Short Communication

Molecular diversity of peanut-nodulating rhizobia in soils of Argentina

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RSα sequencing is a valuable tool for identification of bacterial strains, and for evaluating the genetic structure of indigenous rhizobial populations. The purpose of this study was to evaluate, qualitatively, the presence or absence of RSα fragment in peanut-nodulating strains isolated from plants grown at four sites in central Argentina. RSα fragment was found in only three of 26 indigenous strains, and in one of three inoculant strains analyzed. In contrast to results from studies of other symbiotic nitrogen-fixing bacteria, such as soybean-nodulating strains, no correlation was found between generation time and presence of RSα sequence. Phylogenetic analysis of the *16S rRNA* gene sequence grouped peanut-nodulating strains into two clusters, *Bradyrhizobium japonicum* vs. *B. elkanii*, and showed divergence among strains positive for RSα sequence. Our results confirm the genetic diversity previously reported for various peanut-nodulating rhizobial strains, and indicate that the RSα fragment is not applicable as a marker or tool for competition assays at the field or ecological level.

Keywords: *16S rRNA* / RSα sequence / *Bradyrhizobium* / Peanut / Rhizobial phylogeny

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Introduction

Rhizobia are bacteria whose distinguishing characteristic is the establishment of a symbiotic relationship with legume plants, by formation of new structures called nodules on the root system of the plant. Inside the nodules, differentiated bacteroids fix atmospheric nitrogen to reduced forms, which become available to the plant. Peanut, *Arachis hypogaea* L., is an important legume crop worldwide, whose seeds are utilized mainly as raw material in the oil and confectionery industries. Production of peanut in Argentina is localized in the central region of Córdoba province. An important genus of rhizobia that nodulates peanut is *Bradyrhizobium* [1–3]. A traditional scheme classifies rhizobia as fast- or slow-growing based on growth behavior in yeast extract mannitol agar medium supplemented with bromothymol blue (YEMA AB). Strains that turn the indicator medium yellow (acidification) or blue (alkalinization) are considered fast- or slow-growing, respectively [1]. However, this is not a reliable criterion for judging growth velocity. A more accurate technique is to prepare a growth curve and estimate generation time for each strain. Rhizobia isolated from soybean have been classified as slow- vs. fast-growing based on a generation time greater or less than 6 h [4]. The symbiotic process of biological nitrogen fixation is a "natural" way to provide nitrogen to agricultural crops [5]. Application of a more efficient strain in an inoculant can improve nitrogen fixation for legume crops. Peanut is considered a "promiscuous" species in that it is nodulated by rhizobia that also nodulate a variety of other legumes. An indigenous (native) population of soil rhizobia is generally present, having the potential ability to nodulate an introduced peanut crop. Such indigenous rhizobia may present a competition barrier to establishment of inoculant strains, and cause inoculation failure [6].

RSα sequences are repeated DNA segments with structural properties similar to those of insertion sequences (IS). IS elements are discrete DNA segments having the capacity to transpose to different sites on bacterial plasmids and chromosomes, resulting in in-

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creased number of copies and rearrangement of genome. The RSα sequence can be used to discriminate soybean-nodulating strains, through amplification reaction of the segment with specific primers. For example, *B. japonicum* was found to contain the RSα sequence, whereas fast-growing strains such as *Sinorhizobium fredii* and *S. xingiangensis* did not [7]. Taxonomic characterization of indigenous peanut-nodulating rhizobial strains, by methods based on analysis of genes coding for 16S rRNA, has been proposed [8]. Lateral transfer of portions of the gene could theoretically be a source of phylogenetic distortion, by creating mosaic genes in which different segments have different ancestries [9]. However, *16 rRNA* sequencing is still the most important criterion in bacterial taxonomy [10].

The goals of the present study were to: (i) amplify in qualitative mode the RSα sequence in DNA isolated from indigenous and reference strains, and determine its significance as a potential tool for studies of genetic diversity, comparative growth velocity, and competition assay; (ii) carry out taxonomic characterization of peanut-nodulating rhizobia by analyzing *16S rRNA* sequences.

Materials and methods

Microorganisms and culture media

Indigenous rhizobia were isolated from root nodules of peanut plants grown in soils taken from four sites in Córdoba province: La Aguada, Cabrera, Mackenna, and Río Cuarto. Nodules were surface-sterilized, pierced, and their contents were spotted on YEM agar plates [1]. For each rhizobial strain, a "nodulating assay" (of ability to nodulate sterilized seedlings of *A. hypogaea* L. on sterile vermiculite) was performed. In addition, we analyzed *Bradyrhizobium* sp. USDA 4438, USDA 3180, and C-145 (TAL 1371), which are recommended as inoculants for peanut, *B. japonicum* USDA 110, and *S. fredii*. Each strain was grown on YEM medium [11] for 4–6 days at 28 °C. Generation times were calculated in the exponential phase of growth by measurement of optical density (OD) at 600 nm.

DNA extraction

Colonies were suspended in 500 µl sterile physiological saline solution and centrifuged at 10,000 rpm for 10 min. Supernatant was removed, and the pellet was suspended in 500 µl InstaGene Matrix (Bio-Rad, Hercules, CA, USA) [12]. The suspension was incubated for 30 min at 56 °C, then heated for 10 min at 100 °C. The supernatant was used as a bacterial DNA template for PCR reaction.

16S rRNA gene analysis

Direct PCR was performed utilizing 1 µl DNA template in 20 µl PCR reaction mixture containing the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′) [13]; numbering is based on the *Escherichia coli 16S rRNA* gene [14]. Amplification was conducted for 35 cycles, at 94 °C for 45 sec, 55 °C for 60 sec, and 72 °C for 60 sec. Purified PCR products of ~1,400 bp were sequenced with an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA) by Macrogen Inc. Laboratories (Korea). The *16S rRNA* gene sequences were subjected to BLAST search program (National Center for Biotechnology Information) [15] to find identities (similarities) between sequences. Phylogenetic analyses were conducted using *MEGA* version 4 in order to produce a phylogenetic tree reflecting the evolutionary relationship between peanut-nodulating strains and reference strains by the neighbor-joining method [16], using the Kimura 2-parameter model.

Nucleotide sequence accession numbers

Nucleotide sequences of the *16S rRNA* gene from peanut-nodulating strains C-145, USDA 4438, USDA 3180, PC27, PC30, PR1, P1, P5, P7, P8, 26LA, 27LA, 20AG, 45AG, 55AG, 62AG, V80, and V62 determined in this study have been deposited in the GenBank nucleotide sequence database under accession numbers FJ418929, FJ418928, FJ418916, FJ418927, FJ418921, FJ418926, FJ418917, FJ418922, FJ418918, FJ418919, FJ418912, FJ418913, FJ418911, FJ418914, FJ418915, FJ418925, FJ418923, and FJ418924, respectively.

Amplification of RSα fragment

Amplification of RSα was performed with primers forward SAL1 (5′-AGCGGGCGCGGATAGTTCTGTTG-3′) and reverse SAR2 (5′-GGCTCGGCTCTGTCGTTGTATGC-3′). Polymerase chain reaction (PCR) was performed as described previously [7]. Products obtained were subjected to horizontal electrophoresis on 2% agarose gel stained with ethidium bromide. Gels were exposed to UV light in a transilluminator, and products were visualized and photographed.

Results and discussion

The indigenous strains isolated in this study were identified as *Bradyrhizobium* sp. by microbiological tests, and identifications were confirmed by *16S rRNA* gene sequence analysis. All the strains analyzed ($n = 29$) were able to form effective nodules in peanut ~4 weeks after

inoculation, and were slow-growing in terms of observed generation time and behavior on YEMA AB (Table 1). Inoculation of peanut is not a common practice because this legume is considered to be well nodulated by indigenous rhizobia. However, the symbiotic efficiency of indigenous populations is often lower than that of inoculant strains. In our previous study, inoculation assays indicated good nodulation (which is not the same as efficiency) by indigenous rhizobia characterized as *Bradyrhizobium* sp. strains [17]. Thus, in the field, attempts at inoculation often fail because soils contain indigenous (less efficient) populations of rhizobia which are already established, and out-compete the inoculant strain. Competition for nodulation is a complex and poorly understood phenomenon. Our previous studies showed that spontaneous antibiotic-resistant mutants and ERIC-PCR experiments provide useful markers to evaluate nodule occupancy after seed or infurrow inoculation of peanut grown in soils with indigenous rhizobial populations [18].

Useful markers for competition assays must be simple, accurate, and economical. The RSα sequence can be used to discriminate soybean-nodulating strains [7]. One purpose of this study was to determine if RSα fragment can be used as a tool to evaluate nodule occupancy of peanut in competition assays. Amplification of RSα fragment was conducted for peanut-nodulating strains using the primers described by Pastorino *et al.* [7]. When the RSα fragment exists, the expected size of the amplification product was ~900 bp. Results are shown in Fig. 1, with *B. japonicum* USDA 110 and *S. fredii* used as positive and negative controls, respectively. Of the 29 strains analyzed, only four had RSα sequence: one inoculant strain (C-145), and three indigenous strains (PR1, V62, and 55AG). Regarding the agriculture history of the four experimental sites, we learned that soybean had been grown previously in certain portions of Mackenna and Cabrera. Native strains that tested positive for the RSα fragment were isolated from peanut plants grown at three sites, Río Cuarto (PR1), Mackenna (V62), and La Aguada (55AG), but not from plants grown at Cabrera. Therefore, we could not conclude that peanut-nodulating rhizobia acquired this gene by lateral transfer from the populations of soy-

Table 1. Rhizobial isolates from nodules of peanut, and recommended strains, used in this study.

Strain	Origin	Source	GT	YEMA AB Reaction
Recommended as inoculants				
C-145 (TAL 1371)		NifTAL, USA	14,2 ± 0,9	AL
USDA 4438		ARS, USDA, USA	14,7 ± 0,8	AL
USDA 3180		ARS, USDA, USA	12,7 ± 1,1	AL
Native Isolates				
PC27	Cabrera	This study	12,9 ± 0,9	AL
PC29	Cabrera	This study	13,1 ± 0,9	AL
PC31	Cabrera	This study	7,4 ± 0,6	AL
PC32	Cabrera	This study	21,4 ± 1,7	AL
PC33	Cabrera	This study	14,3 ± 1,0	AL
PC34	Cabrera	This study	12,3 ± 0,5	AL
PC3	Cabrera	This study	15,6 ± 0,7	AL
PC4	Cabrera	This study	10,9 ± 0,8	AL
20AG	La Aguada	This study	14,5 ± 0,7	AL
45AG	La Aguada	This study	11,3 ± 1,1	AL
55AG	La Aguada	This study	14,8 ± 1,2	AL
62AG	La Aguada	This study	11,9 ± 0,5	AL
26LA	La Aguada	This study	13,0 ± 0,8	AL
27LA	La Aguada	This study	12,3 ± 0,5	AL
P1	Río Cuarto	Bogino *et al.* [17]	15,6 ± 0,9	AL
P5	Río Cuarto	This study	19,9 ± 1,4	AL
P7	Río Cuarto	Bogino *et al.* [17]	24,1 ± 1,6	AL
P8A	Río Cuarto	Bogino *et al.* [17]	16,3 ± 1,0	AL
P8B	Río Cuarto	This study	13,1 ± 0,8	AL
PR1	Río Cuarto	Bogino *et al.* [17]	14,9 ± 0,7	AL
PC30	Río Cuarto	Bogino *et al.* [17]	12,4 ± 1,2	AL
VM30	Mackenna	This study	27,6 ± 1,8	AL
VM45	Mackenna	This study	20,5 ± 1,5	AL
VM50	Mackenna	This study	14,3 ± 1,4	AL
V62	Mackenna	This study	14,8 ± 1,0	AL
V80	Mackenna	This study	17,9 ± 1,3	AL

Data for GT are mean ± SD of four independent determinations. AL: alkaline reaction.

bean rhizobial strains. Because RSα fragment is present on both inoculant and indigenous strains, RSα sequence analysis is not applicable as a marker or tool for competition studies at the field or ecological level. However, amplification of RSα fragment can be used in lab assays of competition for nodule occupancy between identified strains with vs. without RSα fragment (e.g., C-145 vs. P1, PR1 vs. 27LA). In order to confirm the RSα fragment, amplified sequences of strains C-145, PR1, V62, and 55AG were re-amplified with PCR experiments using primers located on internal regions designed based on previously published sequences [19, 20]. The DNA fragments obtained by PCR were observed on agarose gel stained with ethidium bromide. The sizes obtained were consistent with values expected from gene sequences deposited by authors of the above studies as rhizobia RSα sequences in GenBank, under accession numbers X02581 and AY283374, respectively (data not shown). All indigenous peanut-nodulating rhizobia are slow-growing. Their generation times ranged from ~7 hr (PC31 strain) to ~27 hr (VM30 strain), with an average of ~15 hr (Table 1). The RSα fragment was identified in only three out of 26 isolates. The three recommended strains are also slow-growing, and only one (C-145) has RSα (Fig. 1). Thus, presence of the RSα sequence is not correlated with growth rate of peanut-nodulating strains. In contrast, in a study of soybean-nodulating rhizobia, the slow-growing strain *B. japonicum* was found to contain RSα sequence, while fast-growing strains such as *Sinorhizobium fredii* and *S. xingiangensis* did not [7].

To elucidate taxonomic relationships of the isolates, we sequenced the *16S rRNA* gene. Nucleotide sequences of nearly full-length *16S rRNA* gene (average ~1,400 bp) from our isolated strains were deposited in the GenBank database, and compared to available sequences of known rhizobia (Fig. 2). As expected, all strains in our collection were grouped within the *Bradyrhizobium* cluster. The peanut-nodulating strains were clearly divided into two groups. The first group was related to strains P1, PR1, P5, and P7, which show major identity with members of the *B. elkanii* cluster. The second, larger group was related to the *B. japonicum* and *B. liaoningense* clusters. Strains within the latter group showed high (≥98%) identity in their gene sequences. This observed separation of peanut-nodulating bradyrhizobia into *B. japonicum* vs. *B. elkanii* clusters is consistent with earlier studies [8, 21]. In this analysis, internal branches

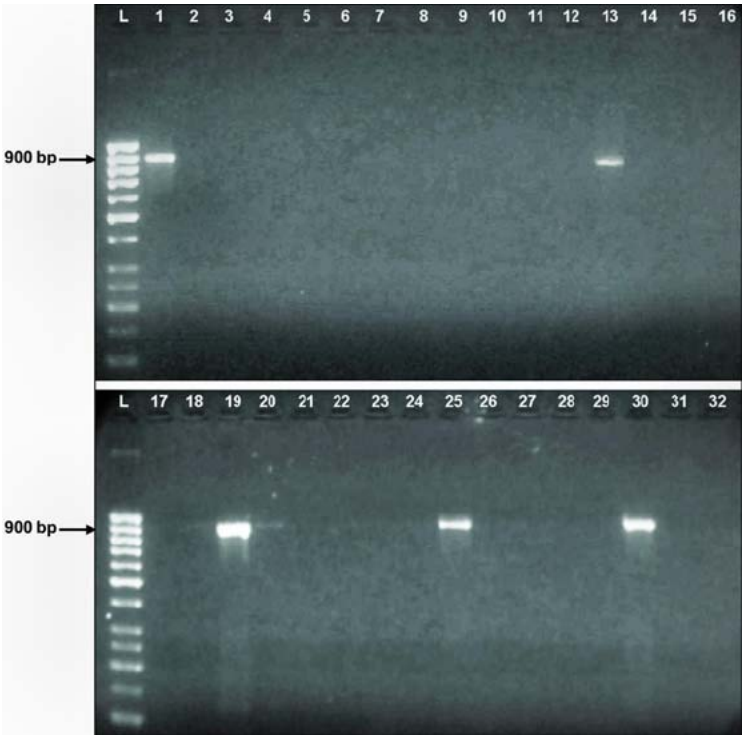

Figure 1. Reaction products of RSα fragment obtained from genomic DNA isolated from peanut-nodulating strains. Lane 1: USDA110, Lane 2: *S. fredii*, Lane 3: PC27, Lane 4: PC29, Lane 5: PC31, Lane 6: PC32, Lane 7: PC33, Lane 8: PC34, Lane 9: PC3, Lane 10: PC4, Lane 11: 20AG, Lane 12: 45AG, Lane 13: 55AG, Lane 14: 62AG, Lane 15: 26LA, Lane 16: 27LA, Lane 17: USDA3180, Lane 18: USDA4438, Lane 19: C-145, Lane 20: P1, Lane 21: P5, Lane 22: P7, Lane 23: P8A, Lane 24: P8B, Lane 25: PR1, Lane 26: PC30, Lane 27: V30, Lane 28: V45, Lane 29: V50, Lane 30: V62, Lane 31: V80, Lane 32: negative control. L: Gene Ruler 50 bp DNA Ladder (Fermentas).

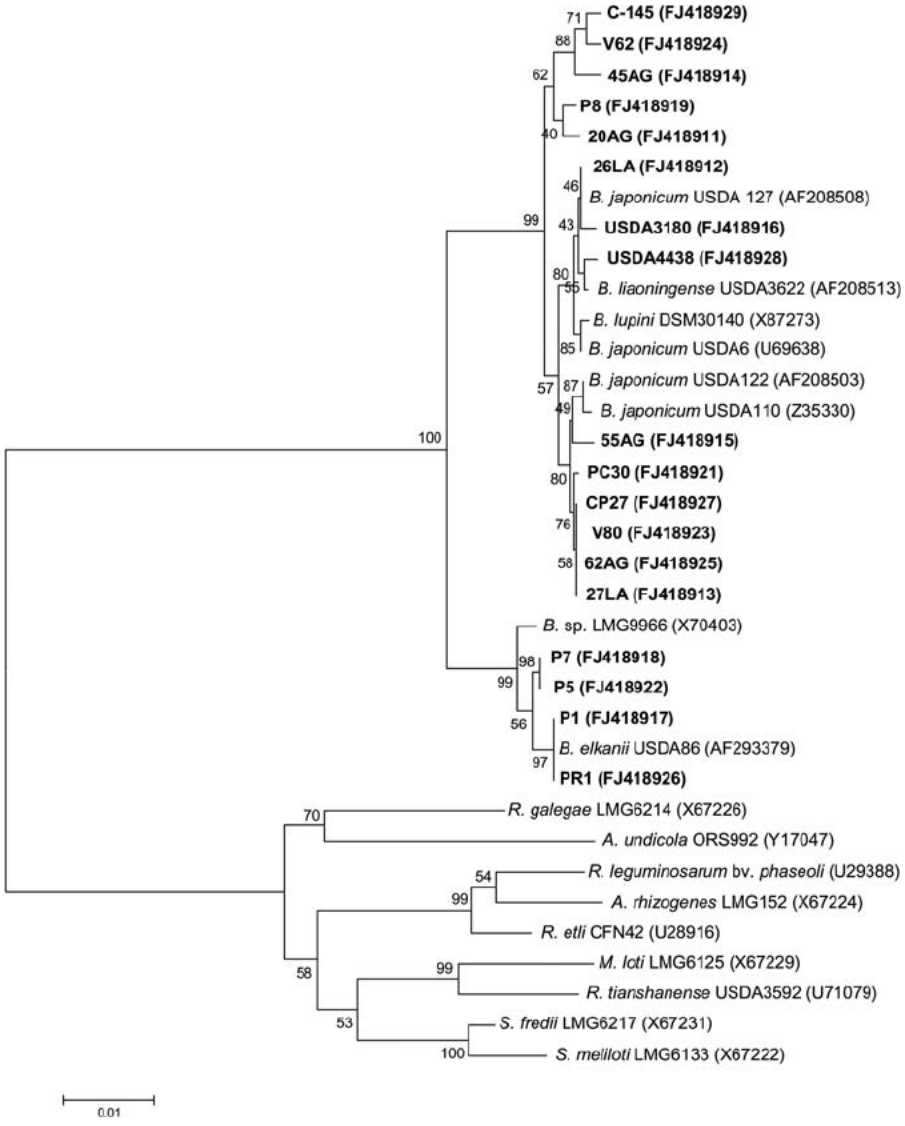

Figure 2. Phylogenetic tree of strains isolated from *A. hypogaea* and representative members of rhizobial groups, based on aligned *16S rRNA* gene sequences. Multiple alignments and Neighbor-Joining method were used to construct the trees, using MEGA 4.0.2 software. Peanut-nodulating strains are indicated by boldface. Bootstrap values, expressed as a percentage of 1000 replications, are given at the nodes. Sequence accession numbers are listed in parentheses. Scale bar: 1 nt substitutions per 100 nt.

linking the species to known–rhizobial species were supported above the 70% level by 1,000 bootstrap replications, and are thus expected to represent true clades [22]. We investigated degree of divergence in *16S rRNA* gene sequence by measuring distances from tree branches among the strains. An interesting finding is that sequence divergence between strains in the two groups was greater than 3.0%, the level considered necessary to differentiate between species [10], i.e., 3.7% between strains C-145 and P1, and 3.4% between strains PC30 and P1. Consistent with our previous findings using ERIC-PCR fingerprinting [18], native populations of peanut-nodulating rhizobia appear to be highly diverse, although all were within the *Bradyrhizobium* cluster (Fig. 2). Peanut-nodulating strains in the *B. japonicum* cluster were assigned to three subgroups not reflecting geographical origin: (i) recommended strains USDA 3180 and USDA 4438, and a native strain from La Aguada (26LA); (ii) several native isolates with different origins (27LA, PC30, PC27, V80, 62AG); (iii) recommended strain C-145 and indigenous strains from La Aguada (20AG, 45AG), Río Cuarto (P8A), and Mackenna (V62A).

We also studied divergence in *16S rRNA* gene sequence among strains carrying the RSα sequence. Except for C145 and V62 (0.4% divergence), RSα-carrying strains showed high levels of *16S rRNA* gene sequence

divergence, e.g., 3.6% in the case of C-145 and PR1. In conclusion, our results do not indicate correlation between RSα-carrying strains and high identity in the *16S rRNA* gene. The presence of RSα fragment in 11.5% of indigenous strains studied, and its occurrence at three different field sites, suggest that peanut-nodulating rhizobia have high genetic diversity, as confirmed by the observed high divergence in *16S rRNA* gene (in some cases close to 4%). These findings are consistent with many previous reports of high diversity and heterogeneity of rhizobial strains isolated from peanut [2, 3, 18], although this is the first study of RSα sequence in such strains. Analysis of such biodiversity is essential to improve our knowledge on indigenous populations of rhizobia with ability to nodulate peanut, a prerequisite for potential applications in agriculture and environmental protection.

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