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Phenotypic and genotypic diversity of *Genista saharae* microsymbionts from the infra-arid region of Tunisia*

M. Mahdhi[1], A. Nzoué[2], F. Gueye[2], C. Merabet[2], P. de Lajudie[2] and M. Mars[1]

1 Laboratoire de Biotechnologies végétales Appliquées à l'Amélioration des cultures, Faculté des Sciences de Gabès, Cité Erriadh Zrig Gabès, Tunisia

2 UMR 113 Symbioses Tropicales et Méditerranéennes INRA/AGRO-M/CIRAD/IRD, Campus International de Baillarguet, Montpellier Cedex, France

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Correspondence
M. Mars, Faculté des Sciences de Gabès, Cité Erriadh Zrig 6072 Gabès, Tunisia.
E-mail: mohamedmars2005@yahoo.fr

*Nucleotide sequence data reported here are available in the GenBank database under the following accession numbers: STM4020 (EF100513), STM4015 (EF100514), STM4016 (EF100515), STM4018 (EF100516), STM4021 (EF100517), STM4022 (EF100518), STM4023 (EF100519), STM4024 (EF100520), STM4025 (EF100521), STM4026 (EF100522), STM4027 (EF100523), STM4028 (EF100524), STM4029 (EF100525), STM4030 (EF100526), STM4031(EF100527), STM4032 (EF100528), STM4033 (EF100529).

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Abstract

Aims: *Genista saharae*, indigenous of Sahara, is a spontaneous shrub that plays an important ecological role for the preservation and fertility of poor and eroded soils. This legume has not been examined for its root nodule bacteria. The taxonomic diversity of bacteria from root nodules of *G. saharae* growing in the infra-arid region of Tunisia was investigated.

Methods and Results: A total of 28 bacterial strains isolated from root nodules of *G. saharae* grown in Tunisian soil were characterized using a polyphasic approach including phenotypic characteristics, PCR-RFLP of 16S rDNA and 16S rRNA gene sequencing. It was found that new isolates are diverse and affiliated to *Ensifer* (75%), *Rhizobium* (10%) and *Phyllobacterium* (15%). The *Phyllobacterium* isolates lacked the capacity for nodule formation on this plant.

Conclusions: *Genista saharae* formed nodules with diverse rhizobia in Tunisian soils. Furthermore, our results support the presence of non-nodulating commensal strains *(Phyllobacterium)* in legumes nodule.

Significance and Impact of the Study: This study is the first report on the characterization of *G. saharae* microsymbionts in Tunisia.

Introduction

Desertification affects vast areas in Tunisia particularly in arid lands. Desertification processes result in the degradation of the soil and vegetation cover, deforestation and exposure of the arable lands to erosion. Trees and herbaceous plants in particular, because of their longevity and capacity to develop powerful root systems, were used to stop the current degradation process of vegetation cover and to preserve eroded soils (Ferchichi 1996). In this context, indigenous legumes of arid regions such as *Genista saharae*, because of their nitrogen fixing symbiosis with legume nodulating bacteria (LNB), collectively called rhizobia, contribute to soil fertility by enhancing soil nitrogen content and organic matter. They provide high-quality animal fodder, prevent erosion and contribute to soil stabilization and ecosystem restoration (Fagg and Stewart 1994).

The plant genus *Genista* includes 87 species, of which only few have been characterized for their bacterial microsymbionts. The microsymbionts of *Genista tinctoria*, *Genista monspessulana* and *Genista linifolia* (Fernando and Jesus 1998; Kalita and Malek 2004) have been classified as *Bradyrhizobium* species. Zakhia *et al.* (2004) found that two strains isolated from *Genista microcephala* grown in infra-arid region of Tunisia were *Rhizobium*.

Genista saharae, growing in Sahara, is a spontaneous shrub legume belonging to the Fabaceae. This evergreen legume is well adapted to drought stress and it is a good contributor to dune stabilization. Until now, no report on the symbiotic and taxonomic characterization of the nodulating bacteria associated to *G. saharae* has been examined. The aim of this study was to investigate, using phenotypic and genetic properties, the taxonomic diversity of a collection of 28 rhizobial bacteria isolated from root nodules of *G. saharae* sampled in the infra-arid region of Tunisia.

Materials and methods

Bacterial isolation and growth conditions

Bacteria were isolated from naturally occurring root nodules sampled on *G. saharae* plants growing in Nafta (34°49′59″N, 7°42′7″E). Isolation of bacteria from nodules was carried out as described by Vincent (1970). The purity of each isolate was ensured by repeated streaking of single colonies onto yeast extract mannitol agar plates (YMA). The isolates and reference strains used in this study are listed in Table 1.

Nodulation test

The *G. saharae* seeds, collected from spontaneous plant growing in Nafta, were surface-sterilized in 98% sulphuric acid for 1 h, washed thoroughly and germinated in petri dishes. Germinated seedlings were aseptically transferred to glass tubes containing Fahraëus seedling agar (Vincent 1970). Inoculation was performed 48 h after transfer with 1 ml of the appropriate isolate of rhizobial broth culture containing approximately 10^9 cells ml^{-1}. Controls, not inoculated, were included. For root nodulation trials, three plants were routinely tested with each isolate. Three weeks later, plants were examined for root nodulation.

Phenotypic characterization

Acid and alkali production was determined in YMA medium with bromothymol blue indicator (0·0025% w/v). The modified-YMA medium (Somasegaran and Hoben 1994) was used to analyse the ability of isolates to use carbohydrates (1% w/v: glucose, galactose, fructose and sucrose) and amino acids (0·1% w/v: L-arginine, L-proline, L-leucine and L-tyrosine) as a sole carbon and nitrogen sources, respectively. Determination of NaCl-tolerance (1, 2, 3, 4 and 5% w/v); maximum growth temperature (15, 28, 35, 40 and 45°C); antibiotic resistance (ampicillin, 60 and 100 $\mu g\ ml^{-1}$; streptomycin, 60 and 100 $\mu g\ ml^{-1}$; kanamycin, 60 and 100 $\mu g\ ml^{-1}$ and nalidixic acid, 20, 50 and 100 $\mu g\ ml^{-1}$) and growth at different pH (4·0, 6·0, 7·0, 9·0 and 12·0) were assessed on YMA as described by Mohamed *et al.* (2000).

PCR-RFLP of 16S rRNA gene

DNAs extracted according to Mhamdi *et al.* (2002) were used as templates. Primers FGPS6 and FGPS1509 (Normand *et al.* 1992), and procedures described previously (Mahdhi and Mars 2006) were used for PCR amplification of 16S rRNA gene. PCR products were checked by electrophoresis on 0·9% agarose gel. Aliquots (6 μl) of PCR products were digested with restriction enzymes. The following enzymes were used: *Rsa*I, *Msp*I, *Alu*I, *Taq*I, *Hin*fI and *Nde*II. Restriction DNA was analysed by horizontal electrophoresis in 4% agarose gel at 4 V cm^{-1} for 2 h.

16S rRNA gene sequencing and sequence analysis

Seventeen isolates, choosen as representative for different 16S rDNA type, were grown and subcultured in YMA broth. Genomic DNA was extracted and the 16S rRNA gene region was amplified as described above. The PCR amplification products were purified from agarose gel using QIAquick GEL extraction Kit (Qiagen, Courtaboeuf, France) and sequenced by using the primers FGPS6, FGPS1509 and 16S-370f. Sequence reactions were performed using the ABI Prism BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol and analysed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Sequences were assembled using ChromasPro version 1.34 software and aligned with ClustalX and Genedoc software packages. Initially a blast search conducted using the National Center of Biotechnological Information (NCBI) website was carried out to find the closest relatives on the basis of sequence similarity. 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 evolution and support of internal branches was assessed using 1000 bootstrap replications.

Results

Nodulation test and phenotypic characterization

For the 28 isolates, only four isolates, which were classified as *Phyllobacterium* by 16S rRNA gene sequencing analyses (see below), failed to nodulate their host plant of origin. The other isolates formed three to six nodules per plantlet after 3 weeks (Table 1).

Table 1 Isolates and reference strains used in this study and their relevant characteristics

Isolates and reference strains	Growth at 40°C	NaCl tolerance (3%)	Utilization of carbohydrates				Utilization of amino acids				Antibiotics resistance				Nodulation test (nodule number per plant)†	16S rDNA type
			Glucose	Galactose	Fructose	Sucrose	L-leucine	L-arginine	L-tyrosine	L-proline	Am (100 μg ml^{-1})	Str (100 μg ml^{-1})	Ka (100 μg ml^{-1})	Na (100 μg ml^{-1})		
GN34 = STM4025	+	+	+	+	+	+	+	+	−	+	−	−	+	+	6 ± 0·326	1
GN27	+	+	+	+	+	+	+	+	+	+	+	+	+	+	5 ± 0·326	1
GN2	+	+	+	+	+	+	+	+	+	+	+	+	−	+	4 ± 0·326	1
GN56	+	−	+	+	+	+	+	+	+	+	+	+	−	+	3 ± 0·326	1
GN51	+	−	+	+	+	+	+	+	+	+	+	−	−	+	3 ± 0·326	1
GN68	+	+	+	+	+	+	+	+	+	+	−	+	+	+	3 ± 0·653	1
GN54 = STM4030	+	+	+	+	+	+	+	+	+	+	+	+	−	+	4 ± 0·326	1
GN5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	5 ± 0·326	1
GN7	+	−	+	+	+	+	+	+	+	+	+	−	−	+	4 ± 0·653	1
GN22 = STM4020	+	+	+	+	+	+	+	+	+	+	+	+	−	+	3 ± 0·565	1
GN76 = STM4033	−	+	+	−	−	−	+	+	−	+	+	+	+	−	3 ± 0·864	1
GN49	+	+	+	+	+	+	+	+	+	+	+	+	−	+	3 ± 1·177	1
GN10 = STM4016	+	−	+	+	+	+	+	+	+	+	+	+	−	+	3 ± 0·326	2
GN64 = STM4032	−	+	+	+	+	+	+	+	+	+	+	+	−	+	3 ± 0·864	2
GN59	+	+	+	+	+	+	+	+	+	+	+	+	−	+	4 ± 0·864	2
GN46	+	+	+	+	+	+	+	+	+	+	+	+	−	+	5 ± 0·326	2
GN26 = STM4022	+	+	+	+	+	+	+	−	+	+	−	+	−	−	0	3
GN33 = STM4024	+	+	+	−	−	+	−	+	−	+	+	+	−	−	0	3
GN25 = STM4021	+	+	−	−	+	−	+	−	−	−	+	−	−	−	0	4
GN44 = STM4028	−	+	+	+	+	+	+	+	+	+	−	−	+	+	5 ± 0·979	5
GN36 = STM4026	+	−	+	+	+	+	+	+	+	+	+	+	−	+	4 ± 0·326	6
GN9 = STM4015	+	−	+	+	+	+	+	+	+	+	+	+	−	+	4 ± 0·326	7
GN55 = STM3031	+	+	+	+	+	+	+	−	+	+	−	+	−	+	3 ± 0·565	8
GN38 = STM4027	+	+	+	−	−	+	+	+	+	+	+	+	+	+	3 ± 0·864	9
GN15 = STM4018	+	+	+	+	+	+	+	+	+	+	+	+	−	+	0	10
GN50 = STM4029	+	+	+	+	+	+	+	−	−	+	−	+	−	−	5 ± 0·326	11
GN24	+	+	+	+	+	+	+	+	+	+	+	−	−	−	3 ± 0·653	12
GN29 = STM4023	+	+	+	+	+	+	+	+	+	+	+	+	+	+	6 ± 0·326	12
B. japonicum NZP5549^{T},= LMG6138^{T}	−	−	−	−	+	−	+	−	+	+	−	−	−	−	Nt	13
R. mongolense STM246^{T} = LMG1941^{T}	+	−	+	+	+	+	+	+	+	+	−	−	−	+	Nt	14
R. etli CFN42^{T} = USDA9032	−	−	+	+	+	+	+	−	+	+	−	+	−	+	Nt	15
R. galegae HMBI540^{T} = LMG6214^{T}	+	−	+	+	+	+	+	+	+	+	−	−	−	−	Nt	16
R. tropci IIB CIAT899^{T} = HAMBI1163^{T}	+	−	+	+	+	+	+	+	+	+	+	+	−	+	Nt	17
M. loti ORS664 = LMG6125^{T}	−	−	+	−	+	+	+	−	+	−	−	−	−	−	Nt	18
M. mediterraneum ORS2739^{T} = LMG17148^{T}	+	+	+	+	+	+	+	+	+	+	+	+	−	+	Nt	19
E. meliloti ORS665^{T} = LMG6133^{T}	+	+	+	+	+	+	+	+	+	+	−	−	−	−	Nt	1

Am, ampicillin; Str, streptomycin; Ka, kanamycin; Na, nalidixic acid; Nt, not tested.
^{T}Strain type.
† Data are the mean of three replicates ± SEM.

Phenotypically, all isolates are fast-growers, acid producers and are able to use a wide range of carbon and nitrogen sources (Table 1). The majority of the isolates are able to grow at pHs between 6·0 and 12, but none could grow at pH 4·0. Most of the tested isolates tolerate NaCl concentrations from 1 to 3%. One isolate (STM4031) is able to grow in the presence of 4% NaCl. The majority of isolates are able to grow at temperatures between 28 and 40°C, but not at 45°C. Four isolates (GN2, GN27, GN34 and GN50) are able to grow at 15°C. Most of the isolates are resistant to 100 μg ml^{-1} of ampicillin and streptomycin, but not to kanamycin (100 μg ml^{-1}) except seven isolates. The majority of isolates (21 isolates) were resistant to 100 μg ml^{-1} of Nalidixic acid (Table 1).

PCR-RFLP analysis of amplified 16S rRNA gene

All strains produced a single band of the expected size of 1500 bp. The analysis revealed three to five different restriction patterns per enzyme. So, 19 16S rDNA types were distinguished among the 28 isolates and eight reference strains. 12 rDNA types were identified among the isolates. Each 16S rDNA type comprising 1 to 12 isolates (Table 1). Only the 16S rDNA type 1 included both new isolates (11 isolates) and a reference strain (*Ensifer meliloti* LMG6133^T).

16S rDNA sequencing

The nearly full length sequences of 16S rDNA (16 isolates) and partial 16S rDNA sequence (one isolate) were obtained for 17 isolates selected to represent the 12 rDNA types: four isolates from type 1, two from types 2 and 3 and one from the other rDNA types. Sequences were compared with the 16S rDNA sequences available in GenBank. In the reconstructed phylogenetic tree (Fig. 1), six isolates (STM4020, STM4025, STM4026 STM4028, STM4030 and STM4033) were grouped with *E. meliloti* LMG6133^T and five isolates (STM4015, STM4016, STM4027, STM4031 and STM4032) were closely related to *Ensifer* sp. ORS1400. One isolate (STM4023) representing 16S rDNA type 12 grouped with *Rhizobium.* sp. ORS1465. Three isolates (STM4021, STM4022 and STM4024) grouped together and were close to *Phyllobacterium leguminum* ORS1419^T. Strain STM4018 is peripheral to the *Phyllobacterium* branch. In addition, partial 16S rDNA sequence of strain STM4029 (1060 bp), representing 16S rDNA type 11 is similar to *Rh. huautlense* Se128.

Discussion

In this study, we characterized 28 isolates associated to *G. saharae* by a polyphasic approach (Vandamme *et al.* 1996) including phenotypic, genotypic and phylogenetic

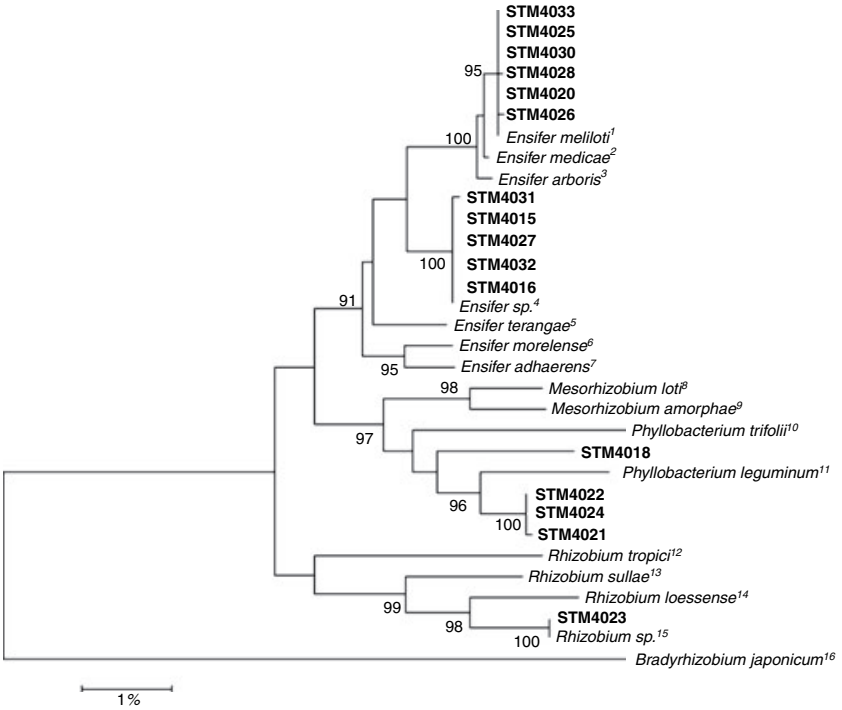

Figure 1 16S rRNA gene sequence-based dendrogram obtained by neighbour-joining method, showing the phylogenetic positions of *Genista saharae* isolates. Significant bootstraps (>80%) are indicated as percentages (1000 replications). Bar = 1% nucleotide divergence. Following are designation and accession number of each reference strain used: 1, LMG6133^T (X67222); 2, A321^T (L39882); 3, HAMBI1552^T (Z78204); 4, ORS1400 (AY500255); 5, LMG7834^T (X68388); 6, Lc04^T (AY024335); 7, ATCC33212^T (AF191739); 8, LMG6125^T (X67229); 9, ACCCC19665^T (AF041442); 10, PETP02^T (AY786080); 11, ORS1419^T (AY785323); 12, IIBLMG9517^T (X67234); 13, IS123^T (Y10170); 14, CCBAU7190B^T (AF364069); 15, ORS1465 (AY500261); 16, LMG6138^T (X66024).

analyses. All isolates except STM4021, STM4022, STM4024 and STM4018 are able to induce nodules in *G. saharae*. Thus, these four isolates can be considered as opportunistic as already proposed (Tan *et al.* 1999; Zakhia *et al.* 2006). Phenotypically, all of the isolates are resistant to high temperature and most of them continued to grow at 40°C. In addition, the majority of the isolates are able to grow in a medium containing high NaCl concentration (3%). This may be a specific adaptation to the high soil temperatures and salinity in arid regions as described by Karanja and Wood (1988). By genotypic analysis, we found that about 75% of the isolates are related to genus *Ensifer*. Some of them (16S rDNA types 2, 7, 8 and 9) are closely related to *Ensifer* sp. ORS1400 isolated from root nodule of *Argyrolobium uniflorum* growing in Tunisia (Zakhia *et al.* 2004). Phylogenetic analysis based on other genes, namely housekeeping ones, should be performed to clarify their taxonomic status. 16S rDNA sequence of representative isolate for 16S rDNA type 12 (STM4023) is also closely related to another Tunisian legume nodule bacteria corresponding to *Rhizobium* sp. ORS1465 isolated from root nodule of *Anthyllis henoniana* (Zakhia *et al.* 2004).

In this study, we also obtained four *Phyllobacterium* isolates. All of them failed to induce nodules on *G. saharae* roots and are similar to *P. leguminum* ORS1419^T, which originated also from Tunisian soils (Mantelin *et al.* 2006). These strains may represent a new species to be further characterized by complementary taxonomic techniques. This finding confirmed that reported by Mantelin *et al.* (2006) with *Phyllobacterium* strains isolated from root nodules of *Astragalus algerianus* growing in Tunisian soil. The non-nodulation *in vitro* of *G. saharae* by *Phyllobacterium* strains originally isolated from this host may indicate that this legume is not the natural host of these strains. Howover, these strains may also be considered as commensal bacteria that lack the capacity to induce nodule development on any legume. It would now be interesting to demonstrate the existence of nod genes in these strains by southern hybridization before the status of these strains as nodules symbionts could be considered.

Our results showed that Tunisian *Genista*-rhizobia belong to *Ensifer* and *Rhizobium* species. No *Bradyrhizobium* strains were recovered. This finding confirms those reported by Mahdhi and Mars (2006) with *Retama raetam* nodulating bacteria in Tunisia. Zakhia *et al.* (2004) mentioned that among 69 strains isolated from wild legumes in infra-arid zone of Tunisia, only two strains were assigned to *Bradyrhizobium*. However, previous studies (Fernando and Jesus 1998; Kalita and Malek 2004) found that other *Genista* species from Poland and Spain were nodulated by only *Bradyrhizobium* spp. This diversity of rhizobia nodulating *Genista* species in nearby locations (Poland, Spain and Tunisia) may be in relation with climatic and edaphic conditions.

In summary, our study is the first report on the characterization of *G. saharae* microsymbionts in Tunisia. We evidenced a novel biodiversity among bacteria isolated from *Genista* species. Three genera, *Ensifer, Rhizobium* and *Phyllobacterium* were represented among the *G. saharae* isolates, most of which were related to previously described LNB in Tunisian soils. Some isolates may represent new species. Further characterization (e.g. DNA–DNA hybridization, sequencing of others genes) is required to ascertain their taxonomy.

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