Quorum Sensing is essential for an effective symbiosis in *R. leguminosarum* UPM791.

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ABSTRACT
The implications of Quorum Sensing in the establishment of a successful symbiosis of *Rhizobium leguminosarum* bv. *viciae* (*Rlv*) with legume plants are discussed in this work. In order to analyze the significance and regulation of the production of AHL signal molecules, mutants deficient in each of the two QS systems present in *Rlv* UPM791 were constructed. A detailed analysis of the effect of these mutations on growth, AHL production, biofilm formation and symbiosis with pea, vetch and lentil plants has been carried out.

INTRODUCTION
Rhizobium leguminosarum is a soil alpha-proteobacterium that establishes a diazotrophic symbiosis with different legumes. This bacterium presents a multipartite genome harboring a large inventory of genes to maximize growth and survival in the soil, together with the additional genetic requirement imposed by the plant host. Very few rhizobial complete genomes have been obtained so far, despite their availability would help us to understand the interaction of rhizobia with their corresponding legume host. In the first stages of this interaction there is a rise in rhizobial cell density around the plant roots due to the supply of nutrients by the plant, enabling bacteria to grow in the rhizosphere. One of the factors involved in this symbiotic process is the intercellular communication system known as Quorum Sensing (QS). This mechanism allows bacteria to carry out diverse biological processes in a coordinate way through the production and detection of extracellular signals that regulate the coordinate transcription of different target genes in populations reaching high cell density.

MATERIAL AND METHODS
Sequencing was carried out through 454 massive sequencing (IGS, USA) and Illumina technology (BGI, China). Annotation has been achieved through the Manatee annotation pipeline (IGS, USA). Selectable marker cassettes were introduced by a fusion PCR procedure. AHLs were obtained from spent supernatant from stationary phase cultures, extracted with ethyl acetate and evaporated. Samples dissolved in methanol were subjected to HPLC analysis (C_{18} reverse phase) followed by electrospray-mass spectropmetry. Biofilm formation was assayed by a modification of the protocol described by Mueller and Gonzalez, 2011. Root hair attachment assays were done as described by Zheng *et al*., 2006. Plasmid content was determined by electrophoretic separation according to the technique described by Eckhardt (1978). For RNA preparation, cells were grown in 10 ml of TY and incubated for 24 h at 28°C, until an $OD_{600nm} \approx 0.7–1.0$. Harvested cells were resuspended in 500 µl of TE buffer. Total RNA was isolated with the RNeasy Minikit (Qiagen). cDNAs were obtained from 1 µg of bacterial RNA using SuperScript III Reverse Transcriptase, Invitrogen. qRT-PCR were carried out with Power SYBR Green in 25 µl final volume. Miscroscopic observations were performed using Confocal Leica TCS SP8 microscope. Fluorescence was observed either by DAPI staining or by the introduction of a GFP-encoding plasmid (pHC60).

RESULTS AND DISCUSSION

The genome of *Rlv* UPM791 consisted on a high quality draft of 17 contigs, with an estimated size of 7.8 Mb organized in one chromosome and five extrachromosomal replicons. Analysis of this genome led to the identification of two LuxRI-like systems (*rhiRI* and *cinRIS*) mediated by N-acyl-homoserine lactones (AHLs). HPLC-MS analysis allowed the adscription of C_6-HSL, C_7-HSL and C_8-HSL signals to the *rhiRI* system, encoded in the symbiotic plasmid, whereas the *cinRIS* system, located in the chromosome, produces 3-OH-$C_{14:1}$-HSL. A third synthase (TraI) is encoded also in the symbiotic plasmid, but its cognate regulator TraR is not functional due to a frameshift mutation. Three additional LuxR orphans were also found with no associated LuxI-type synthase.

To analyze the significance and regulation of the production of AHLs in *Rlv* UPM791, mutants deficient in the two QS systems of this bacterium were constructed. This analysis revealed that the *rhiRI* system is required for normal symbiotic performance, since a drastic reduction of symbiotic fitness was observed when *rhiI* is deleted. *rhiR* is essential for nitrogen fixation in the absence of one of the plasmids (pUPM791d).

Mutations in the *cinRIS* had a strong effect on symbiosis. A UPM791Δ*cinRIS* mutant, unable to produce 3-OH-$C_{14:1}$-HSL signal, originated a defective symbiosis with white and ineffective nodules lacking bacteroids. Transcription analysis showed that expression of *rhiRI* depends on the *cinRIS* system in *Rlv* UPM791. UPM791Δ*cinI* mutant, which did not produce AHLs, was unable to form nodules on any legume tested and was also defective in root hair attachment. This mutation is associated to the instabilization of the symbiotic plasmid through a mechanism still uncovered.

Overall, these data indicate the existence of a QS-dependent regulation significantly different to that previously described in other *R. leguminosarum* strains, where no relevant effect of QS systems on symbiotic phenotype had been observed. The defective symbiotic phenotypes of *Rlv* UPM791 suggest a relevant role of QS in the early stages of symbiosis as well as in the progression of nodule formation. The regulation of AHL production in *Rlv* UPM791 is a complex process involving the symbiotic plasmid (pUPM791c) and the smallest plasmid (pUPM791d), with a key role for the 3-OH-$C_{14:1}$-HSL signal.

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