection isolated from root nodules of *A. corrugatus* and *H. areolata* by using polyphasic approach including phenotypic and PCR-RFLP analysis and 16S rRNA gene sequencing.

Experimental

Materials and Methods

Bacterial isolation and growth conditions. Fifty seven isolates and six reference strains (Table I) representing different rhizobial species belonging to *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* were used in this study. Rhizobial bacteria were isolated from naturally occurring root nodules collected in four arid soils of Tunisia (Table I). For rhizobia isolation, healthy nodules dissected from roots were surface sterilized with ethanol (70%) and sodium hypochlorite (2%). Then nodules were separately crushed and the nodule juice was streaked on plates of yeast-mannitol agar (YMA) (Vincent, 1970) and incubated at 28°C for the isolation of the rhizobia. The obtained bacterial colonies were purified by being repeatedly streaked on the same medium. Pure isolates were stored with 25% (wt/vol) glycerol at −80°C.

Nodulation test. To assess nodulation, seeds were surface-sterilized in 98% sulphuric acid for 30 min and germinated on H_2O-agar plates (0.8%) at 25°C. Seedlings were transferred into vermiculite, inoculated with individual isolates and grown in a growth chamber at 25°C with12–16 h photoperiod. Nitrogen-free nutrient solution was used for plant watering (Vincent, 1970). Controls, not inoculated, were included. Four replicates were maintained for each treatment. Four weeks post inoculation, the plants were uprooted and the occurrence of nodulation in each plant was checked.

Phenotypic characterization. All isolates were initially tested for their phenotypic features. For bacterial growth, bacteria were cultivated in 50 ml of YM broth into 250 ml Erlenmeyer flasks and incubated in a gyratory shaker at 180 g and 28°C. Growth was followed by measuring the optical density at 600 nm every 2 h and generation time of each isolate was deduced from the exponential phase of the growth curves.

Growth of the isolates at different temperatures (28, 37, 40, 42, 45°C), the ability to grow in the presence of different NaCl concentrations (1, 2, 3, 4, 5%) and at different pH levels (4, 5, 7, 9, 11) were determined by growth on supplemented YMA as described by Mohamed *et al.* (2000).

The modified-YMA medium (Somasegaran and Hoben, 1994) was used to investigate the ability of isolates to use carbohydrates (1% glucose, galactose, fructose and sucrose) and amino-acids (0.1% L-proline, L-arginine, L-tyrosine and L-leucine) as a sole carbon and nitrogen sources respectively. Production of acid or alkali was determined on YMA supplemented with 0 ± 0025% (w/v) bromothymol blue as pH indicator. All phenotypic tests were performed in triplicate.

PCR amplification and RFLP analysis of 16S rRNA gene. Total genomic DNA was extracted as described by Mhamdi *et al.* (2002). Primers fD1 and rD1 (Weisburg *et al*., 1991), were used for PCR amplification of 16S rRNA gene. PCR was carried out in Gen Amp PCR system 9700 (Applied Biosystems) in a 25 µl containing template DNA extract as described previously by Mahdhi *et al.* (2012). PCR amplification was analyzed by horizontal 1% (w/v) agarose gel electrophoresis stained with ethidium bromide. The amplified DNA fragments of 16S rRNA gene were digested with *Rsa*I, *Hinf*I, *Hae*III, *Cfo*I, *Nde*II and *Msp*I restriction enzymes (Promega products). The restriction patterns were checked by horizontal 4% (w/v) agarose gel electrophoresis stained with ethidium bromide.

Different 16S rDNA types were designed based upon the combined RFLP patterns obtained from the six enzymes, *e.g.* isolates is defined as a unique 16S rDNA type if it has one band different from other isolates in the six digestions

Sequencing of 16S rRNA gene. Bacterial genomic DNA extracted according to Mhamdi *et al.* (2002) was used as templates. For five isolates 16S rDNA gene chosen as representative of different 16S rDNA types, were amplified using universal primers fD1 and rD1 as described above. The PCR products were purified and sequenced using the ABI PRISM BigDye Terminator cycle sequencing kit according to the manufacturer's protocol and analysed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Sequences were assembled using ChromasPro and were aligned with Clustal X. The acquired sequences were deposited

Table I
New isolates and reference strains used in this study.

Isolates	*Site of origin*	*Host plants*	*Nodulation test*	*16SrDNA type*
ACM1	Menzel Habib	*Astragalus corrugatus*	–	1*
ACM2	Menzel Habib	*Astragalus corrugatus*	+(12)	2†
ACM3	Menzel Habib	*Astragalus corrugatus*	+(11)	2†
ACM4	Menzel Habib	*Astragalus corrugatus*	+(10)	2†
ACN1	Nefta	*Astragalus corrugatus*	+(15)	3ϕ
ACN2	Nefta	*Astragalus corrugatus*	+(14)	3ϕ
ACN3	Nefta	*Astragalus corrugatus*	+(11)	3ϕ
ACN4	Nefta	*Astragalus corrugatus*	+(11)	3ϕ
ACN5	Nefta	*Astragalus corrugatus*	+(10)	2†
ACN6	Nefta	*Astragalus corrugatus*	+(10)	2†
ACN7	Nefta	*Astragalus corrugatus*	+(10)	2†
ACN8	Nefta	*Astragalus corrugatus*	+(13)	2†
ACN9	Nefta	*Astragalus corrugatus*	+(15)	2†
ACN10	Nefta	*Astragalus corrugatus*	–	1*
ACN11	Nefta	*Astragalus corrugatus*	+(14)	2†
ACN12	Nefta	*Astragalus corrugatus*	+(14)	3ϕ
ACN13	Nefta	*Astragalus corrugatus*	+(13)	3ϕ
ACN14	Nefta	*Astragalus corrugatus*	+(11)	2†
ACN15	Nefta	*Astragalus corrugatus*	+(11)	2†
ACN16	Nefta	*Astragalus corrugatus*	+(14)	3ϕ
ACZ1	Zarzis	*Astragalus corrugatus*	+(13)	4‡
ACZ2	Zarzis	*Astragalus corrugatus*	+(15)	4‡
ACZa1	Zárate	*Astragalus corrugatus*	+(15)	4‡
ACZa2	Zárate	*Astragalus corrugatus*	+(15)	4‡
ACZa3	Zárate	*Astragalus corrugatus*	+(12)	4‡
HBM1	Menzel Habib	*Hippocrepis areolata*	+(12)	3ϕ
HBM2	Menzel Habib	*Hippocrepis areolata*	+(13)	3ϕ
HBM3	Menzel Habib	*Hippocrepis areolata*	+(14)	3ϕ
HBM4	Menzel Habib	*Hippocrepis areolata*	+(11)	3ϕ
HBM5	Menzel Habib	*Hippocrepis areolata*	+(10)	3ϕ
HBM6	Menzel Habib	*Hippocrepis areolata*	+(10)	3ϕ
HBM7	Menzel Habib	*Hippocrepis areolata*	+(11)	3ϕ
HBM8	Menzel Habib	*Hippocrepis areolata*	+(12)	3ϕ
HBM9	Menzel Habib	*Hippocrepis areolata*	+(13)	3ϕ
HBN1	Nefta	*Hippocrepis areolata*	+(14)	3ϕ
HBN2	Nefta	*Hippocrepis areolata*	+(12)	3ϕ
HBN3	Nefta	*Hippocrepis areolata*	+(11)	3ϕ
HBN4	Nefta	*Hippocrepis areolata*	+(10)	5†
HBN5	Nefta	*Hippocrepis areolata*	+(11)	3ϕ
HBN6	Nefta	*Hippocrepis areolata*	+(12)	5†
HBN7	Nefta	*Hippocrepis areolata*	+(12)	3ϕ
HBN8	Nefta	*Hippocrepis areolata*	+(12)	5†
HBN9	Nefta	*Hippocrepis areolata*	+(13)	3ϕ
HBN10	Nefta	*Hippocrepis areolata*	+(10)	5†
HBZ1	Zarzis	*Hippocrepis areolata*	+(10)	2†
HBZ2	Zarzis	*Hippocrepis areolata*	+(15)	2†
HBZ3	Zarzis	*Hippocrepis areolata*	+(14)	3ϕ

Table I
New isolates and reference strains used in this study.

Isolates	*Site of origin*	*Host plants*	*Nodulation test*	*16SrDNA type*
HBZ4	Zarzis	*Hippocrepis areolata*	+(13)	2†
HBZ5	Zarzis	*Hippocrepis areolata*	+(12)	2†
HBZ6	Zarzis	*Hippocrepis areolata*	+(14)	2†
HBZ7	Zarzis	*Hippocrepis areolata*	+(14)	2†
HBZ8	Zarzis	*Hippocrepis areolata*	+(13)	5†
HBZ9	Zarzis	*Hippocrepis areolata*	+(12)	5†
HBZ10	Zarzis	*Hippocrepis areolata*	+(11)	5†
HBZ11	Zarzis	*Hippocrepis areolata*	–	1*
HBZ12	Zarzis	*Hippocrepis areolata*	+(10)	2†
HBZ13	Zarzis	*Hippocrepis areolata*	+(15)	2†
R. mongolense STM246^T = LMG1941^T	Mongolia, China	*Medicagoruthenica*	Nt	6
R. galegae HMBI540^T = LMG6214^T	Finland	*Galegae orientalis*	Nt	7
M. loti ORS664 = LMG6125^T	New Zealand	*Lotus tenuis*	Nt	8
M. mediterraneum ORS2739^T = LMG17148^T	Spain	*Cicer arietinum*	Nt	9
S. meliloti ORS665^T = LMG6133^T	Virginia, USA	*Medicagosativa*	Nt	3
B. japonicum NZP5549^T = LMG6138^T	Japon	*Glycine max*	Nt	10

Note: STM: collection du laboratoire des Symbioses tropicales et méditerranéennes; HAMBI: Culture Collection of the Department of Microbiology, University of Helsinki, Helsinki, Finland; LMG: Collection of Bacteria of the Laboratorium voor Microbiologie, Universiteit Ghent, Belgium; NZP: Culture Collection of the Department for Scientific and Industrial Research, Biochemistry Division, Palmerston North, New Zealand; ORS: Collection, Laboratoire commun de Microbiologie, BP 1386, Dakar, Senegal; ^{T} type strain. *: isolates grouped in genus *Agrobacterium*; †: isolates grouped in genus *Rhizobium*; ♮: isolates grouped in genus *Sinorhizobium*, ‡: isolates grouped in genus *Mesorhizobium*. + Positive test, – no nodulation. Numbers in parentheses indicate the number of nodules per plant, Nt: not tested

in the GenBank database and were analysed for homologies to related sequences obtained from GenBank. The phylogenetic analyses were performed using mega 3.1 software (Kumar *et al*., 2001). A neighbour-joining tree was constructed using Kimura two-parameter model (Kimura, 1980) of and support of internal branches was assessed using 1000 bootstrap replications. The GenBank accession numbers for the 16S rRNA gene sequences reported in this paper are KR108303 (ACN5), KR108304 (HBN4), KR108300 (ACM1), KR108301 (ACN1) and KR108302 (ACZ1).

Results

Nodulation test and phenotypic characterisation. A nodulation test was performed for all isolates. Result showed that only two *A. corrugatus* isolates (ACM1 and ACN10) and one *H. areolata* isolate (HBZ11) affiliated to *Agrobacterium* by 16S rRNA gene sequencing analysis (see below) failed to nodulate their host plant of origin. The other isolates formed ten to fifteen nodules per plantlet after four weeks post-inoculation (Table I).

Phenotypically, (Table II) all isolates were acid producers, fast growers (Generation times < 6 h). Five *A. corrugatus* isolates (described by the analysis of 16S rRNA sequences as *Mesorhizobium* have a generation time between 4 and 6 h. All tested isolates used all tested compounds as sole carbon and nitrogen sources and were able to grow at pHs between 6 and 9, but none of them could grow at pHs 4 and 11.

The majority of the isolates grew at 28, 37 and 40°C. Only two isolates (ACN1 and ACN4) continued to grow at 42°C, but not at 45°C. Most of the tested isolates tolerated NaCl concentrations from 1 to 3%. Three *A. corrugatus* isolates (ACN1, ACN4, and ACN13) and one *H. areolata* isolate (HBZ1) continued to grow in 4% NaCl and none of them tolerated 5% NaCl.

PCR-RFLP analysis of 16S rRNA gene. The new isolates of 16S ribosomal DNA and reference strains was PCR-amplified and a single band of the expected size of approximately 1500 bp was produced. PCR products were digested with six restriction enzymes *Rsa*I, *Hinf*I, *Hae*III, *Cfo*I, *Nde*II and *Msp*I. Ten 16S rDNA types were distinguished among the 57 isolates and the six reference strains. Each 16S rDNA type comprised 1 to 23 isolates (Table I). Five rDNA types were identified among the new isolates. Types 1, 2, 3 of 16S rDNA included new isolates originating from both *A. corrugatus* and *H. areolata* microsymbionts. The type 4 and 5 of 16S rDNA contained only *A. corrugatus* and *H. areolata* isolates respectively. Type 3 of 16S rDNA consisted of

Table II
Phenotypic characteristics of the isolates

Characteristics		*Sinorhizobium* isolates	*Rhizobium* isolates	*Mesorhizobium* isolates	*Agrobacterium* isolates
Number of isolates		23	26	5	3
Generation time	**G < 4 h**	+	+	–	+
	4 ≤ G < 6	–	–	+	–
Growth at pH	4	–	–	–	–
	9	+	+	+	+
	11	–	–	–	–
Acid production		+	+	+	+
Alkali production		–	–	–	–
NaCl tolerance	2%	+	+	+	+
	3%	+	+ (20)	+	–
	4%	+ (3)	+ (1)	–	–
	5%	–	–	–	–
Utilisation of sugars	glucose	+	+	+	+
	galactose	+	+	+	+
	fructose	+	+	+	+
	sucrose	+	+	+	+
Utilisation of amino acids	L-arginine	+	+	+	+
	L-proline	+	+	+	+
	L-leucine	+	+	+	+
	L-tyrosine	+	+	+	+
Growth at temperature	40°C	+	+ (24)	+ (4)	–
	42°C	+ (2)	–	–	–
	45°C	–	–	–	–

Note: (+) positive growth/ present; (–) no growth/absent;
Number in parentheses indicate the number of positive isolates of the total number of isolates tested

both new isolates and a reference strain *(Sinorhizobium meliloti* LMG6133^T).

Sequencing of 16S rDNA. A total of five *A. corrugatus* and *H. areolata* isolates representing the five different 16S rDNA types were selected to undergo 16S rDNA gene sequencing. New *A. corrugatus* and *H. areolata* strains exhibited 99–100% 16S rDNA sequence similarity with reference species already described in GenBank. In the reconstructed phylogenetic tree (Fig. 1), strain ACZ1 (Representative of 16S rDNA type 4) was phylogenetically related to *Mesorhizobium temperatum* CCNWSX0012-2 and to *Mesorhizobium* sp. LAC831. The two isolates ACN5 and HBN4, representative of 16S rDNA types 2 and 5 respectively, were grouped in *Rhizobium* branch; with ACN5 strain was associated to *Rhizobium* sp. STM 394, while HBN4 strain was affiliated related to *Rhizobium* sp. STM 4037. The sequences of 16S rDNA of the strain ACN1 was closely related to the 16S rDNA sequences of *S. meliloti* LMG6133^T and *Sinorhizobium* sp. STM4038, while the strain ACM1 was phylogenetically related to *Agrobacterium tumefaciens* 2002000903 and *Agrobacterium* sp. STM4035.

Discussion

Currently, in the rhizobia taxonomy the polyphasic approach, based on phenotypic and genomic criteria is used (Graham *et al.*, 1991; Vandamme *et al.*, 1996; Małek and Sajnaga, 1999). Among the phenotypic features characteristic related to the microorganism ecological niches are considered as the most interesting. In the present studies a collection of 57 isolates was obtained from *A. corrugatus* and *H. areolata* root nodules covering four regions of Tunisia and characterized by a polyphasic approach including phenotypic features, PCR-RFLP of 16S rDNA and 16S rDNA sequence analysis. All isolates, except ACM1, ACN10 and HBZ11, re-induce nodules in their host plant. The three non-re-nodulating strains can be considered as opportunistic endophytes as already proposed (Zakhia *et al.*, 2006; Mahdhi *et al.*, 2007; 2012).

Phenotypically (Table II) our results indicate that *A. corrugatus* and *H. areolata* isolates showed heterogenic responses to the different phenotypic features. This heterogeneity may contribute the nodulation of

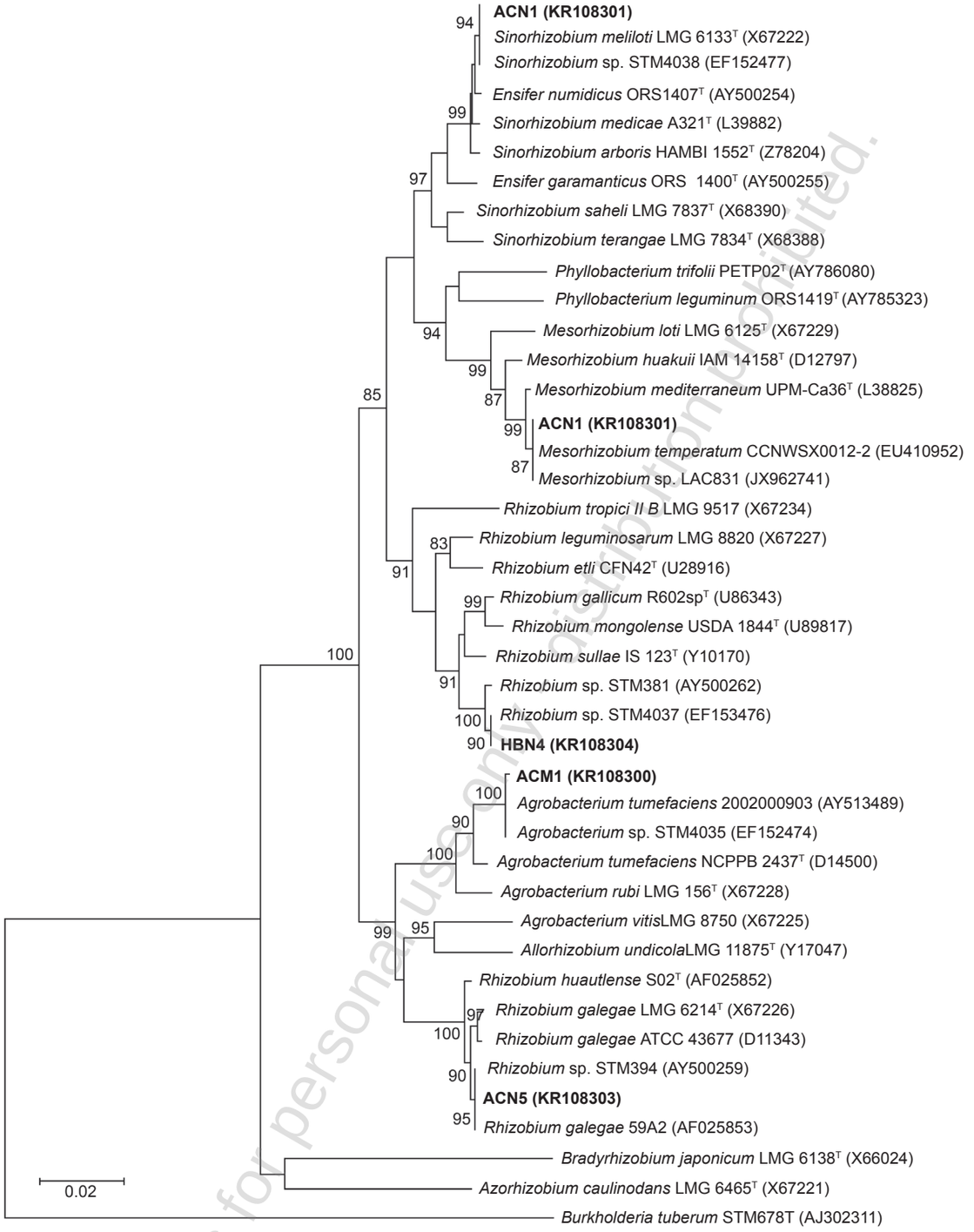

Fig. 1. 16S rRNA gene sequence-based dendrogram obtained by neighbor-joining method showing the phylogenetic positions of *A. corrugatus* and *H. areolata* isolates. Only significant bootstraps (> 80%) are shown (1000 replications). Sequence accession numbers are listed in parentheses.

legumes in different conditions (Wei *et al.*, 2008). All new isolates are acid producers, fast growers (Generation times < 6 h) like *Rhizobium*, *Sinorhizobium* and *Mesorhizobiun* species (Małek and Sajnaga, 1999). All tested isolates are able to use all tested compounds as sole carbon and nitrogen sources. Similar results were reported by Guerrouj *et al.* (2013) for rhizobia nodulating *A. glombiformis* in Eastern Morocco. This ability to use a wide range of carbon sources could be beneficial for the bacterial life cycle in the soil and may be related

to their high competitiveness in a natural environment. Elkan (1992) reported that carbohydrate sources could be used to differentiate fast-growing rhizobia from the slow-growing bradyrhizobia.

As for salinity, temperature and pH tolerance, our results showed that most of the isolates are able to grow at 3% NaCl, at pHs between 6 and 9 and at high temperature (40°C), except of two of them which were continued to grow at 42°C. These results corroborate our earlier reports on the root nodule bacteria isolated from wild legumes in Tunisia (Mahdhi *et al*., 2007; 2008; Rejili *et al*., 2009; Fterich *et al*., 2011). Similarly, Guerrouj *et al.* (2013) reported that rhizobial strains associated to *A. glombiformis* are tolerant to 342 mM NaCl and 40°C in Eastern Morocco. In China, Wei *et al.* (2003) reported that some rhizobial strains nodulating *Astragalus* species tolerate only 2% NaCl. The capacity of new isolates to tolerate high temperatures and high NaCl concentrations could be considered a specific adaptation to high soil temperatures and salinity in arid regions as described by Karanja and Wood (1988).

By using the comparative 16S rRNA gene sequence analysis, the new isolates were grouped on the phylogram in the *Sinorhizobium*, *Rhizobium*, *Mesorhizobium* and *Agrobacterium* genera; with 73% of the new isolates are species within the genera *Sinorhizobium* and *Rhizobium* as are many other indigenous legume symbionts from Tunisia (Zakhia *et al*., 2004; Ben Romdhane *et al*., 2005; Mahdhi *et al*., 2008). By 16S rRNA gene sequencing of isolates ACN5 (16S rDNA type 2), HBN4 (16S rDNA type 5) and ACN1 (16S rDNA type 3) are closely related to Tunisian legume nodulating bacteria belonging to *Rhizobium* sp. STM 394, *Rhizobium* sp. STM 4037 and *Sinorhizobium* sp. STM4038 which were isolated from *Astragalus cruciatus* and *Argyrolobium uniflorum* growing on the same Tunisian soils (Zakhia *et al*., 2004; Mahdhi *et al*., 2008). So, it would now be interesting to test the cross-nodulation capacity of our new isolates on *A. cruciatus* and *A. uniflorum* legumes.

It has been previously reported that *A. corrugatus* is nodulated by strains belonging to the genera *Rhizobium, Sinorhizobium, Brdyrhizobium, Mesorhizobium, Agrobacterium* and *Phyllobacterium* (Wei *et al*., 2003; Gao *et al*., 2004; Zakhia *et al*., 2004; Mantelin *et al*., 2006; Guerrouj *et al*., 2013; Zheng *et al*., 2013; Gnat *et al*., 2014). Our results thus confirm the previous studies showing the large diversity of *A. corrugatus* rhizobia that belong to *Rhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Agrobacterium*. However, no *Phyllobacterium* and *Bradyrhizobium* were recovered in our results. Others studies (Guerrouj *et al*., 2013; Gnat *et al*., 2014) showed that *Astragalus glycyphyllos* in Poland and *A. glombiformis* in Morocco were nodulated by rhizobial bacteria belonging only to the genus *Mesorhizobium*. Among our collection only five *A. corrugatus* isolates (16S rDNA type 5) were phylogenetically grouped in *Mesorhizobium* branch, closely related to *M. temperatum* CCNWSX0012-2 and to *Mesorhizobium* sp. LAC831 nodulating *Lotus creticus* from arid regions of Tunisia (Rejili *et al*., 2009; 2012; 2013). This diversity of rhizobia nodulating *Astragalus* species may be in relation with climatic and edaphic conditions. Our study confirms that the *Astragalus* species nodulated by several rhizobial genomspecies can be qualified as promiscuous and that their rhizobia have very diverse genomic and symbiotic gene backgrounds (Zhao *et al*., 2008; Gnat *et al*., 2014)

Until now, nodulation of *Hippocrepis* species has been poorly documented (Zakhia *et al*., 2004; Muresu *et al*., 2008). In addition, only two strains were included in these studies. In our collection, *H. areolata* isolates are identified as *Rhizobium*, *Sinorhizobium* and *Agrobacterium* genera.

Several studies have reported the presence of *Agrobacterium* strains in nodules of some legumes (de Lajudie *et al*., 1999; Gao *et al*., 2001; Liu *et al*., 2005; Mhamdi *et al*., 2005; Mrabet *et al*., 2006), but all them failed to nodulate their original host plants. Here we isolated three *Agrobacterim* isolates (16S rDNA type 1) that also failed to nodulate their host plants *in vitro*. The sequences of 16S rDNA of one isolated *Agrobacterium* isolate (ACM1) is closely related to the 16S rRNA gene sequence of *Agrobacterium* sp. STM4035, which was isolated by Mahdhi *et al.* (2008) from the root nodules of the pastoral legume *A. uniflorum*.

In conclusion, our study is the first report on the characterisation of *A. corrugatus* and *H. areolata* in Tunisia. Our investigation showed that LNB originating from nodules of these legumes was genetically diverse and affiliated with *Rhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Agrobacterium*. Most of *A. corrugatus* and *H. areolata* isolates were related to previously described LNB in Tunisian soils. However, rhizobia from other locations that were not covered in this study should be investigated to provide further information about the diversity of bacteria nodulating these legumes in Tunisia

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