

Genetic Diversity and Phylogeny of Rhizobia That Nodulate *Acacia* spp. in Morocco Assessed by Analysis of rRNA Genes

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Forty rhizobia nodulating four *Acacia* species (*A. gummifera*, *A. raddiana*, *A. cyanophylla*, and *A. horrida*) were isolated from different sites in Morocco. These rhizobia were compared by analyzing both the 16S rRNA gene (rDNA) and the 16S-23S rRNA spacer by PCR with restriction fragment length polymorphism (RFLP) analysis. Analysis of the length of 16S-23S spacer showed a considerable diversity within these microsymbionts, but RFLP analysis of the amplified spacer revealed no additional heterogeneity. Three clusters were identified when 16S rDNA analysis was carried out. Two of these clusters include some isolates which nodulate, nonspecifically, the four *Acacia* species. These clusters, A and B, fit within the *Sinorhizobium* lineage and are closely related to *S. meliloti* and *S. fredii*, respectively. The third cluster appeared to belong to the *Agrobacterium-Rhizobium galegae* phylum and is more closely related to the *Agrobacterium tumefaciens* species. These relations were confirmed by sequencing a representative strain from each cluster.

Acacia is a legume tree which fixes nitrogen through a symbiosis with rhizobia. In Morocco, *A. raddiana* and *A. gummifera* (native species) are found in arid and Saharan areas. These acacias are generally very sparse and overused by local people, who have no other fuelwood. Regeneration of the trees is made difficult owing to overgrazing, which adds its limiting effects to those of aridity. Therefore, there is a crucial need to rehabilitate these natural populations in order to halt desert encroachment and to restore soil fertility. One possible approach to improve rehabilitation programs is to isolate rhizobia from these areas and obtain data on their diversity and nodulation spectrum.

In the last few years, many studies have investigated rhizobia associated with legume trees (8, 24, 52) and various techniques have been used to identify and describe strains. These studies concluded that there is a large heterogeneity among the strains, and, accordingly, they were often divided into several groups (2, 6, 8, 9, 15, 27, 52). The low similarity among these groups shows that trees can form nodules and fix nitrogen with several different clusters of rhizobia (6, 15, 23, 33).

In eubacteria, rRNA genetic loci include 16S, 23S and 5S genes, which are separated by two internal transcribed spacer (ITS) regions. DNA sequences in the 16S-23S spacer are known to exhibit a great deal of sequence and length variation (17). These variations are used to differentiate genera, species, and strains of prokaryotes (4, 12-14, 26) especially in the alpha division of the proteobacteria (32). In contrast and with a few exceptions only, the rRNA genes (rDNAs) are similar in length throughout the bacterial kingdom and contain highly conserved regions as well as regions that vary according to species and family (48). Consequently, sequence analysis of the 16S rRNA gene, which is the most extensively studied, is a well-

established standard method for phylogenetic (50) and taxonomic (1, 48) studies. These 16S rDNA sequence analyses support the well-established subdivision of rhizobia into species and genera (47, 49, 50). A rapid and versatile method based on restriction endonuclease site differences in PCR-amplified 16S rDNA has been used as an alternative to sequencing (35, 36). In rhizobia, species assignments based on PCR with restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene were consistent with the established taxonomic classification (20).

Our objective was to determine the genetic diversity among rhizobia isolated from different soils and tested on four *Acacia* species and to find their phylogenetic positions within the family *Rhizobiaceae* by using 16S rRNA analysis.

MATERIALS AND METHODS

Bacterial strains. Strains of rhizobia were isolated from root nodules harvested from young seedlings of four *Acacia* species (*A. horrida*, *A. gummifera*, *A. raddiana*, and *A. cyanophylla*) inoculated with 1 ml of a soil suspension. These soil samples had been collected in various regions of Morocco (Fig. 1) and were screened for the presence of rhizobia as described by De Lajudie et al. (8).

All strains of rhizobia used in this study are listed in Table 1. Type or representative strains of rhizobia and agrobacteria were obtained from the Collection des Souches de l'ORSTOM, Dakar, Senegal.

All the rhizobia were maintained on yeast extract mannitol medium (46). **Plant infection tests.** The *Rhizobium* strains were tested for the ability to nodulate *A. horrida*, *A. gummifera*, *A. raddiana*, and *A. cyanophylla*. The *Acacia* seeds were surface sterilized in 1% (wt/vol) calcium hypochlorite for 10 min, rinsed with sterile water, and then scarified. The seeds were germinated for 72 h on 0.7% (wt/vol) agar medium in a growth chamber at 28°C, and the seedlings were placed aseptically in Gibson tubes (12) supplemented with a nitrogen-free plant nutrient solution (3). Each tube was inoculated with a rhizobial suspension from an early-stationary-phase culture. Uninoculated plants and plants to which nitrogen (10 mM KNO₃) was added were used as controls. Three replicates were prepared for each treatment. After 50 days, the plants were harvested and the number of nodules was counted.

DNA extraction. Stationary-phase Tp broth cultures (1.5 ml each) were centrifuged, and the cells were resuspended in sterile distilled water, boiled for 10 min, and immediately cooled on ice to induce lysis. Tp medium contained (per liter of distilled water) 4 g of Bacto Peptone (Difco), 0.5 g of Bacto Tryptone

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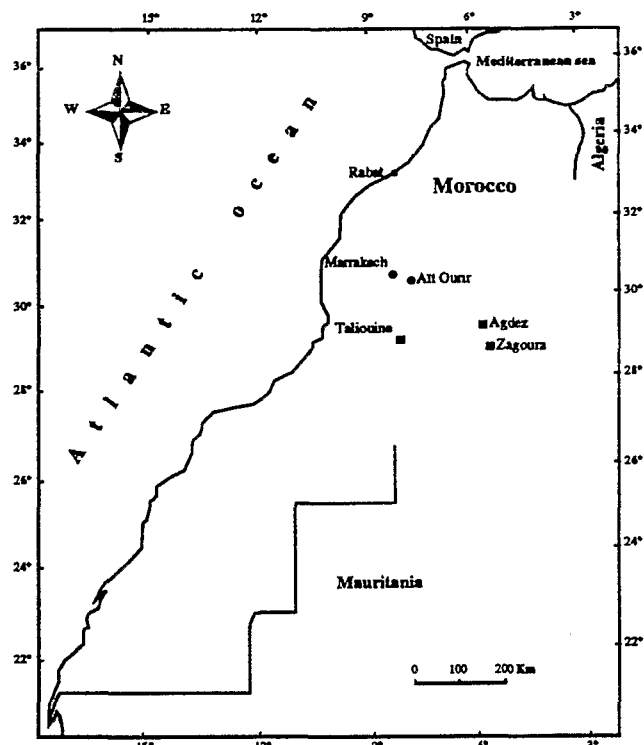


FIG. 1. Locations of soil sampling in Morocco. Symbols: ●, *Acacia gummifera*; ■, *Acacia raddiana*.

(Difco), 0.5 g of yeast extract (Difco), 0.2 g of $MgSO_4 \cdot 7H_2O$, and 0.2 g of $CaCl_2 \cdot 2H_2O$.

16S-23S spacer amplification. The intergenic region between the 16S and 23S rDNAs was amplified by PCR with primers derived from the 3' end of the 16S rDNA (FGPS1490-72; 5'-TGCGGCTGGATCCCCCTT-3') (28) and from the 5' end of the 23S rDNA (FGPL132'-38; 5'-CCGGTTTCCCCATTCGG-3') (35). PCR amplification was performed with a Perkin-Elmer Cetus model 2400 thermocycler. Each reaction mixture contained 5 μ l of lysed cell suspension, 1 \times reaction buffer (Gibco BRL, Cergy-Pontoise, France), 1.5 mM $MgCl_2$, 20 μ M (each) dATP, dTTP, dCTP, and dGTP (Pharmacia-LKB), primers at 0.1 μ M each, and 2.5 U of *Taq* polymerase (Gibco BRL). The PCR temperature profile used was 94°C for 3 min followed by 35 cycles consisting of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, with a final extension step at 72°C for 3 min. Reaction efficiency was estimated by electrophoresis of 5 μ l of the amplification product on a 0.8% (wt/vol) horizontal agarose gel. After migration, the gels were stained in an aqueous solution of ethidium bromide (1 mg/ml) and photographed under UV illumination with Ilford HP5 films.

16S rRNA gene amplification. The conditions for 16S gene amplification were the same as those used for 16S-23S spacer amplification, except that the annealing steps took place at 60°C and the extension periods were 2 min in each cycle. The prokaryotic specific primers used for 16S rRNA gene amplification were FGPS4-281bis (5'-ATGGAGAAGTCTTGATCCTGGCTCA-3') and FGPS 1509'-153 (5'-AAGGAGGGGATCCAGCCGCA-3') (31).

Restriction fragment analysis. Aliquots (8 μ l) of PCR products were digested with restriction endonucleases (Boehringer Mannheim, Meylan, France) as specified by the manufacturer but with an excess of enzyme (5 U per reaction) in a total volume of 10 μ l. The following enzymes were used: *AluI*, *DdeI*, *CfoI*, *HhaI*, *HinII*, *HpaI*, *RsaI*, *Sau3A*, and *TaqI*. The restriction fragments were separated by horizontal electrophoresis in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA [pH 8.0]) with a 2% (wt/vol) MetaPhor (FMC BioProducts, Rockland, Maine) agarose gel containing 1 μ g of ethidium bromide per ml. The gels were run at 80 V for 3 h and immediately photographed with Ilford HP5 films with a 320-nm UV source.

16S rRNA gene sequencing. The 16S rRNA gene of strains MSMC 211, MSMC 310, and MSMC 411 was amplified. The amplified fragments were purified with the QIAEX II kit (Qiagen Inc., Chatsworth, Calif.) and sequenced by the dideoxy chain termination method of Sanger et al. (39). The six primers used for sequencing the whole of the 16S rRNA gene were FGPS4-281bis (5'-ATG GAGAAGTCTTGATCCTGGCTCA-3'), FGPS485-292 (5'-CAGCAGCCGCGG TAA-3'), FGPS1047-295 (5'-ATGTTGGGTTAAGTC-3'), FGPS505'-313 (5'-GTATTACCGCGCTGCTG-3'), FGPS910'-270 (5'-AGCCTTGGCGCCGT

ACTCCC-3'), and FGPS1509'-153 (5'-AAGGAGGGGATCCAGCCGCA-3') (31).

DNA sequence analysis. The 16S rDNA sequences were aligned and analyzed with the sequence editor Phylo-Win (11). Phylogenetic analysis was inferred by using the neighbor-joining method (38) calculated by the Kimura (18) and parsimony (19) methods. A bootstrap confidence analysis was performed with 1,000 replicates (10). The resulting tree was drawn with the NPlot software of Perrière and Gouy (34). The 16S rRNA sequences of the following organisms belonging to the *Rhizobiaceae* were used for comparison (GenBank accession numbers are in parentheses): *R. leguminosarum* ATCC 14482 (U29386), *R. etli* CFN 42 (U28916), *R. tropici* type IIa CFN 299 (X67234), *R. tropici* type IIb CIAT 899^T (X67233), *Mesorhizobium ciceri* UPM-Ca7^T (U07934), *M. huakuii* IAM 14158^T (D13431), *M. loti* NZP 2213^T (X67229), *M. mediterraneum* UPM-Ca36^T (L38825), *Mesorhizobium* sp. strain (group Ua) ORS 1001 (X68389), *Sinorhizobium fredii* USDA 205^T (X67231), *S. medicae* A321^T (L39882), *S. meliloti* NZP 4027^T (X67222), *S. saheli* ORS 609^T (X68390), *S. teranga* ORS 1009^T (X68388), *R. galegae* HAMB1 540^T (D11343), *Agrobacterium tumefaciens* LMG 187 (D14500), *A. rhizogenes* ATCC 11325 (D14501), *A. rubi* LMG 156 (X67228), *A. vitis* NCPPB 2611 (U45329), *Agrobacterium* sp. strain LMG 230 (D14506), *Agrobacterium* sp. strain LMG 196 (X67223), *Agrobacterium* sp. strain 3-10 (Z30542), *Bradyrhizo-*

TABLE 1. Rhizobia used in this study

Geographic origin and climate	Isolate ^a	Host range ^b	ITS group (PCR with RFLP)
Ait Ourir, arid	MSMC14	<i>A. cyanophylla</i>	1
	MSMC413	<i>A. raddiana</i> , <i>A. horrida</i>	1
	MSMC418	ND	1
Zagora, Saharan	MSMC37	<i>A. raddiana</i> , <i>A. horrida</i> , <i>A. cyanophylla</i>	1
	MSMC38	<i>A. raddiana</i> , <i>A. horrida</i> , <i>A. cyanophylla</i>	1
	MSMC311	<i>A. gummiifera</i> , <i>A. horrida</i> , <i>A. cyanophylla</i>	1
	MSMC313	<i>A. raddiana</i> , <i>A. horrida</i> , <i>A. cyanophylla</i>	1
	MSMC414	<i>A. raddiana</i> , <i>A. horrida</i> , <i>A. cyanophylla</i>	1
	MSMC26	—	6
	MSMC211	—	6
	MSMC45	<i>A. raddiana</i> , <i>A. horrida</i> , <i>A. cyanophylla</i>	1
	MSMC46	<i>A. raddiana</i> , <i>A. horrida</i> , <i>A. cyanophylla</i>	1
	MSMC47	<i>A. raddiana</i> , <i>A. horrida</i> , <i>A. cyanophylla</i>	1
	MSMC49	<i>A. raddiana</i> , <i>A. horrida</i> , <i>A. cyanophylla</i>	1
	MSMC419	ND	1
	MSMC28	<i>A. raddiana</i> , <i>A. gummiifera</i> , <i>A. horrida</i> , <i>A. cyanophylla</i>	3
	MSMC21	<i>A. cyanophylla</i>	4
MSMC25	—	4	
MSMC31	<i>A. raddiana</i> , <i>A. horrida</i> , <i>A. cyanophylla</i>	5	
MSMC32	<i>A. raddiana</i> , <i>A. horrida</i> , <i>A. cyanophylla</i>	5	
MSMC210	—	7	
Agdez, arid	MSMC411	<i>A. raddiana</i> , <i>A. cyanophylla</i>	2
	MSMC111	<i>A. horrida</i> , <i>A. cyanophylla</i>	1
Taliouine, arid	MSMC112	<i>A. horrida</i>	1
	MSMC114	<i>A. raddiana</i> , <i>A. horrida</i>	1
	MSMC115	<i>A. raddiana</i> , <i>A. cyanophylla</i>	1
	MSMC310	<i>A. horrida</i> , <i>A. cyanophylla</i>	1
	MSMC48	ND	1
	MSMC29	ND	3
	MSMC116	<i>A. raddiana</i>	8
	MSMC24	—	9
	MSMC33	—	10
	Marrakech, arid	MSMC312	<i>A. horrida</i>
MSMC314		<i>A. raddiana</i> , <i>A. gummiifera</i> , <i>A. horrida</i> , <i>A. cyanophylla</i>	1
MSMC315		<i>A. raddiana</i> , <i>A. horrida</i>	1
MSMC11		<i>A. raddiana</i> , <i>A. cyanophylla</i>	2
MSMC12		<i>A. raddiana</i> , <i>A. gummiifera</i> , <i>A. cyanophylla</i>	2
MSMC412		<i>A. raddiana</i> , <i>A. horrida</i> , <i>A. cyanophylla</i>	2
MSMC416		ND	2
MSMC417	ND	2	

^a MSMC, Moroccan Symbiotic Microorganisms Collection.

^b —, no nodulation; ND, not determined.

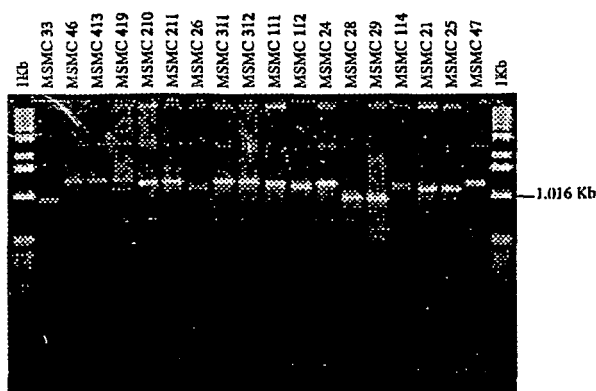


FIG. 2. Electrophoresis of PCR products obtained with the universal primers FGPS1490-72 and FGPL132'-38, which target the ribosomal ITS of *Rhizobium* strains that nodulate *Acacia* spp.

bium elkanii USDA 76 (U35000), *B. japonicum* USDA 110 (D13430), and *Azorhizobium caulinodans* ORS 571^T (D11342).

Nucleotide sequence accession numbers. The newly determined 16S rRNA sequences were deposited in the EMBL Data Library under accession no. AJ004859 to AJ004861.

RESULTS

Plant infection tests. We tested 34 strains of rhizobia on four *Acacia* species (Table 1). *A. raddiana*, *A. cyanophylla*, and *A. horrida* appeared to belong to the same group of host plant, since the majority of the strains tested could induce root nodules on them besides their original host. Only a few strains (less than 15% of the total) could induce a few nodules on *A. gummifera*, indicating that this plant probably requires special growth conditions for nodulation. Some strains (about 17%) did not nodulate any of these plants, even their original host.

16S-23S rDNA spacer analysis. To further investigate the genetic differences among the 40 rhizobia isolated from *Acacia* species in arid and Saharan areas of Morocco, we analyzed the 16S-23S rDNA spacer by PCR with RFLP analysis. Electrophoresis of nondigested PCR products revealed that each isolate possesses one band (Fig. 2). The length of the ITS amplified region, which included about 200 bp of the 16S and 23S genes, was not the same for all strains tested (it was between 1 and 1.4 kb). Therefore, we were able to identify 10 groups corresponding to 10 different bands in this natural population of rhizobia (Table 1). Group 1 contains the majority (22 of 40) of the isolates, which are characterized by an ITS of 1.3 kb. Group 2 contains six isolates, which have an ITS slightly longer than 1.3 kb. The two group 3 isolates have an ITS of 1.2 kb. The two group 4 isolates have an ITS of slightly more than 1.1 kb. The two group 5 isolates have an ITS of 1.1 kb. The two group 6 isolates have an ITS slightly longer than 1.0 kb. Group 7, a heterogeneous group, contains four isolates which each showed a specific length of ITS.

PCR amplification of 16S rRNA genes. The 16S rDNAs of 20 strains, which represent the seven groups identified above, were amplified, as well as those of type strains belonging to different rhizobia and agrobacteria. The stringency conditions of PCR allowed the amplification of a single band of about 1,500 bp for all the strains tested except *R. tropici* type IIa strain CFN 299, which possesses an insertion of 72 nucleotides (46), yielding a single band of about 1,600 bp (data not shown).

RFLP analysis of amplified 16S rRNA genes. The PCR product derived from each strain was digested separately by nine 4-base-cutting enzymes, and the resulting fragments were

separated by electrophoresis (data not shown). The length of PCR product estimated by summing the sizes of the restricted fragments was shorter than or equal to 1,500 or 1,600 bp for CFN 299. Between 4 and 13 distinct restriction patterns were detected with each of the nine endonucleases in the strains tested in this study. All the patterns of reference strains were confirmed by computer-simulated RFLP analysis of their 16S rDNA published sequences (30).

A total of 20 different combinations, each corresponding to one genotype, were identified among the 44 strains analyzed by RFLP in this study. Only three genotypes were distinguished when the 16S rDNA polymorphism was analyzed for the unclassified rhizobia nodulating *Acacia* spp. in Morocco. The patterns of these three genotypes were compared with those of strains belonging to recognized species. One group (group A) showed restriction profiles closer to those of *S. meliloti*. This group includes strains which are clustered in all the different ITS groups except group 1. The second group (group B) comprises strains which belonged to group 1 and showed restriction patterns identical to those of *S. fredii*. The third group (group C) contains three strains, which cannot renodulate any of the four *Acacia* species after their first isolation. This group is different from *A. tumefaciens* B₆ by only the *Nde*II and *Msp*I restriction patterns.

16S rDNA sequence analysis. The 16S rDNA sequences of the following strains representing the three identified *Rhizobium* genotypes were determined: MSMC 411 from group A, MSMC 310 from group B, and MSMC 211 from group C (data not shown). These sequences were aligned and compared with the 16S rDNA sequences of other members of the family *Rhizobiaceae* available in the GenBank database. Figure 3 is a dendrogram which shows the phylogenetic relationships of these unclassified rhizobia and the previously named species of *Rhizobiaceae*. MSMC 411 and MSMC 310, and their respective groups, are included in the *Sinorhizobium* lineage and exhibit a high level of relatedness to *S. meliloti* (99.7% sequence similarity, corresponding to three nucleotide substitutions and one deletion for a comparison of 1,440 bases) and *S. fredii* (99.8% sequence similarity, corresponding to three nucleotide substitutions for a comparison of 1,440 bases), respectively. In contrast, strain MSMC 211, which represents group C, fell in the *Agrobacterium-R. galegae* lineage and is most closely related to *A. tumefaciens* (98.9% sequence similarity, corresponding to 15 nucleotide substitutions for a comparison of 1,440 bases).

DISCUSSION

We decided to make a collection and database of rhizobia that nodulate acacias. Zerhari et al. (51) have isolated more than 50 isolates from seedlings of four *Acacia* species and established their infective potential as well as their host range and their phenotypic diversity.

We have used PCR with RFLP analysis to analyze 16S-23S spacer variation among these rhizobia. The ITS between the 16S and 23S rRNA genes may be an effective marker for detecting genetic differences at the intergeneric level and also at the interspecific level. These differences are partly due to variations in the number and type of tRNA genes found in these spacers (17).

We found great diversity in the length of the amplification bands among the rhizobium strains. Therefore, we were able to distinguish 10 groups on the basis of ITS length. Classification of rhizobium strains to any of these ITS groups appeared to be independent of the host plant and site of origin. The high level of ITS size heterogeneity is consistent with the results of Zerhari et al. (51) in an extensive study of 52 phenotypic charac-

showing that a high proportion of tree-nodulating rhizobia are more closely related to *Sinorhizobium* species (8, 24, 45).

In contrast, group C belongs to the *Agrobacterium-R. galegae* lineage and is more closely related to *A. tumefaciens*. This group includes three isolates, each showing a specific 16S-23S spacer and unable to renodulate any of the four *Acacia* species. In a further investigation, no PCR amplification could be observed with specific primers for a conserved region in the *nodD* gene, thus suggesting that these strains do not carry *nod* genes.

Two hypotheses concerning the presence in nodules of these rhizobia resembling *Agrobacterium* can be advanced. First, these strains lacking all or part of their symbiotic information could penetrate together with other infective rhizobia and co-exist with them. In fact, nonsymbiotic *Rhizobium* strains lacking symbiotic information have been found in soils (21, 42, 44). Alternatively, these strains could originally be infective, penetrate into the nodule, and subsequently lose their symbiotic information, or at least their *nod* genes, during symbiosis or after isolation and cultivation on agar plates, as was found by Macheret (25) for *R. leguminosarum* bv. phaseoli.

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