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Endophytic occupation of peanut root nodules by opportunistic *Gammaproteobacteria*

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Abstract

Several bacterial isolates were recovered from surface-sterilized root nodules of *Arachis hypogaea* L. (peanut) plants growing in soils from Córdoba, Argentina. The 16S rDNA sequences of seven fast-growing strains were obtained and the phylogenetic analysis showed that these isolates belonged to the Phylum *Proteobacteria*, Class *Gammaproteobacteria*, and included *Pseudomonas* spp., *Enterobacter* spp., and *Klebsiella* spp. After storage, these strains became unable to induce nodule formation in *Arachis hypogaea* L. plants, but they enhanced plant yield. When the isolates were co-inoculated with an infective *Bradyrhizobium* strain, they were even found colonizing pre-formed nodules. Analysis of symbiotic genes showed that the *nifH* gene was only detected for the *Klebsiella*-like isolates and the *nodC* gene could not be amplified by PCR or be detected by Southern blotting in any of the isolates. The results obtained support the idea that these isolates are opportunistic bacteria able to colonize nodules induced by rhizobia.

Keywords: *Arachis hypogaea*; *Bradyrhizobium*; *Pseudomonas*; *Klebsiella*; *Enterobacter*; Endophytes from nodules

Introduction

Plants constitute an extremely diverse niche for microorganisms. Internal plant tissues are colonized by a large diversity of endophytic microorganisms that become protected from external biotic and abiotic factors. In leguminous plants, a new organ called nodule is induced and occupied by soil bacteria collectively known as rhizobia.

Until 2001, all bacteria known to be isolated from nodules were restricted to genera within the *Alphaproteobacteria*. However, this began to change when isolates belonging to the *Betaproteobacteria* were discovered as nodule-forming or nodule-associated bacteria [5,6,15,21]. More recently, some reports have provided information on *Gammaproteobacteria* (genera – *Pseudomonas*, *Escherichia*, *Leclercia*, *Pantoea*, and *Enterobacter*) associated with nodules in legumes. In addition, three isolates, which belong to the genus *Bacillus*, were obtained from soybean root nodules but, in the absence of *Bradyrhizobium japonicum*, they were unable to nodulate this legume [2]. Thus, the phenotypic traits ruling interaction with legumes seem to be shared by phylogenetically unrelated taxa.

Dart [7] reported that *Stylosanthes* and *Arachis* (both infected via crack-entry) exhibit less stringent requirements towards their microsymbiotic partners. In addition, the universal ''lock-and-key'' hypothesis formulated for the legume–rhizobia interaction based on the ubiquitous presence of *nod* genes and Nod factors [3], has been disassembled by recent results demonstrating that the canonical common nodulation genes and thus, typical Nod factors, are not required for the symbiotic interaction of photosynthetic bradyrhizobia strains with

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Aeschynomene sensitiva [9]. Thus, taking into consideration these results, it is now easier to speculate that the first recognition step initiated by Nod factors would be bypassed in some legume plants, especially those allowing infection via crack-entry.

Bacteria nodulating peanut in natural environments have been classified as *Bradyrhizobium* (*Arachis*) sp., but species names have not been defined yet. To assess their diversity in the peanut producing area of the province of Córdoba (Argentina), we have previously characterized a collection of isolates obtained from peanut nodules [19]. It was interesting that, besides the slow-growing *Bradyrhizobium* spp., 13 fast-growing bacteria were also obtained. This fast-growing bacterial group was further genetically characterized and ARDRA results revealed that they grouped into 12 different profiles. The sequences of 16S rDNA from two of these isolates (named NET30 and NCHA22) were further analyzed and our results demonstrated that they had 16S rDNA alleles with high identity to those of species *Rhizobium giardinii* and *Rhizobium tropici*, respectively [20]. In the present study isolates that grouped into another seven ARDRA profiles were identified, and their ability to invade peanut nodules was assessed, along with the significance of nodule colonization for plant growth promotion.

Materials and methods

Collection of bacterial isolates

The procedure for the isolation of bacteria used in this study has been described previously [19,20]. Bacteria were isolated directly from field peanut root nodules or from soil using peanut as a trap host. Plants or soil were collected from 15 geographically distant sites in the central and southern region of Córdoba, Argentina (latitude: 32–34°; longitude: 63–65°). The isolates used in this work are listed in Table 1.

DNA preparation

The method described by Walsh et al. [23] was followed. DNA concentration of the samples was approximately 5 ng ml^{-1}. For hybridization assays, genomic DNA was extracted following the standard procedure described by Meade et al. [14].

ERIC–PCR analysis

The DNA sequences of enterobacterial repetitive intergenic consensus (ERIC) primers used in this study have been reported by de Bruijn [8]. The ERIC amplification products in 6 µl sub-samples were separated according to molecular size by horizontal electrophoresis on 1.8% (w/v) agarose gels stained with ethidium bromide.

PCR-amplification and sequencing of 16S rRNA and nifH genes

The nucleotide sequence of the nearly full-length 16S rDNA was directly obtained by Macrogen Laboratories (Korea). The presence of the *nifH* gene in the isolates was assessed by performing a nested PCR using the degenerated primers described by Widmer et al. [24]. PCR conditions were as described by Bürgman et al. [4]. Taking into account the information obtained from the 16S rDNA sequences, the *nifH* gene was also screened

Table 1. Bacterial isolates and reference strains.

Isolates	Geographical origin		Original host plant	Antibiotic resistance profile
	Locality	Department		
TT001	Río Cuarto	Río Cuarto	*Arachis hypogaea* L.	Cm^RNm^S
NCHA33	Charras	Juárez Celman	*Arachis hypogaea* L.	Cm^RNm^S
NCHA35	Charras	Juárez Celman	*Arachis hypogaea* L.	Cm^RNm^S
NMAN11	Manfredi	Río Segundo	*Arachis hypogaea* L.	Cm^RNm^S
NONC13	Oncativo	Río Segundo	*Arachis hypogaea* L.	Cm^RNm^S
NTI31	Ticino	San Martín	*Arachis hypogaea* L.	Cm^RNm^S
NVAM24	Villa María	San Martín	*Arachis hypogaea* L.	Cm^RNm^S
Reference strains				
Bradyrhizobium SEMIA 6144			*A. hypogaea* L. (IPAGRO)	Cm^RNm^R
Rhizobium etli CFN42			Phaseolus vulgaris L. (Lab. Dr. O.M. Aguilar)	

Cm^R: chloramphenicol resistant; Nm^S: neomycin sensitive; Nm^R: neomycin resistant.