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Letter to the Editor

LETTER TO THE EDITOR – Environmental Microbiology

The transcriptional factor TtsI is involved in a negative regulation of swimming motility in *Mesorhizobium loti* MAFF303099

Cecilia M. Duarte, Laura A. Basile, Andrés Zalguizuri and Viviana C. Lepek*

Instituto de Investigaciones Biotecnológicas "Dr. Rodolfo A. Ugalde", Universidad Nacional de San Martín (IIB-UNSAM), CONICET, Av. 25 de Mayo y Francia, San Martín, Buenos Aires 1650, Argentina

*Corresponding author: Instituto de Investigaciones Biotecnológicas, Universidad Nacional de San Martín (IIB-UNSAM), Av. 25 de Mayo y Francia, Gral San Martín, Provincia de Buenos Aires, B1650HMP, Argentina. Tel: +54-11-4006-1500 (int. 2114); E-mail: vlepek@iib.unsam.edu.ar
One sentence summary: Motility regulation in *Mesorhizobium loti* MAFF303099.
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ABSTRACT

Mesorhizobium loti MAFF303099 has a functional Type III secretion system (T3SS) that is involved in the determination of competitiveness for legume nodulation. Here we demonstrate that the transcriptional factor TtsI, which positively regulates T3SS genes expression, is involved in a negative regulation of *M. loti* swimming motility in soft-agar. Conditions that induce T3SS expression affect flagella production. The same conditions also affect promoter activity of *M. loti visN* gene, a homolog to the positive regulator of flagellar genes that has been described in other rhizobia. Defects in T3SS complex assembly at membranes limited the negative regulation of motility by the expression of TtsI.

Keywords: rhizobia; secretion system; swimming motility; VisN; T3SS; flagella

INTRODUCTION

T3SS is a protein secretion system present in several plant and animal pathogenic bacteria that allows the translocation of effector proteins into the eukaryotic host cells (Galán 2001; Cornelis 2002; Alfano and Collmer 2004). This system is evolutionary related to the flagellar export system (Gophna, Ron and Graur 2003; Abby and Rocha 2012). T3SS gene cluster in *Mesorhizobium loti* MAFF303099 is localized in the chromosomal symbiotic island and contains all the genes that code for the conserved components of T3SS complex (Kaneko *et al.* 2000a,b). T3SS functionality is involved in the determination of *M. loti* nodulation competitiveness on some legumes (Sánchez *et al.* 2009, 2012; Okazaki *et al.* 2010) and in the determination of range of host (Hubber *et al.* 2004). Four proteins with capacity to be secreted through *M. loti* T3SS were identified (Hubber *et al.* 2004; Sánchez *et al.* 2009).

Expression of *M. loti* T3SS genes is regulated by the activity of the transcriptional factor TtsI, as occurs in other rhizobia (Sánchez *et al.* 2009). TtsI binds to *tts* box consensus sequences upstream of some of the T3SS genes (Wassem *et al.* 2008). TtsI expression is induced by the coordinated action of the transcriptional regulator NodD and the specific flavonoid exuded by the legume (Krause, Doerfel and Gottfert 2002; Marie *et al.* 2004). Both elements bind to the *nod* box consensus sequences localized in *ttsI* promoter region and also in the *nod* promoter regions, which genes code for proteins involved in Nod factors synthesis (*nod* genes) (Kobayashi *et al.* 2004).

An opposite regulation between T3SS expression and the flagellar system was described in *Erwinia amylovora* and *Pseudomonas syringae* (Cesbron *et al.* 2006; Ortíz-Martín *et al.* 2010). It was determined that HrpL, the positive key regulator of T3SS in these phytopathogenic bacteria, has a role in the

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downregulation of motility (Cesbron *et al.* 2006). Motility regulation has been studied in *Rhizobium leguminosarum* (Tambalo *et al.* 2010) and *Sinorhizobium meliloti* (Sourjik *et al.* 2000). A hierarchy in the regulation of motility-related genes in these bacteria was found, where the global activators VisN and VisR regulate the expression of Rem, which positively controls the expression of genes of the flagellar regulon, including those coding for flagellin (Sourjik *et al.* 2000; Tambalo *et al.* 2010). *Mesorhizobium loti* presents swimming motility in soft-agar (D'Antuono *et al.* 2005), but its regulation has not yet been studied. The genetic organization of flagellar genes in *M. loti* MAFF303099 is similar to that of *R. leguminosarum*, being *visN*, *visR* and *rem* genes present in the flagellar gene cluster of this bacteria (Kaneko *et al.* 2000a,b). In the present work, we demonstrate the participation of TtsI in the inhibition of swimming motility in soft-agar for *M. loti* MAFF303099.

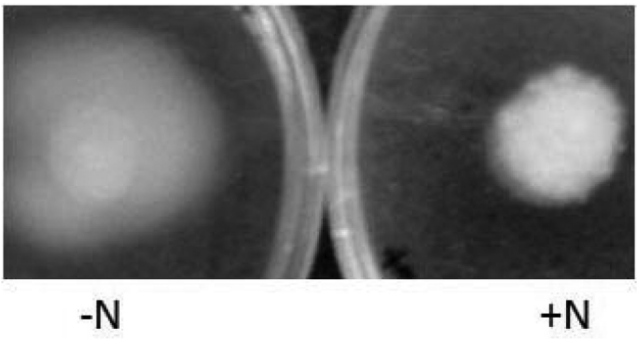

Figure 1. Swimming motility of *M. loti* pMP2112 strain. Bacteria were inoculated by puncture in soft-agar plates, with (+N) or without (–N) naringenin, and observed at 10 dpi.

MATERIALS AND METHODS

Plasmids, bacterial strains and growth media

Bacterial strains and plasmids used in this study are listed in Table S1 (Supporting Information). *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium. *Mesorhizobium loti* strains were grown at 28°C in AB minimal medium (Douglas *et al.* 1985) supplemented with sucrose (0.5% wt/vol). When necessary, antibiotics were added to the following final concentrations: gentamicin (Gm), 30 μg/ml; ampicillin (Amp), 100 μg/ml; spectinomycin (Sp), 100 μg/ml and tetracycline (Tc), 10 μg/ml for *E. coli* or 1 μg/ml for *M. loti*. For T3SS induction, naringenin was added to plates at a final concentration of 1 μM.

Creation of *Mesorhizobium loti ttsI* mutant strain

Oligonucleotide primer pairs (Table S1, Supporting Information) were designed to amplify flanking regions of *mlr6334* gene (also named *ttsI* gene). Details for mutant creation and complementation are given in Appendix S1 (Supporting Information).

Motility assay

Swimming motility was evaluated by puncture inoculation of late exponential phase cells in the center of a soft-agar (0.2% agar) motility plate with AB minimal medium. Only for quantification analysis, culture at late exponential phase was centrifuged, bacterial pellet was resuspended in 50 μl of AB minimal medium and 1 μl was stabbed in the center of the motility plate. Plates were incubated at 28°C and the diameter of the motility halo was measured at the indicated days post-inoculation (dpi). Statistical analysis of replica results was made using the Student's *t*-test.

Determination of surface flagellin levels

A late exponential bacterial culture was inoculated by puncture in the center of each motility plate containing 0.2% of agar with AB minimal medium. After 15 days, incubation bacteria were harvested from plate by resuspension in AB minimal medium. The agar–bacteria mixture was centrifuged, and pelleted cells were washed with 50 mM Tris-hydrochloride buffer (pH 7.0). Samples to be compared were adjusted to the same bacterial concentration based on optical density. Flagellin was prepared according the method previously described (Cesbron *et al.* 2006). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 7.5%) and stained with Coomassie blue.

Flagella staining

A flagellar-staining protocol was carried out according the method described in Merritt, Danhorn and Fuqua (2007), and observed by phase-contrast microscopy using a 100× oil immersion objective.

Analysis of transcriptional expression of lacZ-fusions

Construction details and methodology are given in Appendix S1 (Supporting Information).

RESULTS

Swimming motility of *Mesorhizobium loti* MAFF303099 is negatively affected in the presence of T3SS induction media

As the flavonoid that specifically induces the activity of *M. loti* promoters containing the *nod* box is unknown, we induced the T3SS expression in *M. loti* MAFF303099 by the introduction of the plasmid pMP2112 expressing the transcriptional factor NodD of *Rhizobium leguminosarum* and by adding its specific flavonoid, naringenin, to the culture media (Sánchez *et al.* 2009, 2012; Mercante *et al.* 2015). This heterologous system has been proposed to induce *nod* genes expression in *R. loti* (López-Lara *et al.* 1995), and was also used to study T4SS expression in *M. loti* R7A (Hubber, Sullivan and Ronson 2007). Motility assay in soft-agar (0.2%) was carried out by inoculating by puncture *M. loti* MAFF303099 strain containing pMP2112 plasmid (*M. loti* pMP2112) in media with and without naringenin. Figure 1 shows that motility was altered in the presence of naringenin. It presented minor halo and greater proportion of cell aggregates instead of the swimming ring. When the same assay was made with a strain without pMP2112 plasmid, no inhibition was observed in presence of naringenin (data not shown). These results indicate that conditions that induce T3SS expression, which are NodD together with its specific flavonoid, were responsible for the alteration of swimming motility in semi-solid media of *M. loti* MAFF303099.

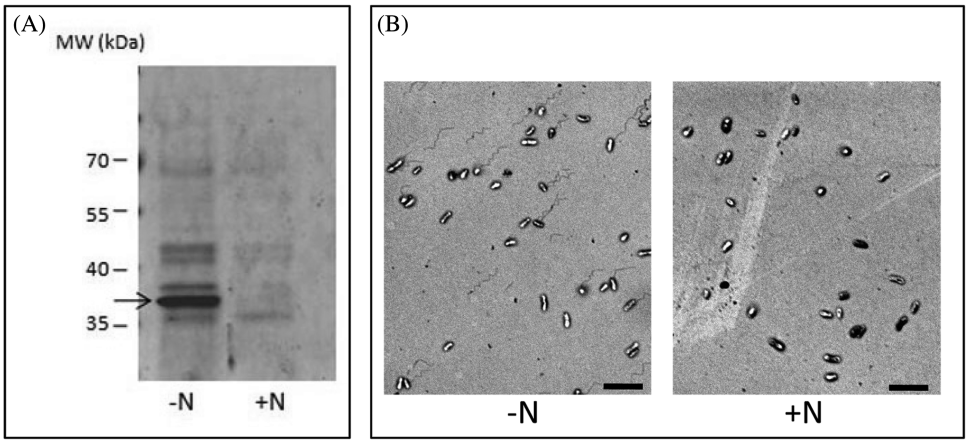

Figure 2. Analysis of flagella levels. (**A**) Analysis of proteins obtained by flagellin extraction methodology. *Mesorhizobium loti* pMP2112 was inoculated by puncture in soft-agar plates with (+N) or without (–N) naringenin. At 14 dpi bacteria were harvested from plates and subjected to a method applied for flagellin recovering from bacterial surface as described in Materials and Methods. Proteins were subjected to 7.5% SDS-PAGE and stained with Coomassie blue. Arrow indicates the differential band. (**B**) Optical microscopy (at ×100) of bacteria recovered from motility plates with (+N) and without (–N) naringenin at 14 dpi. Bacteria were stained as described in Materials and Methods. Bar = 5μm.

Flagella levels are negatively affected in the presence of T3SS induction media

Mesorhizobium loti MAFF303099 presents two genes (*mlr2925* and *mlr2927*) that code for flagellin (FlaA) (Kaneko *et al.* 2000a,b). They code for proteins with a theoretical molecular weight around 33 and 36 kDa, respectively.

Flagellin was extracted from bacteria recovered from motility plates according to a method previously applied to obtain flagellin-enriched fraction from *Erwinia amylovora* (Cesbron *et al.* 2006). By Coomassie blue staining, a major clearly differential band with an MW between 35 and 40 kDa was detected in the fraction corresponding to bacteria incubated under non-T3SS-inducing conditions (Fig. 2A). Based on its MW and the methodology used for its isolation, this differential band could correspond to one of the *M. loti* flagellins. As loading control, aliquots of the respective samples used for flagellin isolation were subjected to SDS-PAGE (Fig. S1, Supporting Information). Figure 2B shows the optical microscopy visualization of bacteria recovered from the motility plates and stained according to Merritt, Danhorn and Fuqua (2007). Flagella only are observed in non-T3SS-inducing conditions. These results confirm that flagella production in MAFF303099 is negatively affected under T3SS-inducing conditions in soft-media.

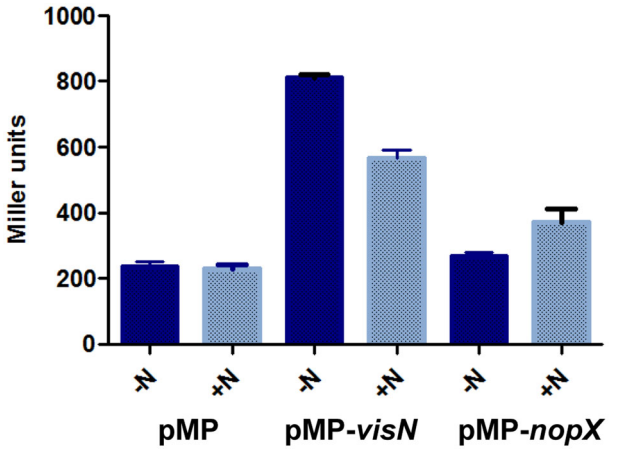

Figure 3. Expression of *visN* and *nopX* promoters fused to *lacZ*. *Mesorhizobium loti* pMP2112 containing the empty vector pMP220 (pMP), the vector pMP220 with *visN* promoter fused to *lacZ* (pMP-*visN*) or the vector pMP220 with *nopX* promoter fused to *lacZ* (pMP-*nopX*) were inoculated by puncture in soft-agar plates with (+N) and without (–N) naringenin. Bacteria were harvested from plates at 11 dpi and β-galactosidase activity was measured. Values are indicated in Miller units as previously described (Sánchez *et al.* 2009). Each result is the average of data corresponding to three replicas.

VisN expression is negatively affected in the presence of T3SS induction media

VisN, a LuxR family transcriptional regulator, was described as a regulator of flagellar genes expression in *Sinorhizobium meliloti* and *R. leguminosarum* (Sourjik *et al.* 2000; Tambalo *et al.* 2010). *Mesorhizobium loti* has a homolog to VisN coded by the *mlr2896* gene (Kaneko *et al.* 2000a,b). As T3SS-inducing conditions affect negatively flagella levels, we proceed to analyze if this negative effect occurs at a transcriptional expression level. A pMP220 plasmid containing *lacZ* gene under the regulation of *visN* promoter (pMP-*visN*) was introduced in *M. loti* pMP2112 strain. Bacteria were recovered from semi-solid media containing or not naringenin, at 11 dpi, and β-galactosidase activity was analyzed. The activity of *nopX* promoter region was also assayed under the same conditions. *nopX* gene belongs to the T3SS genes cluster and presents a *tts* box sequence in its promoter region (see pMP-*nopX* in Table S1, Supporting Information). Figure 3 shows that meanwhile *nopX* promoter activity increases at inducing conditions, the *visN* promoter activity suffers a decrease. This result indicates that T3SS induction conditions in semi-solid media have, directly or indirectly, negative effects on the *visN* gene expression.

Alteration of swimming motility is due to *ttsI* expression

T3SS induction conditions (NodD plus flavonoid), besides inducing TtsI expression, also induce the expression of the *nod* genes coding for the proteins involved in Nod factors production. To discard the involvement of factors independently of TtsI in the inhibition of motility observed, a mutant in *ttsI* gene was constructed. The effect on swimming motility in semi-solid media

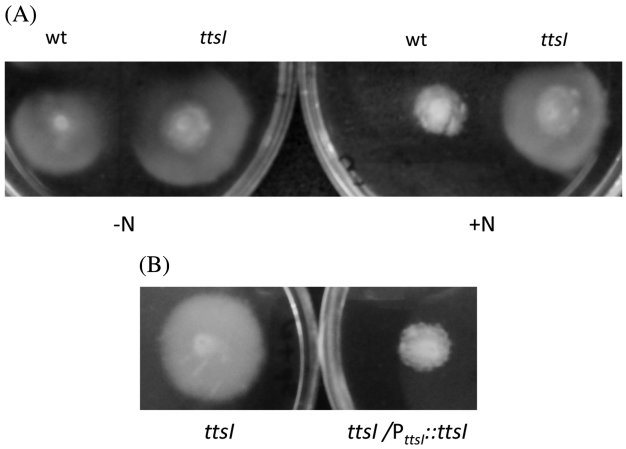

Figure 4. Effect of *ttsI* mutation on swimming motility. (**A**) Swimming motility of *M. loti* wild-type (wt) and *ttsI* mutant (*ttsI*) strains. Bacteria containing pMP2112 plasmid were inoculated by puncture in soft-agar plates, with (+N) or without (–N) naringenin. Motility was observed at 10 dpi. (**B**) Swimming motility in the presence of naringenin of *M. loti ttsI* mutant (*ttsI*), and *ttsI* mutant carrying pMP220 plasmid containing *ttsI* gene expressed under its own promoter (*ttsI*/P_{ttsI}::*ttsI*).

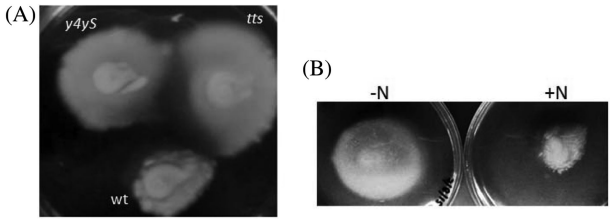

Figure 5. Comparison of motility phenotype of wild-type and mutant strains. (**A**) Wild-type and mutant strains in the presence of T3SS-inducing conditions at 10 dpi. wt: *M. loti* pMP2112, *tts*: *ttsI* mutant strain with pMP2112 plasmid, *y4yS*: *y4yS* mutant strain with pMP2112 plasmid. (**B**) Motility phenotype observed at 13 dpi of *y4yS* mutant strain containing pMP2112 plasmid and complemented with *y4yS* gene expressed under a constitutive promoter. +N: with naringenin, –N: without naringenin.

was assayed with the *ttsI* mutant strain containing the pMP2112 plasmid. Figure 4A shows that the negative effect on motility under inducing conditions evidenced by the wild-type strain was reverted in this mutant strain. The *ttsI* mutant was complemented with the *ttsI* gene under its own promoter (P_{ttsI}::*ttsI*), and cloned into the pMP220 plasmid. Figure 4B shows that the complemented mutant again presents motility alteration in the presence of naringenin. These results confirm that the expression of TtsI is involved in the inhibition of swimming motility in semi-solid media.

We proceeded to analyze the effect of T3SS-inducing conditions on swimming motility in another *M. loti* mutant strain affected in the T3SS. *Mesorhizobium loti y4yS* strain has a mutation in the *mlr8765* gene, that codes for a protein (Y4yS protein) involved in the localization of *M. loti* T3SS secretin at membranes and therefore in the T3SS complex assembly (Mercante *et al.* 2015). *y4yS* gene is localized in a gene cluster under the activity of a promoter region containing a *tts* box. In the absence of the transcriptional factor TtsI, the expression of Y4yS protein does not occur (Mercante *et al.* 2015). Swimming motility of the *y4yS* mutant strain in the presence and absence of T3SS induction conditions is shown in Fig. 5A. The *y4yS* mutant strain showed, as the *ttsI* mutant strain, greater motility than the wild-type strain in the presence of T3SS-inducing conditions. This result indicates that Y4yS protein has a role in the inhibition of *M. loti* swimming motility in T3SS-inducing conditions. A *y4yS* mutant strain complemented with plasmid expressing *y4yS* gene under the constitutive P*lac* promoter (Mercante *et al.* 2015) was assayed for motility. The complemented mutant showed wild-type phenotype (Fig. 5B). These results indicate that the role of Y4yS in the T3SS complex assembly, or at least its correct localization in membranes under T3SS-inducing conditions, is involved in the motility inhibition. Figure 6A shows a quantitative analysis comparing the motility of the wild strain with that of the mutant strains, *tts1* and *y4yS*. It was observed that the *y4yS* mutant strain presents a partial reversal of the motility inhibition phenotype showed by the wild-type strain under T3SS-inducing conditions, while the reversion of inhibition is total in the mutant strain *ttsI*. This result could be in concordance with the idea that the T3SS complex at the membranes is responsible for the motility inhibition. That is, in the mutant strain *ttsI* where there is no T3SS complex, no inhibition of motility occurs, and in the *y4yS* mutant strain in which there are defects in the assembly of complex, inhibition of motility occurs at lesser extent than in the wild-type strain. However, there is a possibility that the transcriptional factor TtsI is responsible for the inhibition of motility independently of T3SS complex assembly at membranes, but that defects in this assembly have a negative feedback effect on TtsI levels. We proceeded to analyze the activity of a promoter region containing a *tts* box, which depends on the levels of TtsI. This promoter region was transcriptionally fused to β-galactosidase gene and introduced into wild-type and *y4yS* mutant strain. The results show a reduction in promoter activity over the time of the experiment, but this reduction occurs in both strains (Fig. 6B). This result rules out the possibility of a negative feedback effect on levels of TtsI by defects in the formation of T3SS complex. It indicates that alteration in the complex assembly in *y4yS* mutant strain is involved in the determination of motility phenotype, independently of TtsI levels. This is consistent with the idea that the effect of TtsI on motility occurs through the assembly of T3SS complex at membranes.

DISCUSSION

The present results indicate the existence of a negative regulation of the swimming motility in semi-solid media for *Mesorhizobium loti* MAFF303099 in conditions that induce *M. loti nod* box-containing promoters. Neither NodD alone, nor naringenin alone is responsible of the motility alteration. It is necessary the presence of the two components to see the altered motility phenotype. It is possible that along the experimentation time some degradation of naringenin occurs. In spite of this, differences in motility halo between absence and presence of naringenin for *M. loti* wild-type strain expressing the naringenin-specific NodD are observed. Defects in motility, in T3SS-inducing conditions, were due to the absence of flagella. Results obtained with the *ttsI* mutant strain indicate that the alteration of motility phenotype in the presence of NodD and naringenin is due to the specific expression of the transcriptional factor TtsI.

The present work is the first demonstration of the existence of an inverse regulation between TtsI expression and swimming motility in rhizobia. A negative regulation of swimming motility in plate in the presence of T3SS induction media for two phytopathogenic bacteria, *Erwinia amylovora* (Cesbron *et al.* 2006) and *Pseudomonas syringae* (Ortíz-Martín *et al.* 2010) was previously

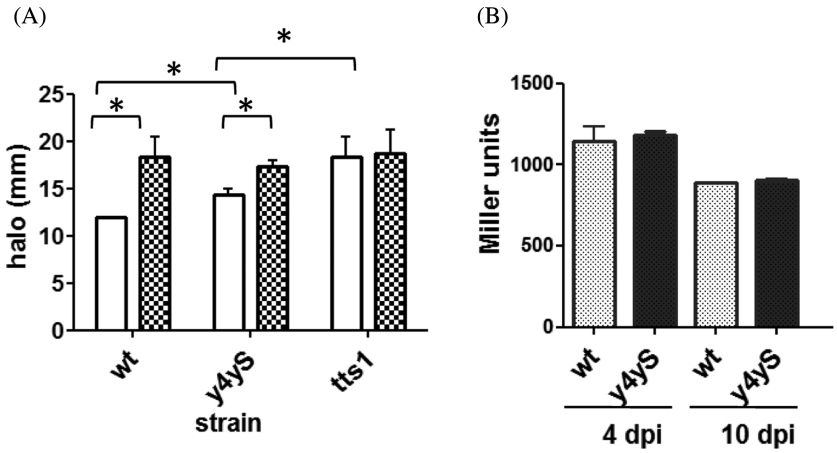

Figure 6. Analysis of *y4yS* mutation effect. **(A)** Quantitative comparison of swimming motility of wild-type (wt) and mutant strains. For better quantification, same volumes of bacterial cultures, at the same OD at 600 nm, were inoculated by stabbing in soft-agar plates with (open rectangle) or without (filled rectangle) naringenin. All assayed bacteria contain pMP2112 plasmid. Halo diameter was measured at 7 dpi. Each result is the average of data corresponding to three replicas. Asterisk indicates significant difference according to Student's *t* test. **(B)** Expression of a *tts* box containing promoter activity in wild-type (wt) and *y4yS* mutant strain. *nopX* promoter region was transcriptionally fused to *lacZ* gene in the pMP220 plasmid (pMP-*nopX*) and introduced into wild-type and *y4yS* mutant strains (both containing pMP2112 plasmid). Bacteria were inoculated by puncture in motility plates containing naringenin. At two different dpi bacteria were harvested and β-galactosidase activity was measured. Each result is the average of data corresponding to two replicas.

described. There, a negative effect of HrpL protein on swimming motility in plates was demonstrated. HrpL is a transcriptional factor that induces the expression of *hrp* genes (genes coding for T3SS components) in the corresponding bacteria (Cesbron *et al.* 2006; Ortíz-Martín *et al.* 2010). The switch off of flagellar system during plant infection may be vital for the pathogenic bacteria because flagellins are considered elicitors of antimicrobial plant defense response (Cesbron *et al.* 2006).

Results obtained from motility analysis with another T3SS mutant strain, *M. loti y4yS*, suggests that motility inhibition by TtsI expression occurs through the assembly of T3SS complex. The presence of T3SS complex at membranes could be a signal for a negative regulation cascade of flagellar genes or could physically interfere with the flagella assembly. In the case of *Pseudomonas syringae*, where HrpL expression negatively affects the motility, also it was observed that a mutant affected in the T3SS assembly (*rhcC* mutant strain) present greater motility than the wild-type strain (Ortíz-Martín *et al.* 2010). In this case, it was proposed that defects in T3SS assembly have a negative feedback effect on HrpL expression (Ortíz-Martin *et al.* 2010). Our results rule out the possibility that the greater motility observed in the *y4yS* mutant strain with respect to the wild-type strain in T3SS-inducing conditions is due to a negative feedback effect on TtsI protein levels by defects in assembly of T3SS complex at membranes.

Mesorhizobium loti MAFF303099 has a homolog for the transcriptional factor VisN, which was described as responsible for the cascade activation of the flagellar genes transcription in *Rhizobium leguminosarum* and *Sinorhizobium meliloti* (Sourjik *et al.* 2000; Tambalo *et al.* 2010). Promoter activity analysis for the upstream region of *M. loti visN* gene demonstrated its inhibition in T3SS-inducing conditions.

Flagella were described as required for the bacterial movement to the roots proximity and even for attachments to the hairy roots in some rhizobia–legume symbiotic process (Smit, Kijne and Lugtenberg 1989; Zheng *et al.* 2015). During the symbiotic process, bacteria sense root-exuded flavonoids and induce the expression of the genes coding for Nod factors production and for the transcriptional factor TtsI. Once bacteria is trapped into the curled root hair tip and became the infection of plant cells by multiplication insight the infection threads (Gage 2004), it could be speculated that motility is no longer required. In particular, the flagellum of rhizobia is not considered as a plant defense elicitor (López-Gómez *et al.* 2011), but the fact that it is no longer required inside the infection threads could support the conservation of this inverse regulation through evolution. Expression of the flagellar protein FlgH of *M. loti* MAFF303099 was reported to be suppressed in the bacteroid state inside the nodule (Uchiumi *et al.* 2004). Also, proteome analysis determined the absence of flagellar proteins in bacteroids (Tatsukami *et al.* 2013). Regulatory gene *visN* of *R. leguminosarum* was described to be significantly downregulated inside the nodules (Tambalo *et al.* 2010). The existence of an inverse regulation between TtsI expression and motility suggests that in *M. loti* MAFF303099, expression of TtsI could be the signal, in the early nodulation steps, for the downregulation of motility.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

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Conflict of interest. None declared.

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