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Dual effect of *Mesorhizobium loti* T3SS functionality on the symbiotic process

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Abstract

Mesorhizobium loti MAFF303099 has a functional type III secretory system (T3SS) involved in the nodulation process on *Lotus tenuis* and *Lotus japonicus*. Four putative *M. loti* T3SS effectors (Mlr6358, Mlr6331, Mlr6361, and Mlr6316) have been previously described, and it has been demonstrated that the N-terminal regions of Mlr6361 and Mlr6358 mediate the secretion via a T3SS. Here, we demonstrate the capacity of Mlr6316 and Mlr6331 N-terminal regions to direct the secretion of a translational fusion to a reporter peptide through T3SS. By using single, double, and triple mutants, we demonstrated the positive and negative participation of some of these proteins in the determination of competitiveness on *Lotus* spp. Low competitiveness values correlated with low nodulation efficiency for a mutant deficient in three of the putative *M. loti* effectors. Our data suggest that the net effect of *M. loti* T3SS function on symbiotic process with Lotus results from a balance between positive and negative effects.

Introduction

Type III secretion systems (T3SSs) are present in several pathogenic bacteria (Cornelis, 2002). These systems are multiprotein complexes through which effector proteins are delivered into the host cell where they can modulate various cellular functions (Galán, 2001; Cornelis, 2002; Alfano & Collmer, 2004).

Various rhizobium species also have a T3SS through which several proteins are secreted (Viprey *et al.*, 1998; Krause *et al.*, 2002; Lorio *et al.*, 2004; de Lyra *et al.*, 2006). Three of these proteins, NopA, NopB, and NopX, constitute the pili, whereas NopX has been postulated to act as the translocator protein (Saad *et al.*, 2008). Other secreted proteins, including NopD, NopJ, NopL, NopM, NopP, and NopT, have been described as effector proteins (Bartsev *et al.*, 2004; Skorpil *et al.*, 2005; Rodrigues *et al.*, 2007; Dai *et al.*, 2008; Kambara *et al.*, 2009).

Nodule formation and nitrogen fixation in legume roots are the result of a symbiotic process characterized by a complex exchange of signals between the plant and the bacterium. Plant flavonoids induce the production of rhizobial Nod factors responsible for the first morphological and physiological events that trigger nodule development. As for Nod factors, expression of rhizobial T3SS components and effectors is induced by flavonoids and NodD as the gene encoding the TtsI transcriptional factor contains a *nod* box consensus sequence in its promoter region (Krause *et al.*, 2002; Marie *et al.*, 2004). TtsI binds to *tts* boxes in the promoter regions of genes encoding T3SS components, inducing their transcription (Wassem *et al.*, 2008). *Mesorhizobium loti* forms a symbiotic association with *Lotus* spp. The sequencing of the *M. loti* MAFF303099 genome has revealed the presence of all the genes required to encode a T3SS (Kaneko *et al.*, 2000a, b). Regulation of the *M. loti* MAFF303099 T3SS is the same as in other rhizobia because its *ttsI* homolog is preceded by a *nod* box. Using a bioinformatic approach, we have previously searched for other *tts* box-controlled genes. We identified three new T3SS putative effectors in *M. loti* MAFF303099 (proteins encoded by *mlr6331*, *mlr6358*, and *mlr6361*) (Sánchez *et al.*, 2009) and determined the NodD/flavonoid-transcriptional regulation for another putative effector previously described for *M. loti* MAFF303099 (a protein encoded by *mlr6316*) (Hubber *et al.*, 2004). We also determined that the N-terminal

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region of Mlr6361 and Mlr6358 directs the secretion of the protein through T3SS (Sánchez *et al.*, 2009).

We have previously found that a *M. loti rhcN* mutant strain is negatively affected in nodulation competitiveness on *Lotus glaber* (now called *Lotus tenuis*) (Sánchez *et al.*, 2009). The *rhcN* gene encodes a protein similar to RhcN of *Rhizobium* sp. strain NGR234, a T3SS protein that shares characteristics of ATPase and whose mutation abolishes T3SS secretion (Viprey *et al.*, 1998). Okazaki *et al.* (2010) analyzed the nodulation efficiency of an *M. loti* mutant which lacked a region of the chromosome-containing genes encoding for both structural components of T3SS and putative secreted proteins and demonstrated that the presence of T3SS affected nodulation positively on *Lotus corniculatus* and *Lotus filicaulis* but negatively on *Lotus halophilus*, *Lotus peregrinus*, and *Lotus subbiflorus*. Okazaki *et al.* also observed no significant differences, between this mutant and the wild-type strain, in nodulation ability on *Lotus japonicus* Gifu B-129. Transcriptional analysis applied to *Lo. japonicus* Gifu B-129 inoculated with the *M. loti rhcN* mutant strain showed alterations in the expression level of genes normally upregulated during the nodulation process (Sánchez *et al.*, 2009). *Mesorhizobium loti* induced small white 'tumor-like' growth on *Leucaena leucocephala*, but a mutant in a conserved structural component of T3SS (the *M. loti rhcJ* mutant strain) formed large pink nodules (Hubber *et al.*, 2004). Little is known about the role of each of the putative *M. loti* T3SS effectors. The protein encoded by *mlr6316* has been described to have a partial negative effect on *Le. leucocephala* nodulation (Hubber *et al.*, 2004), whereas the protein encoded by *mlr6361* has been described to have a negative role in *Lo. halophilus* nodulation (Okazaki *et al.*, 2010). However, it has not yet been determined which of the proteins secreted by the *M. loti* T3SS are involved in the positive nodulation effects observed in some *Lotus* spp.

The aim of this work was to determine whether the N-terminal regions of proteins encoded by *mlr6316* and *mlr6331* are able to direct the protein secretion via *M. loti* T3SS and to determine the involvement of the different T3SS putative effectors in the symbiosis with two different *Lotus* species.

Materials and methods

Plasmids, bacterial strains, and growth media

Bacterial strains and plasmids used in this study are listed in Supporting Information, Table S1. MAFF303099 strains were grown at 28 °C in AB minimal medium (Douglas *et al.*, 1985) supplemented with sucrose (0.5% w/v). When necessary, antibiotics were added to the following final concentrations (μg mL^{-1}): gentamicin (Gm), 30; ampicillin (Amp), 100; neomycin (Nm), 100; spectinomycin (Sp), 100; and tetracycline (Tc), 10 for *Escherichia coli* or 1 for *M. loti*. For T3SS induction, naringenin was added to cultures at an optical density 600 nm ($OD_{600\,nm}$) of 0.1 to a final concentration of 1 μM.

Construction of the chromosomally integrated 3xFLAG translational fusions

pBAD-*mlr6331* was constructed as previously described (Sánchez *et al.*, 2009). The oligonucleotide primer pairs used are described in Table S1. Sequences encoding the N-terminal portion of the protein, together with the upstream promoter region, were cut from pBAD-*mlr6361* (59 aa), pBAD-*mlr6316* (160 aa), pBAD-*mlr6358* (160 aa) (Sánchez *et al.*, 2009), and pBAD-*mlr6331* (177 aa), respectively, and cloned into the pK18mobTc vector (Sánchez *et al.*, 2009). The same plasmids were also introduced by biparental mating into an *M. loti rhcN* pMP2112 mutant strain.

Isolation of extracellular and intracellular proteins

Supernatant protein extractions were carried out by direct TCA precipitation as previously described (Sánchez *et al.*, 2009). Supernatant was concentrated approximately 2000 times. For total (intracellular) bacterial protein extractions, 1 ml of the bacterial cultures used above was centrifuged and the pellets dissolved in cracking buffer.

Analysis of proteins by gel electrophoresis

Proteins were separated using SDS-PAGE and then stained using silver nitrate. For immunoblotting, anti-NGR234 strain NopA (Marie *et al.*, 2004) or a commercially available anti-FLAG M2 monoclonal antibody (Sigma) was used.

Creation of *M. loti* mutants

Analyzed mutants and oligonucleotides pairs used for their construction are described in Table S1. Construction details are given in Appendix S1.

Competitive assays

For competitive analysis, the indicated strains were mixed together in equal amounts and used to inoculate lotus plants as described previously (D'Antuono *et al.*, 2005). The proportion of each strain in the mixture was

determined as described previously (Sánchez *et al.*, 2009). Statistical analyses were carried out using ANOVA and the chi-square test.

Nodulation tests

Lotus seeds were surface-sterilized and pregerminated. Nodulation was observed by the agar slant method (Vincent, 1970). Three-day-old seedlings were placed into column tubes containing agar B&D ¼ (Broughton & Dilworth, 1971) (two plants per tube), inoculated with *M. loti* strains at an OD of 0.6 (100 μL), and observed daily for nodule number. Results are the average of three experiments. Statistical analysis was carried out by ANOVA.

Results

N-terminal translational fusions of *mlr6316* and *mlr6331* direct secretion through T3SS

It has been proposed that the signal to be secreted by T3SS resides in the amino acid sequence of the N-terminal region of T3SS effectors (summarized in Gosh, 2004). Experiments using fusion of this region to a reporter protein have been previously carried out to demonstrate the N-terminal region capacity to direct protein secretion through T3SS (Rüssmann *et al.*, 2002; Lorio *et al.*, 2004). Thus, we fused a FLAG epitope at the C-terminus of the truncated proteins by cloning the respective N-terminal regions into the vector pBAD24 3xFLAG (Fig. S1) (Guzman *et al.*, 1995; Spano *et al.*, 2008). To investigate protein secretion through T3SS, we introduced translational constructions into *M. loti* MAFF303099 already containing pMP2112, which constitutively expresses *nodD* of *Rhizobium leguminosarum*. Because the flavonoid that specifically induces the expression of *M. loti* promoters containing the *nod* box is unknown, we used this heterologous system (as proposed by López-Lara *et al.*, 1995) to induce flavonoid-controlled genes in MAFF303099 with naringenin.

We have previously described that the N-terminal regions of *mlr6361* and *mlr6358* are able to direct the secretion of a reporter peptide through the T3SS of *M. loti* (Sánchez *et al.*, 2009). As strains carrying plasmid-borne translational fusions of *mlr6316* and *mlr6331* were growth defective, we decided to analyze the secretion of the N-terminal translational fusions of *mlr6316* and *mlr6331* as single copies integrated into the *M. loti* MAFF303099 chromosome (MAFF6316SRpMP2112 and MAFF6331SRpMP2112). We also assayed the *mlr6358* (MAFF6358SRpMP2112) and *mlr6361* (MAFF 6361SRpMP2112) secretion capacity. When the assay was carried out in the presence of naringenin, secretion of the fused protein into the supernatant was observed in small amounts (data not shown). It has been previously described for pathogenic animal bacteria (Boyd *et al.*, 2000; Lee *et al.*, 2001; Deng *et al.*, 2005), that secretion of effectors proteins by T3SS could be induced by lowering the calcium concentration of the culture medium. To test whether a similar culture condition could trigger secretion in *M. loti*, we assayed the same translational fusions by growing the bacteria in induction medium without calcium. Secretion of the translational fusions corresponding to the four proteins was easily detected under these conditions (Fig. 1a). Another band, probably due to the degradation of the fused Mlr6331, was detected only in the pellet, indicating that the presence of the complete fused protein in the supernatant was not because of bacterial lysis. Fusions were also integrated into the chromosome of the *rhcN* mutant strain already containing pMP2112 (*rhcN*6316SRpMP2112, *rhcN*6331SRpMP2112, *rhcN*6358SRpMP2112, and *rhcN*6361SRpMP2112). No secretion was observed for any of them (Fig. 2b). These results demonstrate that secretion of the translational

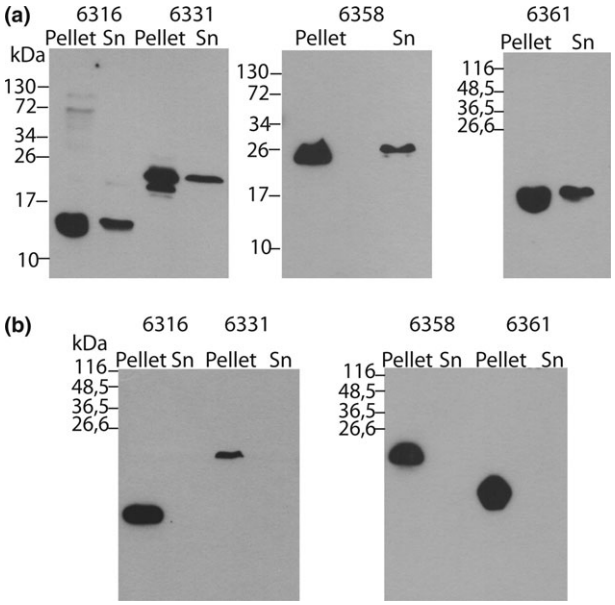

Fig. 1. Secretion of FLAG amino terminal fusions of Mlr6316, Mlr6331, Mlr6358, and Mlr6361. Supernatants (Sn) and intracellular proteins (pellet) were isolated from the MAFF303099 (a) and *rhcN* mutant (b) strains containing the respective chromosomally integrated fusions. The left blot in (a) corresponds to Mlr6316 and Mlr6331 fusions, the middle blot to Mlr6358 fusion, and the right blot to Mlr6361 fusion. The left blot in (b) corresponds to Mlr6316 and Mlr6331 fusions and the right blot to Mlr6358 and Mlr6361 fusions. Bacteria were grown for 30 h in inducing conditions in the absence of calcium. Proteins were separated by 15% SDS-PAGE, immunoblotted, and probed with an anti-FLAG antibody. Positions of size markers loaded onto the gels are shown (in kDa).

fusions corresponding to *mlr6361*, *mlr6358*, *mlr6316*, and *mlr6331*, chromosomally integrated in the wild-type (wt) strain, occurs in a T3SS-dependent manner.

Symbiotic phenotype of *M. loti* T3SS mutants

Previous reports have indicated that mutations in protein secretion systems in *M. loti* affect symbiotic competitiveness in lotus (Hubber *et al.*, 2004; Sánchez *et al.*, 2009). *Mesorhizobium loti* MAFF303099 *rhcN* mutant was less competitive than the wt strain with regard to nodulation on *Lo. tenuis* cv. Pampa INTA (Sánchez *et al.*, 2009). Because it has been reported that the *M. loti* T3SS mutant has different nodulation efficacies on different *Lotus* species (Okazaki *et al.*, 2010), we decided to compare the symbiotic competitiveness of the wt with that of *rhcN* mutant strains on *Lo. japonicus* Miyacojima MG-20. As shown in Fig. 2a, the strains showed no differences in competitiveness when they were co-inoculated in this plant. As the two strains differ in their protein secretion capacity, the lack of differences in competitiveness in the co-inoculation assays could be due to phenotypic complementation. We thus performed a nodulation test to compare the nodulation efficiency of the wt with that of *rhcN* mutant strains on *Lo. japonicus* MG-20 and found no significant differences between strains (Fig. 2b). Also, we analyzed the competitiveness of the wt and *rhcN* mutant strains on *Lo. tenuis* cv. Esmeralda, and in contrast to that observed on *Lo. tenuis* cv. Pampa INTA, the mutant was more competitive than the wt strain in this variety (Fig. 2a). This result indicates that the inability to secrete some effectors, or to surface-expressed T3SS pili components, favors the *M. loti's* competitive ability on *Lo. tenuis* cv. Esmeralda. To determine the role of the four *M. loti* T3SS putative effectors in the nodulation process, we performed nodulation competitive assays on *Lo. tenuis* cv. Esmeralda and *Lo. japonicus* MG-20 with the wt and single, double, and triple mutant strains. Co-inoculation experiments were carried out using different combinations of the strains analyzed. Surprisingly, the mutant deficient in three of the putative T3SS effectors (*M. loti mlr6358/mlr6361/mlr6316*, hereafter triple mutant) showed a significant decrease in competitiveness compared to the wt strain on both *Lo. tenuis* cv. Esmeralda (Fig. 3a) and *Lo. japonicus* MG-20 (Fig. 3c). These differences were not the consequence of different growth rates as both strains showed similar growth curves in minimal medium (data not shown). The *M. loti* triple mutant also showed a significantly lower competitive ability when co-inoculated with the *rhcN* mutant strain (Fig. 3a).

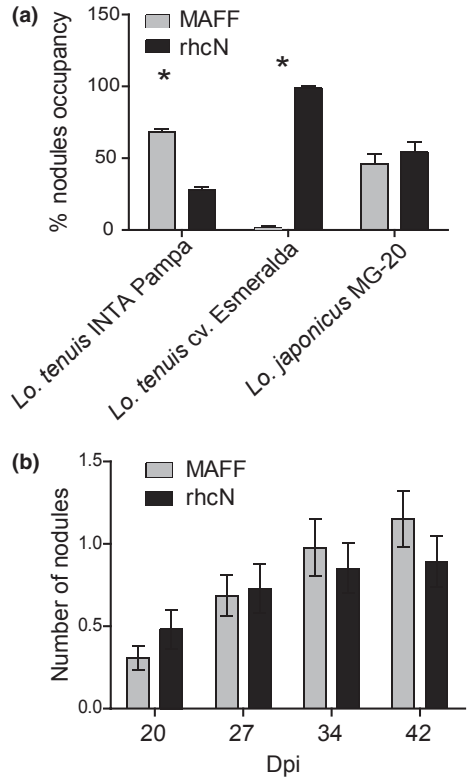

Fig. 2. (a) Symbiotic phenotype of the *rhcN* mutant strain on different Lotus plants. Plants were co-inoculated with an equal mixture of wt and *rhcN* mutant strains. The percentages of nodules occupied by each bacterial strain 6 weeks postinoculation are shown. Data corresponding to *Lotus tenuis* cv. Pampa INTA were previously reported by Sánchez *et al.* (2009). The asterisk indicates that the relative nodule occupancy differed significantly ($P \leq 0.05$) from the null hypothesis of 50%:50% occupation applying the chi-square test and that using ANOVA, the difference between relative percentages is significant ($P \leq 0.05$). (b) Nodulation kinetics of *Lotus japonicus* MG-20 inoculated with the *Mesorhizobium loti* wild-type and *rhcN* mutant strains. The number of nodules was counted daily and recorded for at least 20 plants per strain per experiment. The values are means of three nodulation tests for each strain, and the error bars indicate standard deviations. Statistically significant differences ($P < 0.05$) were analyzed by applying the ANOVA test. Dpi, days postinoculation.

Different independent experiments (Fig. 3a) indicated a positive role for the protein encoded in *mlr6316* in the symbiotic competitiveness on *Lo. tenuis* cv. Esmeralda: The wt strain showed a slightly higher competitiveness than the *mlr6316* mutant, and the same difference was observed when the double mutant *mlr6358/mlr6361* was co-inoculated with the triple mutant.

The comparison between the results obtained when the wt strain was co-inoculated with the *mlr6316* mutant and those obtained when the wt strain was co-inoculated with the triple mutant indicates that the triple mutation affects competitiveness more drastically than the single mutation in *mlr6316* (Fig. 3a and b). This suggests the possibility that the protein encoded in *mlr6358* and/or the protein

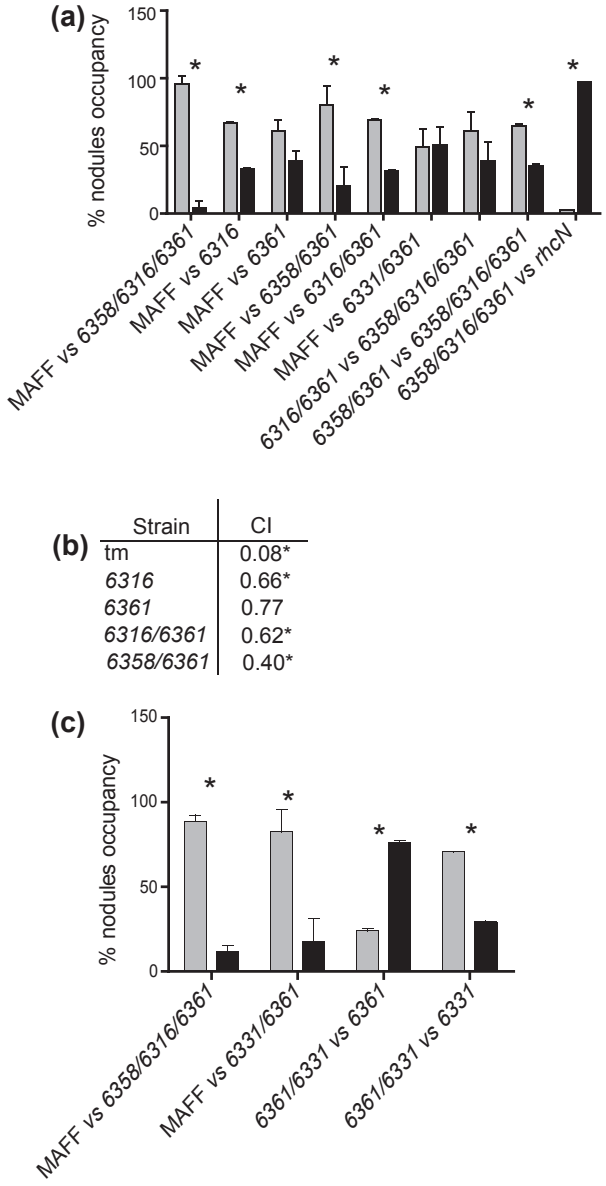

Fig. 3. Competition assays on *Lotus tenuis* cv. Esmeralda and *Lotus japonicus* MG-20. The strains were co-inoculated at equal concentrations. For each *x* vs. *y* assay, the gray bars correspond to the percentage of nodules occupied by strain *x*, and the black bars correspond to percentage of nodules occupied by strain *y*. The asterisk indicates that the relative nodule occupancy differed significantly ($P \leq 0.05$) from the null hypothesis of 50%:50% occupation applying the chi-square test and that using ANOVA, the difference between relative percentages is significant ($P \leq 0.05$). (a) Competitiveness on *Lo. tenuis* cv. Esmeralda; (b) competitive index (CI) for each mutant strain co-inoculated with the wt strain (MAFF) on *Lo. tenuis* cv. Esmeralda. CI is defined as the number of singly occupied nodules formed by the mutant strain as a proportion of all nodules formed, divided by the proportional representation of viable cells of that strain in the inoculate mixture according to McDermott & Graham (1990); (c) competitiveness on *Lo. japonicus* MG-20. tm, triple mutant *Mesorhizobium loti mlr6358/mlr6361/mlr6316*.

encoded in *mlr6361* play a positive role in the symbiotic competitiveness. Consistent with this, the double mutant *mlr6358/mlr6361* was less competitive than the wt strain (Fig. 3a). The triple mutation in *mlr6358*, *mlr6361*, and *mlr6316* also caused a more drastic effect on competitiveness than the combined *mlr6316/mlr6361* mutation (Fig. 3a and b). This indirectly indicates that Mlr6358 has a positive effect on competitiveness on *Lo. tenuis*.

No statistically significant differences were observed in competitiveness on *Lo. tenuis* cv. Esmeralda between the wt and the *mlr6361* mutant or between the wt and the double mutant *mlr6331/mlr6361* (Fig. 3a). However, the mutant affected in both Mlr6361 and Mlr6331 showed decreased competitiveness compared with the wt strain on *Lo. japonicus* MG-20 (Fig. 3c). To determine which of the two proteins are responsible for the positive effect on this plant, co-inoculation assays of the double mutant with each of the single mutants were performed. Results indicate that the double mutant was less competitive than the single mutant affected in *mlr6361* but more competitive than the single mutant affected in *mlr6331* (Fig. 3c). This indicates that Mlr6331 has a positive effect and that Mlr6361 has a negative effect on the competitiveness on *Lo. japonicus* MG-20.

We determined the nodulation kinetics for the *M. loti* wt, the *rhcN* mutant, and the triple mutant on *Lo. tenuis* cv. Esmeralda (Fig. 4). Although the *rhcN* mutant showed greater competitive ability on this plant (Fig. 3a), its nodulation kinetics was negatively affected when compared with the wt strain. On the other hand, in concordance with the competitiveness results, the *M. loti* triple mutant presented a kinetic phenotype significantly negatively affected compared with the wt strain and a delayed nodulation kinetics compared with the *rhcN* mutant strain (Fig. 4).

Discussion

Chromosomal integration of the *mlr6331* and *mlr6316* N-terminal regions fused to the reporter peptide allowed us to assess their ability to direct protein secretion in a T3SS-dependent manner. The secretion was more efficient in induction media in the absence of calcium. In animal pathogenic bacteria, a decrease in calcium concentrations has been proposed as one of the signals that trigger T3SS secretion of T3SS effectors (Lee *et al.*, 2001; Deng *et al.*, 2005).

Although no canonical T3SS signal sequence is present in Mlr6316, we demonstrated that its N-terminal region (160 aa) directs secretion in a T3SS-dependent manner. The homologous Mlr6316 protein expressed by *M. loti* R7A is encoded by the *msi059* gene and is translocated into the host cell through a type IV secretion system

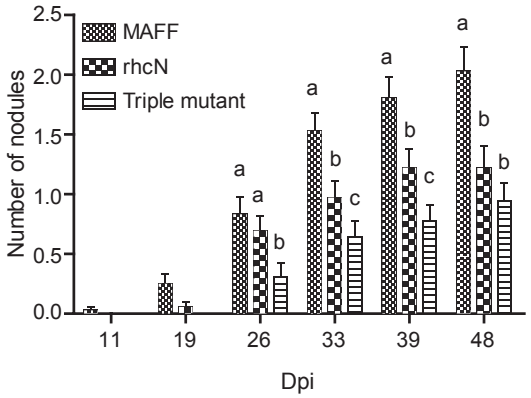

Fig. 4. Nodulation kinetics of *Lotus tenuis* cv. Esmeralda plants inoculated with the *Mesorhizobium loti* wt and the *rhcN* and triple mutant strains. Nodulation tests for each strain were performed three times, and 20 plants were tested each time. Error bars correspond to standard deviations from the mean. Means with the same letter, corresponding to each indicated time, are not significantly different ($P \leq 0.05$). Dpi, days postinoculation.

(T4SS) (Hubber *et al.*, 2004). It has been suggested that an RxR motif in the C-terminal region forms part of the T4SS signal (Hubber *et al.*, 2004). Mlr6316 and the protein encoded by *msi059* (Msi059) share 88% of amino acid identity, and very few differences have been observed between their respective N-terminal regions. Both Msi059 and Mlr6316 also have an RxR motif in their C-terminal region. It is possible that the two proteins conserve the capacity to be secreted both by T3SS and T4SS.

The case of *mlr6331* is similar to that of *mlr6316* as it does not have the characteristic amino acid pattern present in T3SS substrates. However, Yang *et al.* (2010) applied a computational prediction of type III secreted proteins in Gram-negative bacteria and found that the protein encoded by *mlr6331* is a putative T3SS substrate.

Competitive experiments were carried out to analyze the participation of *M. loti* T3SS or putative *M. loti* T3SS effectors in the symbiotic process. Competitive assays have been used in several works to analyze the changes in the symbiotic phenotype (Lagares *et al.*, 1992; Vinuesa *et al.*, 2002; Hubber *et al.*, 2004). This method has the advantage that the symbiotic capacities of two bacterial strains are compared on the same plant, and this could improve the sensitivity for the detection of a subtly altered phenotype.

The results presented here demonstrate that symbiotic competitiveness on *Lo. tenuis* cv. Esmeralda was negatively affected by a functional T3SS. To determine which proteins were responsible for this effect and taking into account that a particular T3SS effector is often only partially responsible for the overall effect of the T3SS (Kambara *et al.*, 2009), we went on to analyze the nodulation competitiveness phenotype on *Lo. tenuis* cv. Esmeralda using single, double, and triple mutants affected in the potentially secreted *M. loti* T3SS proteins described. Surprisingly, we observed a significantly diminished competitiveness associated with the triple mutant compared to the wt strain. The same phenotype was observed on *Lo. japonicus* MG-20. The results of the nodulation kinetic test indicate that the triple mutant also induced a lower number of nodules than the wt strain on *Lo. tenuis* cv. Esmeralda. These experiments indicate that some of the proteins affected in the triple mutant or a combination of them have a positive role in the nodulation ability on lotus and also suggest that no phenotypic complementation occurred between the co-inoculated strains in competitiveness assay.

We here demonstrated the positive involvement of Mlr6316 in the symbiotic competitive capacity. It has been previously described that the *msi059* mutant (affected in the *mlr6316* homolog in *M. loti* R7A) shows a delayed nodulation on *Lo. corniculatus* (Hubber *et al.*, 2004). From results, we also indirectly conclude a positive participation of Mlr6358 in the bacterial competitiveness on *Lo. tenuis* cv. Esmeralda. No effect on competitiveness was demonstrated for Mlr6361 in co-inoculation experiments on *Lo. tenuis*. However, it could not be discarded that this protein exerts certain effect. In fact, although a positive effect was indirectly demonstrated for Mlr6358, no direct evidence was obtained for this, co-inoculating strains that differed only in the presence of Mlr6358. In general, the functional analysis of type III secreted effectors in phytopathogenic bacteria is hindered by the fact that mutation of effectors genes frequently has very small or no effect on the bacterial phenotype in the interaction with the plant (Grant *et al.*, 2006). A possible reason is the existence of effectors with redundant functions. A functional redundancy for the putative T3SS effectors described in *M. loti* is possible as Mlr6331 is 68% similar to the 2360-aa C-terminal of Mlr6361, and Mlr6358 is 54% similar to the 684-aa N-terminal of Mlr6361 (Sánchez *et al.*, 2009).

In spite of the positive effects attributed to some of the proteins, individually or in combination, on the symbiotic competitiveness on the two lotus species assayed, a mutation in the *rhcN* gene had either no effect or a significant positive effect on this phenotype, depending on the legume examined. The *rhcN* mutant is affected in the protein secretion through T3SS. Our results suggest that some pili components or T3SS-secreted proteins could negatively affect bacterial competitiveness on these plants. The balance between positive and negative effects may determine the role of T3SS in the symbiotic process on the respective legumes (negative on *Lo. tenuis* cv. Esmeralda and no effect on *Lo. japonicus* MG-20). The

results obtained for Mlr6331 and Mlr6361 on *Lo. japonicus* MG-20 also indicate a positive effect for Mlr6331 and a negative effect for Mlr6361. This negative effect was evident only in the absence of Mlr6331. As this condition, was not assayed on *Lo. tenuis* cv. Esmeralda, it could not be discarded that Mlr6361 also has a negative effect on this plant. The fact that the double mutant *mlr6331/mlr6361* was less competitive than the wt strain on *Lo. japonicus* MG-20 seems to indicate that the positive effect of Mlr6331 is stronger than the negative effect of Mlr6361 and that the net balance result is thus positive. The two proteins share a large C-terminal sequence, and double hybrid experiments indicate that interaction occurs between them (Shimoda *et al.*, 2008a, b). Further analysis must be carried out to determine how these characteristics are involved in protein functionality. In the interaction between *Rhizobium* strain NGR234 and *Tephrosia vogelii*, both positive and negative T3SS effectors have been described, resulting in the generation of the 'equilibrium hypothesis', which suggests that the combination of these effects determines whether T3SS acts positively, negatively, or has no effect on nodule formation (Skorpil *et al.*, 2005; Kambara *et al.*, 2009). A dual effect of T3SS effectors also was described for plant-bacterial pathogens (Oh *et al.*, 2010; Boureau *et al.*, 2011). Okazaki *et al.* (2010) attributed a negative effect for Mlr6361 on *Lo. halophilus* nodulation. Our results also indicate a negative effect for Mlr6361 in competitiveness on *Lo. japonicus* MG-20 and could not discard the same on *Lo. tenuis*. However, the negative role of Mlr6361 does not appear to be the only factor responsible for the negative effects of T3SS functionality on both plants. Besides the putative *M. loti* T3SS effectors studied here, several other candidate effectors remain to be analyzed. Some arose from our bioinformatic search of promoter regions containing sequences significantly homologous to the *tts* box (Sánchez *et al.*, 2009). Other candidate effectors arose from the analysis of Yang *et al.* (2010), and by homology to known phytopathogen T3SS effectors, other two putative T3SS proteins in *M. loti* MAFF303099 were identified (Grant *et al.*, 2006).

The results obtained from kinetic nodulation and competitiveness analysis on *Lo. tenuis* cv. Esmeralda also indicate a better performance for the *rhcN* mutant than for the mutant affected in the expression of the three putative T3SS effectors. This is in concordance with the idea that a mutation that affects T3SS functionality prevents both positive and negative T3SS effects. However, the *rhcN* mutant induced a lower number of nodules than the wt strain in spite of the higher competitiveness of the former. This indicates that high competitiveness not necessarily reflects high nodulation capacity and suggests that the participation of the positive and negative effects resulting from T3SS functionality may affect different phenotypes in a different manner.

In conclusion, the results presented here demonstrate the capacity of Mlr6331 and Mlr6316 N-terminal regions to direct secretion through *M. loti* T3SS. The results also show that Mlr6358, Mlr6361, Mlr6331, and Mlr6316, either individually or in combination, play a role in the symbiotic competitiveness on *Lo. tenuis* and/or *Lo. japonicus*. Data also show that the function of T3SS in the symbiotic process with lotus results from a balance between positive and negative effects. Further analysis is needed to identify other *M. loti* T3SS effectors or components involved in T3SS functionality in symbiosis.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Schematic representation of hybrid proteins consisting of the truncated derivatives of the four putative T3SS effectors fused to the reporter peptide 3xFLAG into the pBAD24-3xFLAG.

Table S1. Bacterial strains, plasmids, and primers used in this study.

Appendix S1. Creation of *M. loti* mutants.

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