

# *Phaseolus lunatus* is nodulated by a phosphate solubilizing strain of *Sinorhizobium meliloti* in a Peruvian soil

E. Ormeño<sup>1</sup>, R. Torres<sup>1</sup>, J. Mayo<sup>1</sup>, R. Rivas<sup>2</sup>, A. Peix<sup>3</sup>, E. Velázquez<sup>2</sup> & D. Zúñiga<sup>1</sup>

<sup>1</sup>Laboratorio de Ecología Microbiana Marino Tabusso, Departamento de Biología, Universidad Agraria de la Molina, Lima, Perú. <sup>2</sup>Departamento de Microbiología y Genética, Facultad de Farmacia, Universidad de Salamanca, Salamanca, Spain. <sup>3</sup>Instituto de Recursos Naturales y Agrobiología (IRNA, CSIC), C/Cordel de Merinas, 40-52, 37008, Salamanca, Spain. <sup>3</sup>Corresponding author\*

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## Abstract

The genus *Phaseolus* includes several species indigenous to American continent that belong to family *Leguminosae*. This genus includes several species, some of them only cultivated in American countries. This is the case of *Phaseolus lunatus*. This plant can be nodulated by fast and slow growing rhizobia. At the moment the fast growing species nodulating *Phaseolus* commonly belong to genus *Rhizobium* and more rarely to *Sinorhizobium fredii*. A strain, LMTR32, isolated from *Phaseolus lunatus* growing in Peru soils showed a high ability to solubilize bicalcium phosphate from YED-P plates. The 16S rRNA sequence of this strain showed a 100% similarity with the type strain of *Sinorhizobium meliloti*. The LMW RNA profile of this strain is identical to that of type strain of *Sinorhizobium meliloti* and confirms that the strain LMTR32 belongs to this species. More studies are necessary in order to establish the prevalence of this species in nodules of *Phaseolus lunatus* in Perú, and, in the future, it will be very interesting to perform wider taxonomic studies of rhizobia nodulating *Phaseolus* in different American countries.

## Introduction

The genus *Phaseolus* is indigenous to American soils and was spread in the world after America discovery. This fact has supported the hypothesis that American endosymbionts of *Phaseolus* belong to different species than those isolated in other geographical locations. Moreover, during decades, the rhizobiologists had classified the rhizobia according to cross-inoculation groups of legumes. Currently, this classification has not been completely forgotten and certain cross-inoculation groups have been maintained and even has been used to classify the species of genus

*Rhizobium* (Jordan, 1984). In this way, the species *Sinorhizobium meliloti* has been considered an exclusive endosymbiont of *Medicago*, *Melilotus* and *Trigonella*, whereas the endosymbionts of *Phaseolus* were classified in species from genus *Rhizobium*. *R. tropici* (Martínez-Romero et al., 1991) and *R. etli* (Segovia et al., 1993) nodulate *Phaseolus* in American soils. *R. gallicum* and *R. giardinii* (Amarger et al., 1997) have been identified from nodules of *Phaseolus* in France. *R. leguminosarum* biovar *trifolii* has been isolated from nodules of *Phaseolus vulgaris* in Spain (Velázquez et al., 2001a). For the moment, only a species of genus *Sinorhizobium* (*S. fredii*), has been identified in *Phaseolus* nodules (Herrera-Cervera et al., 1999; Sadowsky et al., 1988; Velázquez et al., 2001a) and only one species of

\* FAX No: + 34-923-224876.

E-mail: alvarp@usal.es

genus *Rhizobium*, *Rhizobium mongolense*, can nodulate *Phaseolus vulgaris* and *Medicago ruthenica* (van Berkum et al., 1998) that in theory belong to different cross-inoculation groups. All these studies are carried out on *P. vulgaris* because this plant has been spread in many countries and is used in human nutrition. However, the genus *Phaseolus* comprises several species that only are present in South American countries as occurs with *Phaseolus lunatus* that is used as forage in Perú. The endosymbionts of lima bean (*P. lunatus*) have been few studied and commonly belong to genus *Rhizobium* although slow-growing strains have been isolated (Matos and Zúñiga, 2002).

During a study of strains nodulating *Phaseolus lunatus* in Perú we isolated a strain able to induce nodules in this plant and to solubilize phosphate *in vitro*. In this work we have identify this strain using 16S rDNA sequence and LMW RNA profiles and we have analyzed the ability of this strain to mobilize phosphorous to *P. lunatus*.

## Materials and methods

### *Bacterial strains and evaluation of its ability to solubilize phosphate*

The isolation of strain LMTR32 was made according to Vincent (1970) using yeast manitol agar – YMA – (Bergersen, 1961) from young effective nodules. The ability to solubilize bicalcium phosphate was tested in Petri dishes containing YED (yeast extract 0.5%; glucose 1% and agar 2%) supplemented with a 0.2% of bicalcium phosphate (YED-P). A suspension of the strain was inoculated in this medium and the plates were incubated for 7 days until the solubilization zone surrounding the colonies was observed (de Freitas et al., 1997).

### *16S sequencing and analysis*

DNA extraction was carried out as previously described (Rivas et al., 2001). The amplification of 16S rDNA and its sequencing was performed according to the method already described (Rivas et al., 2002). The sequence obtained was compared with those from the GenBank using

the FASTA program (Pearson and Lipman, 1988).

### *LMW RNA extraction and SCE LMW RNA profiling*

LMW RNA extraction was accomplished following the phenol/chloroform method described by Höfle (1988), using cells grew in tryptone-yeast agar, TY (Beringer, 1974). The following commercial molecules from Boehringer Mannheim (Mannheim, Germany) and Sigma (St. Louis, MO, USA) were used as reference: 5S rRNA from *Escherichia coli* MRE 600 (120 and 115 nucleotides) (Bidle and Fletcher, 1995), tRNA specific for tyrosine from *E. coli* (85 nucleotides) and tRNA specific for valine from *E. coli* (77 nucleotides) (Sprinzl et al., 1985). Samples containing 3 µg were added to 5 µg of loading solution (300 mg/mL of sucrose, 460 mg/mL of urea, 10 µL/mL 20% SDS, 1 mg/mL xylene cyanol) and, after 10 min of heating at 70 °C, applied to each well. LMW RNA profiles were obtained using staircase electrophoresis (SCE) which was performed in 400 × 360 × 0.4 mm gels in a vertical slab unit (Poker Face SE 1500 Sequencer, Hoeffer Scientific Instruments, San Francisco, CA, USA). The separating gel contained 14% acrylamide/Bis (acrylamide: *N,N*-methylene bisacrylamide 29:1 (w/w), 7 M urea in TBE buffer: 100 mM Tris, 83 mM boric acid, 1 mM EDTA, pH: 8.5) in TBE buffer, pH: 8.5. Before running the pre-electrophoresis (30 min at 100 V), the system was stabilized at 50 °C. The running buffer (TBE, ×1.2) was recycled at a flow rate of 300 mL/min with a peristaltic pump (MasterFlex, Cole Parmer Instruments, Chicago, Illinois, USA) (Cruz-Sánchez et al., 1997). After electrophoresis, gels were silver-stained according to Haas et al. (1994).

### *Mobilization of phosphorous in plants*

Experiments to study the P mobilization in plants were performed with common bean and were conducted in pots containing vermiculite as sterile support. The pots were placed in a plant growth chamber with mixed incandescent and fluorescent lighting (400 microeinsteins m<sup>-2</sup> s<sup>-1</sup>; 400–700 nm), programmed for a 16 h photoperiod, day–night cycle, with a temperature varying

from 15 to 27 °C (night-day), and 50–60% relative humidity. The experimental design was as follows. Treatment 1: seeds inoculated with the strain LMTR32 and adding 0.2% bicalcium phosphate to the vermiculite. Treatment 2: Control treatment with insoluble phosphate and uninoculated seeds. Fifteen pots were used for each treatment. The seeds were placed in each pot at a depth of 2 cm.

For inoculation, strain LMTR32 was grown in Petri dishes with YMB (Bergersen, 1961) for 5 days. After that, sterile water was added to the plates to obtain a suspension with ca.  $10^8$  cells mL<sup>-1</sup>. For inoculation we added 1 mL of the suspension of strain LMTR32 to each seed placed in Petri dishes. The seeds were dried overnight at room temperature.

At harvest (30 days) the dry weight of the aerial part of the plants of common bean was determined. Plant N, P, K, Ca and Mg content was measured according to the A.O.A.C. methods (Johnson, 1990). The data obtained were analyzed by one-way analysis of variance, with the mean values compared using the Fisher's Protected LSD (Least Significant Differences) ( $P = 0.05$ ).

## Results and discussion

### *Ability to solubilize phosphates in vitro*

The strain LMTR32 isolated from effective nodules of *P. lunatus* in Perú was able to solubilize phosphate in plates containing bicalcium hydrogen phosphate as P source. The diameter of the halo surrounding the colonies was 5 mm. This diameter is lower than that obtained in *Mesorhizobium* strains, but it is higher than those obtained for strains of genus *Rhizobium* (Peix et al., 2001). The type strains of the species from genus *Rhizobium* nodulating *P. vulgaris* do not show phosphate solubilization in plates containing bicalcium phosphate although some strains nodulating this legume have been reported as P solubilizers (Halder et al., 1990, 1993). There are no data about the phosphate solubilization of strains nodulating *P. lunatus* because no studies have been carried out with isolates from this species. For this reason we have identified the strain LMTR32 and we ana-

lyzed the possibility that this strain mobilize phosphorous to the plant.

### *16S rDNA sequence analysis*

The strain LMTR32 was identified in first place using 16S rDNA complete sequence (Accession number AY196963). This sequence showed a 100% similarity with that of *Sinorhizobium meliloti*. At the moment this species has been not identified in nodules of *Phaseolus*, although some authors have reported the existence of strains related with this species that were isolated from *P. vulgaris*. Nevertheless, in these works the strains isolated do not were completely identified and in some cases the identification was based on symbiotic genes and not in ribosomal genes. For example, based on *nodC* and *nifH* sequences and restriction patterns (Mhamdi et al., 2002) have found strains related to *S. meliloti* isolated from *P. vulgaris* in Tunisian soils. Previously, other authors have reported the nodulation of *Phaseolus vulgaris* by strains of *S. meliloti* (Laguerre et al., 2001). In the present study we have used the LMW RNA profiles to identify at species level the strain LMTR32.

### *LMW RNA profiling*

LMW RNA profiles include three zones in prokaryotes: 5S rRNA zone, class 1 and class 2 tRNA. The 5S rRNA zone is characteristic of each genus and the tRNA profile is characteristic of each species from the same or different genus. Therefore, these profiles are molecular signatures for both prokaryotes and eukaryotes microorganisms (Velázquez et al., 2001b). In a previous study we demonstrated that the species that nodulate *Phaseolus vulgaris* can be distinguishable using LMW RNA profiles (Velázquez et al., 2001a). In this study we identified a strain isolated from nodules in South Spain as *Sinorhizobium fredii*. This species had been reported in common bean nodules by other authors (Herrera-Cervera et al., 1999; Sadowsky et al., 1988), but in our work we unambiguously identified this strain using 16S rDNA sequence and LMW RNA profile. For this reason, in the present study we have analyzed the LMW RNA profile of strain LMTR32 to confirm the identification obtained using 16S rDNA sequencing. Figure 1

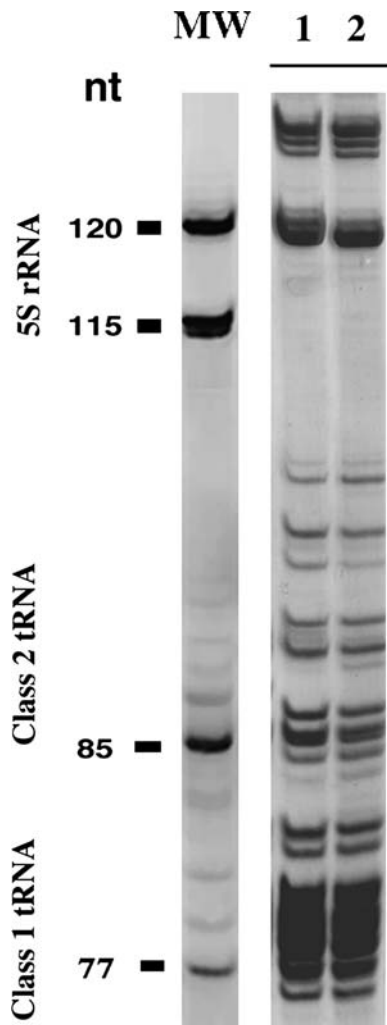


Figure 1. LMW RNA profiles of strain *S. meliloti* ATCC 9930<sup>T</sup> (lane 1) and strain LMTR32 (lane 2).

shows the LMW RNA profiles of the type strains of *S. meliloti* (lane 1), the strain LMTR32 (lane 2). The comparison among these strains shows that the LMW RNA profiles are identical in the type strain of *S. meliloti* and in the strain

LMTR32 and therefore this strain was identified as *S. meliloti*. These results open a new way in the study of symbiotic relatedness because the promiscuity of strains nodulating legumes seems to be very extended affecting to many rhizobial species. Moreover, unlike the type strain of *S. meliloti*, the strain LMTR32 was able to solubilize phosphate *in vitro*.

#### *Mobilization of phosphorous in plants*

The solubilization of phosphate *in vitro* by rhizobia does not involve the mobilization of the P to their hosts. Because of that, we have analyzed if the strain LMTR32 is able to mobilize phosphorous to common bean plants. The results showed that this strain was able to nodulate *P. lunatus* but the number of nodules induced by this strain is low. Moreover the nodules were low effective and therefore dry weight and nitrogen content per plant were also low. Nevertheless, the strain LMTR32 was able to solubilize phosphorous to plants. The increase of the P in plants inoculated with this strain was significantly higher than in the uninoculated plants. Therefore, the strain *S. meliloti* LMTR32 is able to solubilize phosphate *in vitro* and also to mobilize phosphorous to common beans. This result is in agreement with those obtained in the case of other rhizobia (Peix et al., 2001). Concerning to the symbiotic characteristics of this strain, such as nodulation and nitrogen fixation are similar to those presented by strains of *S. fredii* nodulating common bean and they are lower than to those presented by species of genus *Rhizobium* (Rodríguez-Navarro et al., 2000). Nevertheless, the results of this work indicate the great interest of the analysis of bacterial population nodulating legumes in different geographical regions to know the biodiversity of rhizobia that establish relationship with different species and genus of these plants (Table 1).

Table 1. Symbiotic characteristics of strain *S. meliloti* LMTR32 compared to those of *R. etli*

Strain	Number of nodules	Dry weight per plant (mg)	Total N (mg)	Total P (mg)	Total Ca ( $\mu$ g)	Total Mg ( $\mu$ g)	Total K (mg)
Control with insoluble P	0 <sup>a</sup>	470 <sup>a</sup>	9.4 <sup>a</sup>	1.4 <sup>a</sup>	3.1 <sup>a</sup>	6.0 <sup>a</sup>	6.9 <sup>a</sup>
<i>Sinorhizobium meliloti</i> LMTR32	18 <sup>b</sup>	700 <sup>b</sup>	17.4 <sup>b</sup>	3.1 <sup>b</sup>	6.7 <sup>b</sup>	18.9 <sup>b</sup>	8.4 <sup>b</sup>

Values followed by the same letter are no significantly different from each other at  $P = 0.05$  according to Fisher's Protected LSD (Least Significant Differences).

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