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ORIGINAL ARTICLE

The phenotypic, phylogenetic and symbiotic characterization of rhizobia nodulating *Lotus* sp. in Tunisian arid soils

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Abstract Fifteen bacterial isolates, representatives of different 16S rRNA-RFLP genomogroups which were isolated from root nodules of *Lotus creticus* and *L. pusillus* growing in the arid areas of Tunisia were characterized by phenotypic features and 16S rDNA sequences. Phenotypically, all isolates are fast growers with the ability to grow at a pH between 5.5 and 9. Most of the tested isolates tolerate NaCl concentrations from 1.39 to 3.48 %. Phylogenetically, the studied isolates are affiliated into the genera: *Sinorhizobium* (5 strains), *Rhizobium* (2 strains), and *Mesorhizobium* (4 strains). The 16S rDNA sequences of Tunisian *Lotus* sp. nodule isolates: LAC7511, LAC733, and *Mesorhizobium alhagi* (*Alhagi sparsifolia* symbiont) shared 100 % identical nucleotides similar to the 16S rDNA sequences of LAC831, LAC814 and *Mesorhizobium temperatum* CCNWSX0012-2 (*Astragalus adsurgens* symbiont). Non-nodulating bacteria, considered as endophytes of *Lotus* sp. nodules, were also found in our studies and they were classified into the genera: *Phyllobacterium* (2 strains), *Starkeya* (1 strain) and *Pseudomonas* (1 strain). Except for these four endophytic *Lotus* sp. bacteria, all other strains under investigation induce nodules on *Lotus* sp., but they differ in the number of induced root nodules and the effectiveness of atmospheric nitrogen fixation. The *Sinorhizobium* sp., *Mesohizobium* sp. and *Lotus* sp. nodule isolates, forming the most effective symbiosis with the plant host, are potential candidates for inoculants in revegetation programs.

Keywords *Lotus* sp. · Rhizobia · 16S rRNA gene sequence · Tunisia

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Introduction

The plants belonging to the genera *Lotus* are now considered as prospective temperate legumes suitable for a wide application in Europe, Asia, Northern America and Africa. They are increasingly utilized in pastures throughout the world because of their high productivity over a wide range of soils (Blumenthal and McGraw 1999). They provide high quality animal fodder, prevent erosion and contribute to soil stabilization and ecosystem restoration (Fagg and Stewart 1994). The genus *Lotus* includes plants that are adapted to a wide range of habitats from marine environments to high altitudes, and from sandy to heavy saline soils (Heyn and Hernstradt 1967; Montes 1988). These crops have been promoted for low fertility pastures where they are more persistent and exhibit higher yields compared to traditional fodder legumes (Safronova et al. 2004).

Due to their capacity to enter into symbiosis with legume nodulating bacteria (LNB) collectively called rhizobia, *Lotus* sp. could play an important role in the nitrogen cycle. They may be used to restore or increase fertility of degraded and eroded soils. Studies of nodule bacteria isolated from indigenous legumes species growing in Tunisian arid zones showed their high diversity. They have been classified into the genera: *Sinorhizobium*, *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Phyllobacterium* (Zakhia et al. 2004, 2006; Mantelin et al. 2006; Rejili et al. 2009; Mahdhi et al. 2012). Rhizobia that nodulate *Lotus* species include both fast-growing *Mesorhizobium loti* (Jarvis et al. 1997) and slow-growing *Bradyrhizobium* sp. (Jordan 1982). At present, little information

is available about the diversity and symbiotic properties of rhizobium strains nodulating *Lotus* sp. in Tunisia. Zakhia et al. (2004) found that strains isolated from *Lotus creticus* growing in the infra-arid region of Tunisia were the *Rhizobium* species. Recently, our research group in collaboration with a French group identified two new species called *Ensifer numidicus* sp. nov. and *E. garamanticus* sp. nov. which form symbiosis with *Argyrolobium uniflorum* and *L. creticus* (Merabet et al. 2010). Rejili et al. (2009) studied the genetic diversity of 60 isolates from the nodules of *Lotus pusillus* and *L. creticus* growing in arid environments in Tunisia by some molecular approaches including rep/PCR and PCR-RFLP of 16S rRNA gene analysis. *Lotus* sp. isolates are genomically very diverse. Some of them are affiliated to the genera *Sinorhizobium*, *Rhizobium*, and *Mesorhizobium*, but the taxonomic position of many other isolates has not been established. Therefore, the objective of this study is to determine the diversity of *L. pusillus* and *L. creticus* root nodule bacteria isolated by Rejili et al. (2009) and to clarify their taxonomic status by phenotypic and 16S rRNA gene sequence analyses, and also to determine their symbiotic effectiveness.

Materials and methods

Bacterial strains and cultural conditions

Fifteen bacteria, isolated by a standard method (Vincent 1970) from nodules of *Lotus pusillus* and *L. creticus* growing in the arid regions of Tunisia, were used in this study. They were chosen from among those previously characterized by RFLP analysis of 16S rDNA (Rejili et al. 2009). The isolates used in this study are shown in Table 1.

Table 1 Isolates used in this study, their hosts and their geographic origin

Isolate	Host plant	Site of origin
LAC231	*Lotus creticus*	Oueddkouk
LAC241	*Lotus creticus*	Oueddkouk
LAC241	*Lotus creticus*	Oueddkouk
LAC241	*Lotus creticus*	Elkestil
LAC553	*Lotus creticus*	Elkestil
LAC733	*Lotus creticus*	Dhiba
LAC742	*Lotus creticus*	Dhiba
LAC742	*Lotus creticus*	Dhiba
LAC765	*Lotus creticus*	Dhiba
LAC813	*Lotus creticus*	Fjé
LAC814	*Lotus creticus*	Fjé
LAC831	*Lotus creticus*	Fjé
LPS664	*Lotus pusillus*	Dar Dhaoui
LPS715	*Lotus pusillus*	Elhamma
LPS811	*Lotus pusillus*	Elkestil

16S rRNA gene sequencing

Fifteen isolates representing each RFLP-16S rDNA genomo groups of 60 *Lotus* sp. microsymbionts described by Rejili et al. (2009) were cultured in YMA medium (Somasegaram and Hoben 1994). Bacterial DNA was prepared as described by Zakhia et al. (2004) or alternatively by the alkaline lysis method (Baele et al. 2000). The nearly full-length 16S rRNA gene was amplified using primers fD1 and rD1 (Weisburg et al. 1991) as described by Herrera-Cervera et al. (1999). PCR amplification was carried out in a 25-μl reaction mixture according to Rejili et al. (2009). The 16S rDNA amplicons were cleaned with Nucleofast filters. Two forward primers (FGPS6 and 16S-370f) and one reverse primer (FGPS1509) were used to obtain the complete and partial gene sequences. DNA was sequenced in the automated DNA sequencing service of Secugen (Madrid, Spain). DNA sequencing reactions were done in an Applied Biosystems PCR machine using the Big Dye 1.3 kit. After terminator elimination by CleanSeq from Agencourt, samples were electrophoresed in an AB3730 xl DNA sequencing machine. The 16S rDNA sequences of *Lotus* sp. symbionts and those of reference rhizobial species from GenBank were aligned by the Clustal X program and analyzed using Genedoc software package. A neighbor-joining tree was constructed and bootstrapped with 1,000 replications of each sequence using Mega software (Kumar et al. 2001). The GenBank accession numbers for the 16S rRNA gene sequences reported in this paper are JX962731 (LAC241), JX962732 (LAC231), JX962733 (LAC243), JX962734 (LAC513), JX962735 (LAC553), JX962736 (LAC733), JX962737(LAC742), JX962738 (LAC765), JX962739 (LAC813), JX962740 (LAC814), JX962741 (LAC831), JX962742 (LAC7511), JX962743 (LPS664), JX962744 (LPS715), and JX962745 (LPS811).

Phenotypic characterization

Twenty-six phenotypic features were used for characterization of studied isolates. The modified YMA medium (Somasegaram and Hoben 1994) was used to analyze the ability of isolates to use carbohydrates (1 % glucose and fructose) and amino acids (0.1 % L-arginine and L-leucine) as the sole carbon and nitrogen sources, respectively. The tolerance of isolates to pH was assessed by adjusting YMA medium pH to 5.5, 6.0, 7.0, 8.0, and 9.0 by the addition of sterile acid or alkali. The salt tolerance was analyzed by adding YMA medium NaCl to final concentrations of 1–4 %. Maximum growth temperature and antibiotic resistance (ampicillin 60 and 100 μg ml^{-1}, streptomycin 60 and 100 μg ml^{-1}, and kanamycin 60 and 100 μg ml^{-1}) were also assessed in YMA as described by Mohamed et al. (2000).

Acid and alkali production was determined in YMA medium with bromothymol blue indicator (0.0025 %). In all experiments, growth was recorded after 72 h in triplicate.

Nodulation test and symbiotic efficiency

Symbiotic effectiveness of rhizobial strains was expressed in percent of dry weight of the aerial biomass of the plants inoculated with these bacteria to that of control uninoculated but fertilized plants, which were maintained with Jensen's medium containing 0.1 M KNO_3. Seeds were surface-sterilized in 96 % sulfuric acid for 2 h, rinsed thoroughly with sterile distilled water, and germinated in Petri dishes at 20 °C. One seedling was transplanted into sterile plastic pots filled with autoclaved vermiculite. The pots were placed in a growth chamber at 23 °C with a 12- to 16-h photoperiod. Inoculation was performed with 10^8–10^9 cells of each isolate. The uninoculated plants (T_N: N-fertilized and uninoculated plants; and T_0: non-fertilized and uninoculated plants) were included as controls. Plants were harvested 10 weeks after planting and observed for nodulation. Shoots were cut off, dried at 70 °C for 48 h, then weighed. Nitrogen fixing effectiveness of nodules was determined by comparing the dry-shoot weight of inoculated plants with the dry-shoot weight of +N control plants.

Statistical analysis

Statistical analyses were performed with a SAS statistical package. Symbiotic properties were determined for each isolate in eight replications and using pair-wise comparison between the different treatments.

Results

16S rRNA gene sequencing

The nearly full-length sequences of 16S rRNA gene (1,375 bp) of 15 *Lotus* sp. nodule isolates were obtained by PCR reaction with primers fD1 and rD1 (Weisburg et al. 1991). The studied isolates are related to known LNB and are designated to the genera: *Sinorhizobium*, *Mesorhizobium*, *Rhizobium*, *Phyllobacterium*, *Starkeya*, and *Pseudomonas*. In the reconstructed phylogenetic tree (Fig. 1), five isolates (LAC243, LPS811, LAC765, LAC813 and LAC742) are located in the *Sinorhizobium* sp. branch. Strain LAC243 is closely related to *Sinorhizobium meliloti* Rm41. The 16S rDNA sequences of LPS811, LPS765, and *Sinorhizobium meliloti* BeTF2 are identical in 100 %. Strain LAC742 exhibited 99 % 16S rDNA sequence similarity with *Sinorhizobium* sp. CCBAU05593. Four strains, LAC7511, LAC733, LAC831. and LAC814. are grouped together with the *Mesorhizobium* species and all these strains formed on the phylogram common monophyletic cluster with a high bootstrap. The 16S rDNA sequences of LAC7511 and LAC733 strains as well as *Mesorhizobium alhagi* CCNWSX672 shared 100 % identical nucleotides, whereas 16S rDNA sequences of strains LAC831 and LAC814 were identical in 100 % with those of *Mesorhizobium temperatum* CCNWSX0012-2. LAC241 and LAC553 are closely related to *Rhizobium huautlense* RA16. The two *Lotus* sp. isolates, LPS715, LAC231, and *Phyllobacterium* sp. STM4021 indicated 100 % 16S rDNA sequence similarity. The 16S rDNA sequence analysis of LAC513 and LPS664 isolates revealed that these bacteria are phylogenetically related to *Starkeya* sp. ORS1474 and *Pseudomonas* sp. VTAE174, respectively.

Phenotypic analysis

Eleven *Lotus* sp. nodule isolates, identified by 16S rRNA gene sequence analysis as bacteria of the genera *Mesorhizobium*, *Rhizobium*, *Sinorhizobium*, and *Phyllobacterium* were examined for 26 phenotypic characteristics. Phenotypic features of studied strains are shown in Table 2. All tested strains were fast growers and acid producers on YMA medium with studied sugars as a sole carbon sources. They grew at 200, 300, 400, and 500 mM NaCl, except the LAC7511 and LAC733 strains (closely related to *Mesorhizobium alhagi*) which are able to grow at 400 and 500 mM NaCl. Two studied *Sinorhizobium* genus strains, LPS811 and LAC765, and two strains, LAC831 and LAC814, classified as *Mesorhizobium temperatum* exhibited the growth at 42 °C. All tested strains were able to grow at pH 5.5–9.0, except for LAC7511 and LAC733 (described by the analysis of 16S rDNA sequences as *Mesorhizobium alhagi*) which do not tolerate pH 5.5 and 6. Regarding sugar and amino acid utilization, the majority of the tested strains were able to use glucose–fructose and L-arginine–L-leucine as carbon and nitrogen sources, respectively. Exceptions were LPS715 and LAC231 isolates, classified as the genus *Phyllobacterium* strains, which are able to grow in the presence of fructose (as a sole carbon source) and L-arginine (as a sole nitrogen source). All strains were sensitive to streptomycin (100 μg ml^{-1}) and to kanamycin (60 and 100 μg ml^{-1}). Two strains, LPS715 and LAC231, identified by 16S rRNA gene sequences as belonging to the genus *Phyllobacterium*, LAC7511 and LAC733 strains closely related to *Mesorhizobium alhagi*, and LAC831 and LAC814 isolates classified as *Mesorhizobium temperatum* strains were resistant to 60 μg $ml^{-1}$of ampicillin and streptomycin. *Lotus* sp. LAC241 and LAC553 symbionts were only resistant to 60 μg ml^{-1} of ampicillin. The tested strains classified as *Sinorhizobium* strains were diverse for antibiotic tolerance (Table 2).

Symbiotic properties

The studied *Lotus* sp. nodule isolates, identified by 16S rRNA gene sequence analysis as the members of the genera:

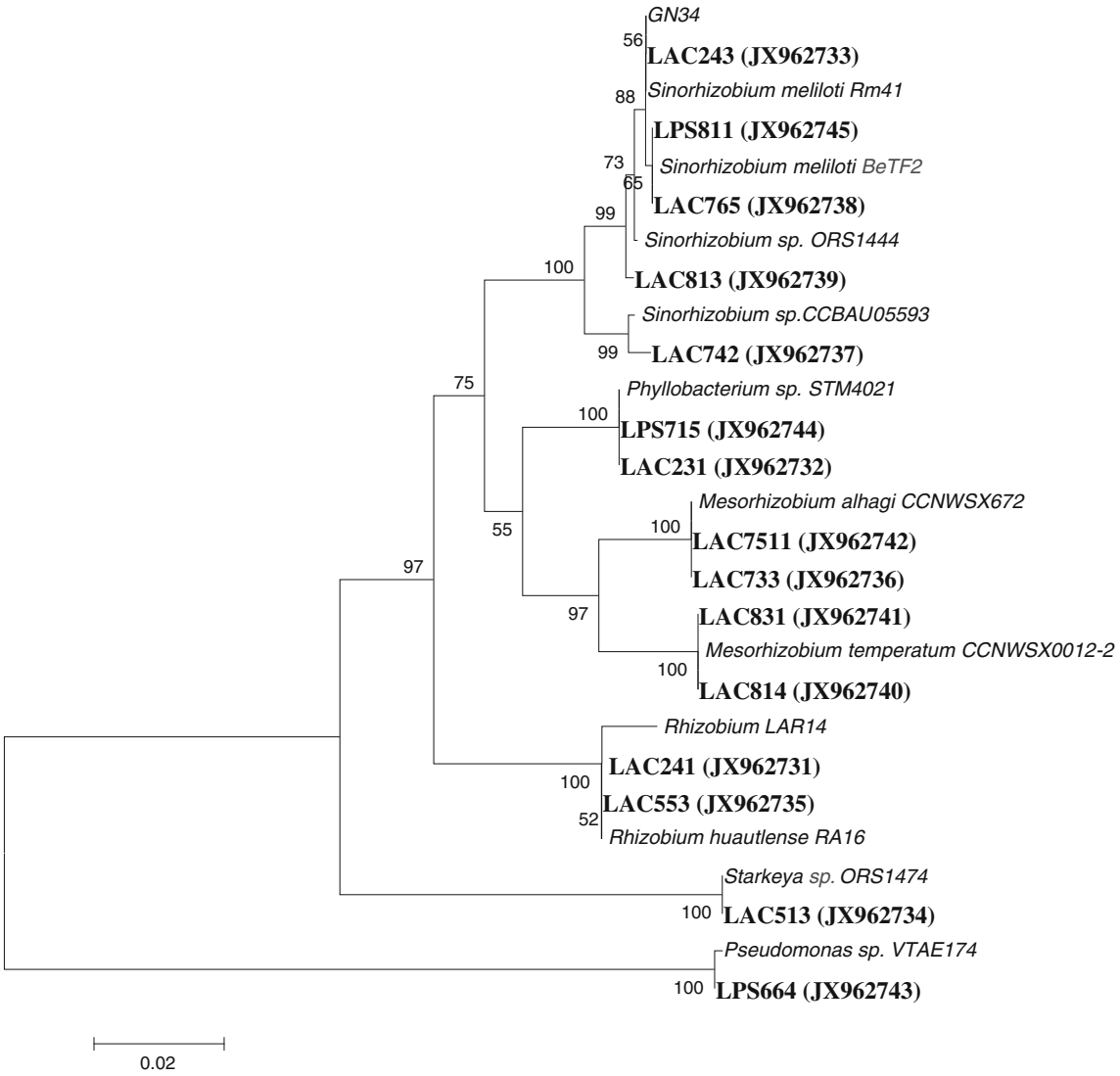

Fig. 1 Phylogenetic analysis of *Lotus creticus* and *Lotus pusillus* nodule isolates based on the 16S rRNA gene sequences. Analyses were conducted using the Neighbor-Joining method, showing the relationships between isolates and reference rhizobial species. Bootstrap values >50 (using 100 replicates) are indicated at branching points. *Bar* 0.2 % estimated substitutions. GenBank accession numbers of the new isolates are shown in parentheses

Mesorhizobium, *Rhizobium*, *Sinorhizobium* (=*Ensifer*), *Phyllobacterium*, *Starkeya*, and *Pseudomonas* were evaluated for their symbiotic potential on their original hosts (Table 3). All isolates, except LAC513, LAC231, LPS715, and LPS664, were able to form symbiosis with plants from which they were isolated. Studied *Lotus* sp. symbionts induced root nodules globular in shape with a pink-red pigmentation on their plant hosts. Two isolates, LAC733 (classified to the genus *Mesorhizobium*) and LAC813 (designated as member of the genus *Sinorhizobium* = *Ensifer*), gave significantly higher nodule numbers per *Lotus* sp. plant than the others (P <0.05). Analysis of dry biomass of plant aerial part, used as an indicator of relative N_2 fixation effectiveness, indicated that strain LAC765 (*Sinorhizobium* = *Ensifer*) was the most effective diazotroph with dry weight biomass of 91.46±0.01 in respect to the T_N control (P <0.05). The least effective isolate in nitrogen fixation was the LAC742 strain with only 62.41±0.05 of the dry biomass with respect to the T_N control. The results of symbiotic performance of studied LAC765, LAC814, LAC243, and LAC7511strains in laboratory plant tests showed that these four isolates enter into effective mutualistic interactions with *Lotus* sp. (relative effectiveness ≥70 %).

Discussion

Knowledge on genetic and functional diversity of root-nodule bacteria associated with the indigenous legume flora is very useful for inoculant strain selection. In this study, 15

Table 2 Results of phenotypic characteristics of *Lotus* sp. isolates (+ positive growth, − no growth)

Characteristic	*Sinorhizobium*					*Rhizobium*		*Mesorhizobium*				*Phyllobacterium*	
	LAC243	LPS811	LAC765	LAC813	LAC742	LAC241	LAC553	LAC7511	LAC733	LAC831	LAC814	LPS715	LAC231
Acid production	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at pH													
5.5	+	+	+	+	+	+	+	−	−	+	+	+	+
6	+	+	+	+	+	+	+	−	−	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+	+
Generation time													
2≤GT≤6													
5≤GT≤6													
NaCl tolerance													
200 mM	+	+	+	+	+	+	+	+	+	+	+	+	+
300 mM	+	+	+	+	+	+	+	+	+	+	+	+	+
400 mM	+	+	+	+	+	+	+	−	−	+	+	+	+
500 mM	+	+	+	+	+	+	+	−	−	+	+	+	+
Growth at temperature													
20 °C	−	−	−	−	−	−	−	+	+	−	−	+	+
28 °C	+	+	+	+	+	+	+	+	+	+	+	+	+
35 °C	+	+	+	+	+	+	+	−	−	+	+	+	+
40 °C	+	+	+	+	+	+	+	−	−	+	+	−	−
42 °C	−	+	+	−	−	−	−	−	−	+	+	−	−
Antibiotic resistance													
Ampicillin 60 μg ml^{-1}	−	+	+	−	−	+	+	+	+	−	−	+	+
Ampicillin 100 μg ml^{-1}	−	−	−	−	−	−	−	−	−	−	−	−	−
Streptomycin 60 μg ml^{-1}	+	−	−	+	+	−	−	−	−	+	+	+	+
Streptomycin 100 μg ml^{-1}	−	−	−	−	−	−	−	−	−	−	−	−	−
Kanamycin 60 μg ml^{-1}	−	−	−	−	−	−	−	−	−	−	−	−	−
Kanamycin 100 μg ml^{-1}	−	−	−	−	−	−	−	−	−	−	−	−	−
Utilization of carbohydrates													
Glucose	+	+	+	+	+	+	+	+	+	−	−	+	+
Fructose	+	+	+	+	+	+	+	+	+	−	−	−	−
Utilization of amino-acids													
L-Leucine	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Arginine	+	+	+	+	+	+	+	+	+	+	+	−	−

Table 3 Results of symbiotic potential of root wild legume nodulating bacteria

Isolate	Number of nodules	Shoot dry weight (g/plant)	Relative effectiveness (% of T_N control)
Lotus creticus			
LAC241	19.00±01.20 b	01.78±0.02 d	67.38±0.02
LAC243	09.67±00.12 g	01.91±0.01 b	75.78±0.21
LAC553	14.00±01.22 f	01.65±0.02 e	66.28±0.02
LAC733	24.32±01.52 a	01.73±0.02 d	65.42±0.02
LAC742	18.30±01.56 c	01.65±0.05 e	62.41±0.05
LAC7511	15.00±01.33 e	01.85±0.05 c	70.07±0.05
LAC765	20.00±00.98 b	02.42±0.01 a	91.46±0.01
LAC813	23.40±00.95 a	01.71±0.05 d	64.85±0,05
LAC814	17.60±00.70 d	02.32±0.01 a	87.68±0.01
LAC831	18.32±01.52 c	01.65±0.02 e	66.28±0.02
Lotus pusillus			
LPS811	08.01±00.27 g	01.87±00.05 c	74.15±00.22

Lowercase letters show statistical differences between isolates; values with the same letter are statistically similar and those with different letters are statistically different

bacterial isolates, representing different 16S rRNA-RFLP genomogroups, which were isolated from root nodules of *Lotus creticus* and *L. pusillus* and studied earlier by Rejili et al. (2009) were characterized by phenotypic features and 16S rDNA sequence analysis.

By using the comparative 16S rRNA gene sequence analysis, *Lotus* sp. isolates are grouped on the phylogram in the *Sinorhizobium* and *Rhizobium* genera cluster, as are many other indigenous legume symbionts from Tunisia (Zakhia et al. 2004; Ben Romdhane et al. 2006; Mahdhi et al. 2012), and also located in the *Mesorhizobium* species branch as described for the first time by Rejili et al. (2009) in the case of *Lotus* sp. nodule isolates derived from Tunisian arid soil. Among our *Lotus* sp. isolates, *Bradyrhizobium* genus strains were not detected; however, Jordan (1982) reported that such slow-growing rhizobia are *Lotus* species symbionts. Similarly to our results, León-Barrios and Donate-Correa (2007) found that rhizobia nodulating *Lotus* sp. derived from Spain belong to the genera: *Mesorhizobium*, *Sinorhizobium*, and *Rhizobium*. In our studies, we showed that 16S rRNA gene sequences of strains LAC7511 and LAC733 as well as *Mesorhizobium alhagi* CCNWSX672 (symbiont of *Alhagi sparsifolia*; Chen et al. 2010) shared 100 % identical nucleotides. The 16S rRNA gene sequences of the two LAC831 and LAC814 strains indicate high similarity rates to 16S rDNA sequence of *Mesorhizobium temperatum* CCNWSX0012-2, (nodulating *Astragalus adsurgens*; Gao et al. 2004). Close phylogenetic relationships between bacteria nodulating *Lotus edulis* and *L. ornithopodioides* as well as *L. cytisoides* and *Mesorhizobium loti* type strain were documented by Safronova et al. (2004) on the basis of 16S rDNA PCR-RFLP analysis. Among *Lotus tenuis* symbionts isolated in Argentina by Estrella et al. (2009) some of them were closely related to *M. amorphae*, *M. mediterraneum*, *M. tianshanense*, and the type strain *M. loti* NZP2213.

In our collection of *Lotus* sp. nodule isolates, studied by 16S rRNA gene sequence analysis, two strains, LPS715 and LAC231, are classified as *Phyllobacterium* and two others, i.e. LPS664 and LAC513, are identified as the members of the genus *Pseudomonas* and *Starkeya,* respectively. The 16S rDNA sequences of two isolated *Phyllobacterium* strains are similar to the 16S rRNA gene sequence of *Phyllobacterium* sp. STM4021, which was isolated by Mahdhi et al. (2007) from the root nodules of *Genista saharae* growing in arid Tunisian soil. Legume endophytes have also been reported among the genus *Pseudomonas* bacteria (Elvira-Recuenco and Van Vuurde 2000). We also isolated the genus *Pseudomonas* strains from the nodules of *Hedysarum spinosissimum* (Mahdhi et al. 2012), similar to Benhizia et al. (2004) and Muresu et al. (2008). Shiraishi et al. (2010) reported that *Pseudomonas* genus strains formed root nodules on black locust with differentiated nodule tissues. Zakhia et al. (2006) found that one strain isolated from root nodules of *Lotus argenteus,* growing in the infra-arid regions of Tunisia, belongs to the genus *Starkeya*, but it failed to nodulate its original plant host in vitro. The genera studied by us the, *Phyllobacterium*, *Pseudomonas*, and *Starkeya* strains, failed to form nodules on *Lotus creticus* and *L. pusillus* in laboratory plant tests, although they were isolated from these legumes. We suppose that these bacteria entered nodules due to co-infection with natural *Lotus* sp. symbionts. Further investigations are needed to explain this phenomenon.

Phenotypically, all tested isolates were acid producers, sensitive to 100 μg ml^{-1} of ampicillin and kanamycin, and able to grow at pH 5.5–9.0, except LAC7511 and LAC733 strains (described as closely related to *Mesorhizobium alhagi*) which do not tolerate pH 5.5 and 6. Cooper (1982) reported that rhizobial strains which nodulate *Lotus* sp. show marked differences in acid tolerance. Among *Lotus* sp. symbionts are moderately slow-growing *Mesorhizobium loti* (Jarvis et al. 1997),

tolerant to pH 4.5, and slow-growing *Bradyrhizobium* sp. (Jordan 1982) which are sensitive to this pH. As for salinity tolerance, our results showed that only two isolates, i.e. LAC7511 and LAC733, are enable to grow at 400 and 500 mM NaCl. Safronova et al. (2004) described rhizobia nodulating *L. edulis*, *L. ornithopodioides*, and *L. cytisoides* which tolerate up to 2 % NaCl. *Lotus* sp. nodule bacteriaI isolated by us and identified as members of the genus *Sinorhizobium*, were even able to grow at 35°–40 °C, and two of them also multiply at 42 °C (Table 2). Safronova et al. (2004) reported that *Lotus* sp. mesorhizobium symbionts cannot grow at temperatures higher than 35 °C. It has been reported that many rhizobia form symbiosis with legumes in Tunisia (Zakhia et al. 2004; Zribi et al. 2004; Rejili et al. 2009; Mahdhi et al. 2012) and most new *Lotus* sp. nodule isolates described in this paper tolerate a high temperature (40 °C) as well as high NaCl concentration (up to 3 %). This may be an effect of specific adaptation to the high temperatures and soil salinity that characterize arid regions (Karanja and Wood 1988; Abdelmoumen et al. 1999).

Lotus sp. LAC765 symbiont from our collection, closely related to *Sinorhizobium* genus bacteria, is the most efficient in nitrogen fixation strain among studied isolates (Table 3). Its ability to grow at high temperature (42 °C) and high NaCl concentration (500 mM) renders this LAC765 isolate as a promising bacterium for *Lotus* sp. inoculation, provided that this strain possess high competitiveness for nodule occupancy.

In conclusion, our investigation showed that LNB originating from root nodules of *Lotus creticus* and *L. pusillus* growing in arid Tunisian soil are genetically diverse and they are designated to the genera: *Sinorhizobium*, *Rhizobium*, and *Mesorhizobium*. Among *Lotus* sp. nodule isolates were bacteria which in laboratory plant tests did not show the ability to induce these root structures. They were classified into the genera *Phyllobacterium*, *Starkeya*, and *Pseudomonas*. In order to provide further information on the diversity of rhizobia forming symbiosis with *Lotus* sp. growing in Tunisia, further studies are necessary and such investigations are in progress.

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