Implication of Symbiotic Regulators on the Expression of Type VI Secretion System in *Sinorhizobium fredii* USDA257

GRADO EN BIOQUÍMICA POR LA UNIVERSIDAD DE SEVILLA

Y LA UNIVERSIDAD DE MÁLAGA

2022/2023

Clara Granado Beltrán

Trabajo de Fin de Grado

Tutor: Francisco de Asís Pérez-Montaño

Codirector: Pedro José Reyes Pérez

Departamento de Microbiología

Universidad de Sevilla

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Abstract

Rhizobia are Gram-negative bacteria that establish symbiosis with legumes and carry out the biological fixation of N_2 facilitating its assimilation by the plants, thus being considered an alternative to chemical fertilizers. Therefore, it is of great interest to understand all the mechanisms underlying this symbiosis. The Type VI Secretion System (T6SS) enables bacteria to translocate effector proteins to other bacteria or eukaryotic cells. This system is present in *Sinorhizobium fredii* USDA257, a model organism used to study plant-microbe interactions. However, its role remains practically unknown. In this work we seek to discern the regulation of this system in *Sinorhizobium fredii* USDA257 via studying the implication of symbiotic regulator genes such as *nodD2*, *nolR*, *syrM* and *mucR*. Furthermore, the determination of inducing and repressing media for this system is key to carry out further research towards a better understanding of the system. We found that the T6SS is activated the most in Minimum Medium, compared to other culture media. In this medium, both gene expression and protein secretion of this system were activated to a high extent. Oppositely, in Tryptone Yeast medium, the T6SS was induced the least. We aimed to obtain non-functional gene mutants to test the effect of the regulators on the expression of the T6SS in both inducing and repressing conditions, obtaining simple recombinant mutants of *syrM* and *mucR*. We expect our assays to be a starting point for more advanced research on this topic, since the understudied T6SS is likely to have the potential of improving rhizobia-legume symbiosis.

Keywords: Rhizobia-legume Symbiosis, Nodulation, Type VI Secretion System (T6SS), *Sinorhizobium fredii* USDA257, Regulators

1. Introduction

1.1. Biological Nitrogen Fixation

Nitrogen is an essential macronutrient for plants and a limiting factor for plant growth since it is a component of both nucleic acids and proteins. Regarding agriculture, nitrogen deficiency in soil causes reduced productivity and crop yield. Nitrogen deficiency is often treated with chemical nitrogen-rich fertilizers; nevertheless, an overage in soil nitrogen can lead to runoffs from the fields, contaminating ground water and resulting in eutrophication (Goyal et al., 2021). This practice is very common; as a matter of fact, in 2021, 190 million tonnes of chemical fertilizers were used in agriculture worldwide, of which 57% were nitrogen fertilizers (FAO 2021).

Despite nitrogen being abundant in the atmosphere as N_2, plants can only assimilate combined nitrogen in the forms of nitrate, nitrite, and ammonium. In this context, legumes have evolved for millions of years into symbiotic partners of rhizobia, Gram-negative bacteria that carry out the biological fixation of atmospheric N_2 to inorganic nitrogen that can be assimilated by the plants (Maitra et al., 2021; Lea & Miflin, 2010).

Rhizobia are Gram-negative bacteria, belonging mainly to the α- and β-proteobacteria classes (Rajkumari et al., 2022), which are facultative symbionts of plants from the family *Leguminosae*. In this symbiosis, rhizobia interact with the plants' roots and induce the formation of specialized structures called nodules (Maitra et al., 2021; Noel, 2009). The nodules provide a favourable environment for the rhizobia to live and reproduce. These bacteria undergo significant morphological and metabolic changes to differentiate into bacteroids, which are adapted to the low oxygen conditions inside the nodule and possess the ability to fix atmospheric nitrogen into ammonia (Maróti & Kondorosi, 2014).

1.2. Symbiotic Dialogue and Nodule Development in Rhizobium-Legume Interaction

The rhizobium-legume interaction is a complex process that requires a coordinated two-way molecular dialogue between the bacterium and the plant, which entails the expression of a variety of genes involved in symbiosis.

This process starts when the bacteria are chemotactically attracted by flavonoids, phenolic compounds derived from the 2-phenyl-1,4-benzopyrone flavone that are released in the plant's exudates (Navarro-Gómez, 2020; Liu & Murray, 2016).

Once rhizobia reach the rhizosphere, they adhere to root hair thanks to adhesive proteins such as plant lectins, bacterial rhicadhesin, and surface polysaccharides (Rodríguez-Navarro et al., 2007). In response to flavonoids, the attached bacteria put into action the NodD protein, a transcriptional activator that binds to *nod* boxes (NB). NB are promoter sequences located upstream of nodulation genes, activating their transcription. The activation of these nodulation genes leads to the synthesis and secretion of lipo-chitooligosaccharides, the so-called Nod factors (NF) (López-Baena et al., 2016; Pérez-Montaño et al., 2016) (Fig. 1).

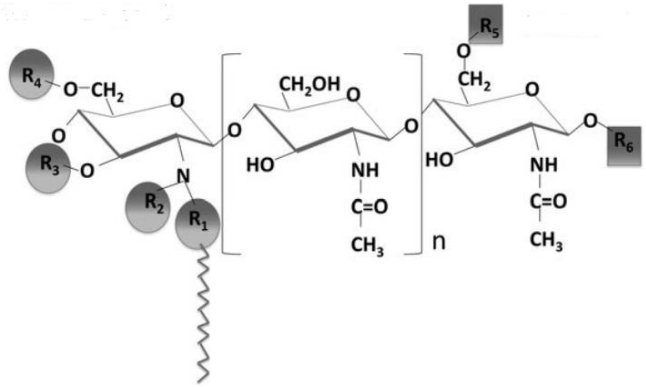

Figure 1. Nod factor general structure. Nod factors' chemical structure is defined by a linear chain of three to five N-acetyl-D-glucosamine units joined by β-1→4 bonds and attached to a fatty acid (Kassaw & Frugoli, 2013).

NF are perceived by specific plant LysM-receptors that trigger a downstream signalling cascade, leading to rhizobium-specific intracellular colonization of the root hair via the infection thread formation and eventual development of nodules on roots (Oldroyd, 2013) (Fig. 2).

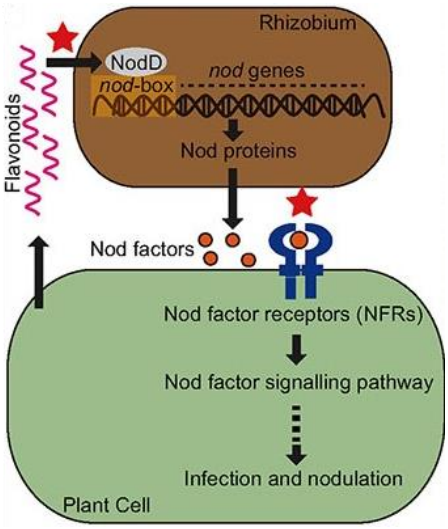

Figure 2. The symbiotic dialogue in the rhizobium-legume symbiosis. Flavonoids are secreted by the plant and activate NodD proteins in the rhizobium, which bind to NB and induce the expression of NF. NF are recognized by LysM-receptors in plant cells. This triggers a signalling pathway that ends in the infection and nodulation of the host. (Wang et al., 2018)

This infection thread is filled with growing and dividing bacteria that eventually reach the radical cortex, where plant cells divide into a meristem that will later become the nodule and will be infected by the bacteria. Once inside the nodule, bacteria differentiate into bacteroids, rhizobial forms specifically adapted to nitrogen fixation (Wang et al., 2018~~b~~; Gage, 2004) (Fig. 3).

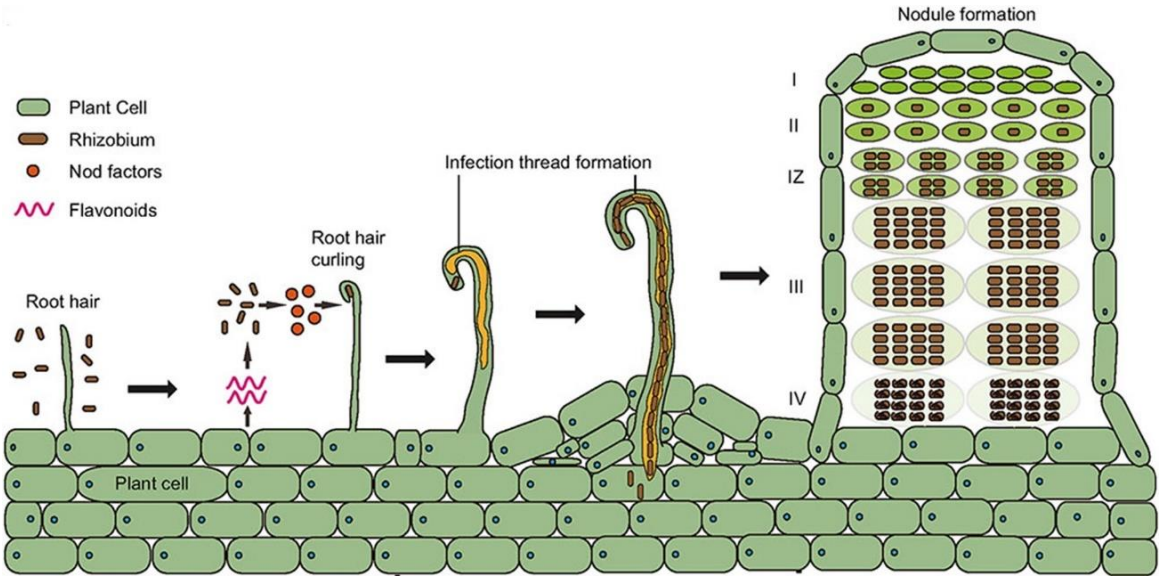

Figure 3. Nodulation process: plant infection and nodule formation. The nodule contains a meristem zone (I), an infection zone (II), an interzone (IZ), a nitrogen fixing zone (III), and a senescent zone (IV) (Wang et al., 2018).

In *Sinorhizobium fredii* USDA257, our model organism that will be described later, NF genes are not only regulated by the transcriptional activator NodD1 but also by other general regulators such as NodD2, NolR, SyrM and MucR, that can act as activators or repressors of nodulation genes, depending on the regulator. These proteins play a critical role in orchestrating the complex interactions between the plant and the bacteria, ensuring that the symbiotic relationship is established and maintained. Interestingly, our department's group has shown that these genes also play an important role in regulating the expression of T3SS in *S. fredii* HH103, a relevant system for the establishment of symbiosis (Acosta-Jurado et al., 2020, 2019, 2016; Jiménez-Guerrero et al., 2018; Pérez-Montaño et al., 2016). Therefore, it is of great interest to study the potential role of these proteins on the T6SS regulation and assembly to discover the role of this system in symbiosis in *Sinorhizobium fredii* USDA257.

1.3. *Sinorhizobium fredii* USDA257

Sinorhizobium fredii USDA257, hereafter USDA257, is a fast-growing rhizobial symbiont that exhibits a broad host range. It was first isolated from soybean root nodules from cultivars in China and it has been used as a model organism for studying plant-microbe interactions in non-commercial varieties of soybean since then (Pueppke & Broughton, 1999). USDA257 possesses two replicons, a chromosome (cUSDA257) and a single plasmid (pUSDA257), sharing a high sequence similarity with that of *S. fredii* HH103 strain (Schuldes et al., 2012). In fact, the global GC content of USDA257 is 62.0%, a similar value to that of HH103 (62.1%). Moreover, the difference in the chromosome size of USDA257 and HH103 is due to the existence of a non-symbiotic plasmid that is in free form in the case of HH103 but inserted in the genome of USDA257. In any case, in both USDA257 and HH103, genes involved in Nod factor production are located on the so-called symbiotic plasmid (pUSDA257 and pSfHH103d respectively). Despite their similarities, a noticeable difference between the two strains is the occurrence of the Type VI Secretion System (T6SS), only present in USDA257 (Vinardell et al., 2015; Schuldes et al., 2012).

S. fredii USDA257 is one of the few *Sinorhizobium* strains in which the T6SS has been found apart from the Type III Secretion System (T3SS) so far (Sugawara et al., 2013). The T3SS of USDA257 has been extensively studied and plays a prominent role

in symbiosis and in the determination of rhizobial host-range (Staehelin & Krishnan, 2015), since recognition of some T3SS effectors by legumes can exert positive (induction of nodulation) or negative (inhibition of nodulation) roles depending on the cultivar. However, the ecological and physiological function of the USDA257 T6SS is practically unknown.

1.4. Type VI Secretion System

The Type VI Secretion System (T6SS) is a molecular machine used by a wide range of Gram-negative bacterial species to translocate effectors or toxins into other cells. The T6SS participates in numerous biological processes, including bacterial antagonism, competition, subversion of host cell defence responses, and niche colonization (Gallique et al., 2017). It functions mainly as a contractile nanomachine to puncture target cells and deliver toxins into prokaryotic cells, being conducive to bacterial competition for space and resources. Nonetheless, in certain cases T6SS effectors target eukaryotic cells and is thus involved in the infective process of eukaryotic hosts (Boyer et al., 2009).

The T6SS is a multi-protein complex constituted by 13 main components. Genes encoding for these proteins are grouped in the same gene cluster and are called *tss* (type six secretion) (Cascales, 2008). Additionally inside the cluster, we can find genes that encode for accessory proteins with complementary functions named *tag* (type VI accessory genes) (Silverman et al., 2012). Regarding the structure of the system, we can distinguish three parts: membrane complex, baseplate, and tail (Fig. 4). The membrane complex is responsible for anchoring the secretion system to the membrane. Once the membrane complex is assembled, the proteins of the baseplate are recruited. This structure stabilizes the joint between the membrane complex and the tail. The tail is constituted by ring-shaped hexamers of the Hcp protein. The PAAR protein, together with the VgrG protein and the Hcp rings, are considered the spear of the system, which is propelled into the target cell. Hcp and VgrG can be detected in the extracellular medium of T6SS-active bacteria, being a useful tool to keep track of the T6SS activity *in vitro* (Bernal et al., 2018). Finally, in the cluster we can also find the kinase-phosphatase PpkA-PppA couple. Even though the PpkA-PppA couple is not a structural component of the system, it is proved to mediate the activation of the T6SS in

Pseudomonas aeruginosa through a phosphorylation cascade that ends with the assembly of structural components (Mougous et al., 2007).

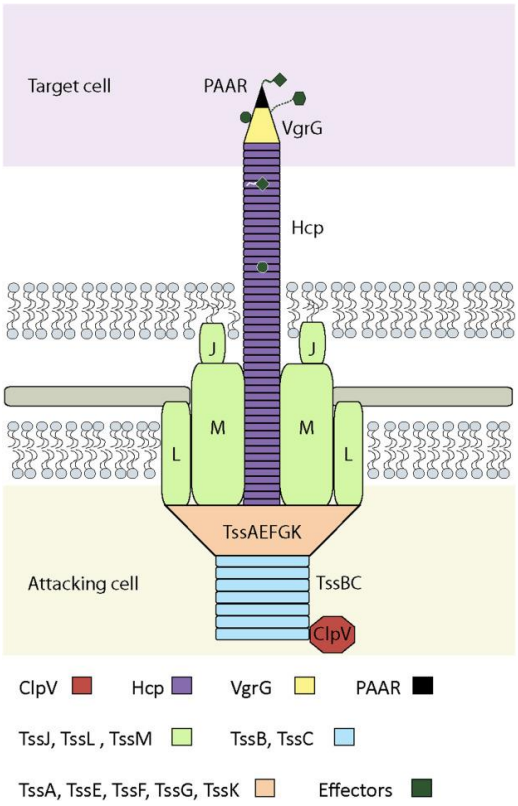

Figure 4. Schematic representation of the T6SS structure. The membrane complex proteins – TssJ, TssL and TssM – are coloured in light green, while the baseplate proteins – TssA, TssE, TssF, TssG and TssK – are represented in light orange and the sheath components – TssB and TssC – in blue (Bernal et al., 2018).

Despite the T6SS being moderately distributed among rhizobia, little is known about its ecological and physiological role. Interestingly, recent studies have demonstrated that rhizobial T6SS is required for efficient nodulation of legumes in both *Rhizobium etli* Mim1 and *Bradyrhizobium* Sp. LmicA16 (De Sousa et al., 2023; Salinero-Lanzarote et al., 2019; Tighilt et al., 2022). Since this area of research is still being investigated, we aim to contribute to a better understanding of the interplay between bacteria competition and symbiosis in rhizobia.

2. Objectives

- Mutation of nodulation regulator genes *nodD2*, *nolR*, *syrM* and *mucR* of *S. fredii* USDA257.
- Determination of inducing and repressing media for the T6SS in *S. fredii* USDA257.

3. Material and Methods

3.1. Culture media and antibiotics

Rhizobia strains were cultivated in the media detailed below. Liquid cultures were incubated in agitation at 180 rpm. Solid media was obtained by adding 20 g L^{-1} of agar to the liquid media.

Table 1. Bacteria culture media

Component	**Concentration (g L^{-1})**
TY Medium (Behringer, 1974)	
Yeast extract	3
Tryptone	5
$CaCl_2 \cdot 2\ H_2O$	0.65
YM Medium (Vincent, 1970)	
K_2HPO_4	0.5
$MgSO_4 \cdot 7\ H_2O$	0.2
NaCl	0.1
Yeast extract	0.4
Mannitol	10 or 3*
Minimum Medium, MM (Robertsen et al., 1981)	
K_2HPO_4	0.3
$MgSO_4 \cdot 7\ H_2O$	0.15
$CaCl_2 \cdot 2\ H_2O$	0.05

$FeCl_3$	0.006
NaCl	0.05
Sodium glutamate	1.1
Mannitol	10 or 3*
Vitamin Solution (1000X)	1 mL

Vitamin Solution (Behringer, 1974)**	
Biotin	0.02
Thiamin	0.01
Sodium pantothenate	0.01

LB Medium (Sambrook et al., 1989)	
Yeast extract	5
Tryptone	10
NaCl	5

*MM was prepared either with 3 g or 10 g of mannitol depending on the desired conditions.

**The Vitamin Solution was sterilized by filtration and 1 mL of vitamin solution was added to 1 L of MM previously autoclaved.

All the media ingredients were dissolved in distilled H_2O. The pH of all media was adjusted to 6.8-7.0 and the media were sterilized in the autoclave for 20 minutes under 1 atm pressure and 121 °C.

When required, the media were supplemented with the appropriate antibiotics as described in (Lamrabet et al., 1999).

Table 2. Antibiotics employed in this work

Selective Agent	Stock Solution (mg mL^{-1})	*S. fredii* (µg mL^{-1})	*E. coli* (µg mL^{-1})
Spectinomycin (Spc)	50	50	100
Kanamycin (Km)	30	60	30
Rifampicin (Rif)	25	50	25
Tetracycline (Tc)	10	1-2	10

Rifampicin was dissolved in methanol, while tetracycline was dissolved in ethanol. The rest of antibiotics were dissolved in distilled water.

3.2. Bacterial strains, plasmids, and constructs

Table 3. Bacterial strains and plasmids used in this work.

Strains and Plasmids	Characteristics	Reference
***Sinorhizobium fredii* USDA257**	Parental strain, Rif^R	(Pueppke & Broughton, 1999)
***Escherichia coli* DH5α**	Competent strain used for cloning. *SupE44*, Δ*lacU169*, *5hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*, Nx^R	(Sambrook et al., 1989)
pRK2013	Helper plasmid for mobilization of non-self-transmissible plasmids, Km^R	(Figurski & Helinski, 1979)
pMP220	Broad-host-range cloning vector containing a promoter-less *lacZ* gene, Tc^R	(Spaink et al., 1987)
pMP220 P_{ppkA}::*lacZ*	pMP220 plasmid containing the promoter of the *ppkA* gene upstream of the *lacZ* gene	Kindly provided by Pérez-Montaño lab.
pMP92	Broad-host-range cloning vector, IncP, Tc^R	(Spaink et al., 1987)
pMP92::*hcp*-HA	Vector containing the hcp gene with a HA tag	Kindly provided by Pérez-Montaño lab
pMUS1232	pK18*mobsacB*Δ*syrM*, Km^R, Suc^S	(Acosta-Jurado et al., 2020)
pMUS1176	pK18*mobsacB*Δ*mucR*, Km^R, Suc^S	(Acosta-Jurado et al., 2016)
pMUS735	pK18*mob* derivative containing HH103 *nolR*::Ω (Km^R, Spc^R)	(Vinardell et al., 2004)
pMUS689	pK18*mob* derivative containing HH103 *nodD2*::Ω (Km^R, Spc^R)	(Acosta-Jurado et al., 2019)

3.3. Triparental Conjugation

Plasmids were transferred from *E. coli* to *Sinorhizobium* strains by triparental conjugation as described by Simon (1984) using pRK2013 as the helper plasmid. The following cultures were mixed: 800 µL of the receptor parental strain of USDA257, 200 µL of each donor *E. coli* strain containing the corresponding plasmid to be transferred and 100 µL of *E. coli* containing the pRK2013 helper plasmid. The mixture was centrifuged for 3 minutes at 5000 rpm, the supernatant was discarded and after adding 800 µL of distilled water the mixture was centrifuged again in the same conditions. The supernatant was discarded, and the pellet was resuspended in the remaining supernatant (around 75 µL). The culture was then plated in the form of a patch. The plate was incubated for 16 hours at 28°C. The cellular mass was harvested and resuspended in 1 mL of TY medium. Next, pertinent dilutions were carried out and plated in TY medium supplemented with the necessary antibiotics to select the transconjugant strains.

3.4. Obtention of *syrM* and *mucR* mutants by deletion

Recombinant DNA techniques were performed according to the general protocols of Sambrook et al. (1989). The method chosen to obtain the mutants by deletion is a two-step recombination process using the pK18*mobsacB* suicide vector. Once the conjugation was carried out according to the protocol described above, the candidate simple recombinant *S. fredii* strain (Km^R) was selected and confirmed by colony PCR. A simple recombinant colony was inoculated in liquid TY medium without antibiotics and incubated in agitation at 28°C for 4 days. After this, 100 µL aliquots derived from serial dilutions of the liquid culture were plated in TY supplemented with sucrose 12.5% (w/v). Colonies resistant to sucrose were picked in TY with and without kanamycin in order to select strains with a second recombination event, in which only one of the mutated or wild-type versions of the gene remains in the genome.

3.5. Obtention of *nodD2* and *nolR* mutants by insertion of the Ω interposon

Recombinant DNA techniques were performed as described in Frey & Krisch (1985). The method selected to mutate the *nodD2* and *nolR* genes was the insertion of

an interposable element, the Ω interposon, into the gene sequences. This method aims for a direct double recombination event in a single step, where the gene and the Ω interposon, but not the suicide plasmid pK18*mob*, are integrated in the genome, replacing the wild-type version of the gene. Transconjugants that were Spc^R (resistance conferred by the interposon) but Km^S (resistance conferred by the vector) are candidates that may have substituted the wild type gene by the mutated gene.

3.6. PCR Amplification

To confirm the mutation of the mutant candidates for the genes *syrM*, *mucR*, *nodD2* and *nolR* we carried out colony PCR following the protocol described by Saiki (1990) with modifications (Table 4).

Table 4. PCR Amplification Protocol

Step	PCR Temperature (°C)	Time (min)	Cycle
Initial denaturation	95	2	1x
Denaturation	95	0.5	35x
Annealing	Depending on the set of primers	0.5	
Extension	72	Depending on the PCR product length	
Final extension	72	5	1x
Storage	4	10	-

Regarding the PCR mixture, one single colony from the mutant candidates was introduced in 50 µL of sterile water and heated at 95°C for 10 min to generate the template DNA solution. For each sample preparation, the PCR mix consisted in the components listed below (Table 5).

Table 5. Sample preparation for Colony PCR

Reagent	Quantity (µL)
Taq DNA Polymerase Master Mix RED (Ampliqon, Denmark)	5
Forward Primer	0.4
Reverse Primer	0.4
Template DNA	1
Nuclease free H_2O	3.2

Primers used in this work are listed in Table 6.

Table 6. Primers used for the amplification of *syrM*, *mucR*, *nodD2* and *nolR* genes.

Primers	Nucleotide Sequence	Reference
syrM-F	5' – TAG AAG CTT AAG TCG CTG ACA CCG GC – 3'	(Acosta-Jurado et al., 2020)
syrM-R	5' – CAC GGA TCC TGC GAT GAC ATG GCA GC – 3'	(Acosta-Jurado et al., 2020)
mucR-F	5' – ATT AAG CTT GAT GGG CAC GCT – 3'	(Acosta-Jurado et al., 2020)
mucR-R	5' – AAA GGA TCC GAC CCG GTC CTG – 3'	(Acosta-Jurado et al., 2020)
nodD2-F	5' – CGA CCC TAC GGT CGA AAA G – 3'	This study
nodD2-R	5' – GCT GAA TGA GAT GGT CTC CC – 3'	This study
nolR-F	5' – CGG TTA ATC GGC ACT TAG C – 3'	This study
nolR-R	5' – GAT GTG AGC GTC AAG TCT G – 3'	This study

3.7. DNA electrophoresis

Electrophoresis was carried out using EtBr-stained Agarose Gels at 1% (w/v) in 0.5x TAE buffer. As the ladder, we used the New England Biolabs (U.S.A) 1 kb ladder. Samples were run at 180 V for 20 minutes using the Mupid-One Electrophoresis System (Advance, Japan). The resulting DNA bands were visualized in

an UV light transilluminator at 260 nm. Images were captured with Gel Doc 2000 (Bio-Rad, U.S.A) and were analysed with Quantity One 1-D Analysis Software (Bio-Rad, U.S.A).

Employed solutions:

- TAE Buffer: 30 mM Tris-HCl, 0.002% (v/v) of 0.5 M EDTA pH 8, 0.00114% (v/v) glacial acetic acid. pH was adjusted to 8.

3.8. β-Galactosidase Activity Assay

The β-galactosidase activity assay is used to quantify gene expression. The promoter of the gene of interest is fused to the *lacZ* gene or a cassette containing the *lacZ* gene is inserted in the gene of interest itself. To quantify this activity, we followed the method described by Miller (1972).

The liquid cultures were incubated for 3 days until stationary phase. The O.D. was measured at 600 nm. 200 µL of each culture was mixed with 800 µL of Z-buffer, 20 µL of chloroform and 10 µL of 0.1% (w/v) SDS. The whole mixture was vortexed for 15 seconds. Then, 200 µL of ONPG in a 4 mg mL^{-1} Z-buffer solution was added to each sample. The induction reaction was stopped when the samples turned yellow by adding 500 µL of 1 M Na_2CO_3 and the time was noted down. Spectrophotometric measures were taken at 420 nm. The β-galactosidase activity was calculated according to the following formula:

$$\beta - Galactosidase\ Activity\ (M.U.) = \frac{Final\ volume\ (mL) \cdot O.D._{420nm} \cdot 1000}{Culture\ volume\ (mL) \cdot t\ (\text{min}) \cdot O.D._{660nm}}$$

Where M.U. are Miller Units, and *t* is the time allowed for the reaction.

Statistically, we represented the data in a barplot and carried out an ANOVA assay using a p-value of 0.05 to determine if the change in gene expression between the different samples was statistically significant.

Employed solution:

- Z-buffer: 60 mM $Na_2HPO_4 \cdot 2\ H_2O$, 60 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl and 1 mM $MgSO_4$. pH was adjusted to 7.

3.9. Intracellular and Extracellular Protein Extraction

We followed the method described by Hachani (2011). Pre-inoculum of all needed bacteria that had been grown at 28 °C in agitation for 3 days was inoculated in new culture media to establish the inoculum. The initial OD_{600} of the cultures was adjusted to 0.1 in 40 mL. Cultures were grown for 2 days at 28 °C in agitation until stationary phase. Extracellular samples were subjected to centrifugation twice at 5000 rpm. for 20 min at 4 °C, discharging the pellet every time and preserving the supernatant. 7.2 mL of the supernatant was distributed into four 2 mL Eppendorf tubes. 200 µL of 10% (v/v) trichloroacetic acid was added to each Eppendorf tube and inverted 3 times in the laboratory fume hood. The samples were incubated in a 4 °C chamber overnight. The samples were then centrifuged for 30 min at 16000 *g* and 4 °C and the supernatant was discarded. 1 mL of cold 90% acetone was added to each Eppendorf, followed by another centrifugation in the same conditions. The pellet was left drying at room temperature for 3 hours and resuspended in 4X Laemmli buffer. For intracellular samples, cultures were centrifuged at 21000 rpm. for 1 min. The supernatant was discarded, and the pellet was resuspended in 100 µL of 4X Laemmli Buffer.

Employed solution:

- Laemmli Buffer: 62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 20% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.05% (w/v) bromophenol blue.

3.10. Unidirectional Protein Electrophoresis

The electrophoresis was carried out in denaturing conditions, separating proteins based on their molecular weight. Samples, both intracellular and extracellular, were heated at 100 °C for 10 min. 15 µL of the samples, together with Laemmli Buffer, were loaded into a Mini-PROTEAN® TGX™ Precast Polyacrylamide Gel with a concentration gradient from 4% to 20% (w/v) (Bio-Rad, U.S.A). The protein ladder used was the Broad-Range SDS-PAGE Standards (Bio-Rad, U.S.A). The gel was run at 150 mV for 1 hour in a Mini-PROTEAN® electrophoresis cell.

Employed solution:

- Laemmli Buffer: 62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 20% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.05% (w/v) bromophenol blue.

3.11. Protein Immunodetection

Once the electrophoresis was completed, proteins were transferred onto a nitrocellulose membrane (Bio-Rad, U.S.A) at 25 V, 1 A for 15 min using the Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, U.S.A). The membrane was immersed in blocking solution for 1 hour. After that, the membrane was incubated overnight at 4 °C in agitation (180 rpm) with the primary antibodies. The secondary antibodies were added to the membrane and left for 1 hour in agitation at room temperature. The antibodies were provided by Cell Signalling Technology (U.S.A).

Table 7. Description of the antibodies used in the protein immunodetection.

Primary antibodies	Dilution	Secondary antibodies	Dilution
Rabbit mAb for the HA epitope, targeting the Hcp protein attached to the HA tag.	1:5000	Anti-rabbit IgG HRP-linked	1:3000
Mouse mAb for the RNA-polymerase	1:1000	Anti-mouse IgG HRP-linked	1:3000

*Antibodies were dissolved in blocking solution.

Employed solutions:

- Transference buffer: 25 mM Tris pH 8.3; 192 mM glycine, 20% (v/v) absolute ethanol.
- 10X TBS: 0.2 M Tris, 1.3 M NaCl. pH was adjusted to 7.6.
- Blocking solution: 1X TBS, 5% (w/v) milk powder.

4. Results

4.1. Symbiotic regulator genes share a high sequence identity in HH103 and USDA257

Since our objective is to mutate the regulator genes of the nodulation process in USDA257 using available constructs designed for HH103 and the mutagenesis techniques employed are based on genomic recombination events, the homology between the two strains must be determined. All genes, *syrM* (locus tag SFHH103_04280 and USDA257_p03090), *mucR* (locus tag SFHH103_0069 and USDA257_c07290), *nodD2* (locus tag SFHH103_04329 and USDA257_p02600) and *nolR* (locus tag SFHH103_02239 and USDA257_c47330) from HH103 were compared to their homologs in USDA257 using BLASTN and ClustalW alignment.

syrM sequences shared 99% identity between them, differing in only 1 out of 1020 nucleotides, due to a C↔T transition in the position 42. There were no gaps and the coverage was 100%.

mucR sequences shared 94% identity between them, differing in 27 out of 432 nucleotides. There were no gaps and the cover query was 100% (Fig. 5).

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mucR_USDA257  ATGAGTGAGAATACGCTCGGTACGAGCAACGAACTCCTGG  40
  mucR_HH103  ATGAGTGAGAATACGCTCGGTACGAGCAACGAACTCCTGG  40

mucR_USDA257  TTGAGCTGACGGCGGAAATCGTTGCCGCCTATGTGAGCAA  80
  mucR_HH103  TTGAGCTGACGGCGGAAATAGTTGCCGCCTATGTGAGCAA  80

mucR_USDA257  CCATGTGGTTCCTGTTGCCGAGTTGCCGACGCTGATAGCC  120
  mucR_HH103  CCACGTGGTTCCGGTTGCCGAGCTGCCGACGCTGATTGCC  120

mucR_USDA257  GATGTCCATTCGGCGCTCAATAACACGACGGCTCCCACGC  160
  mucR_HH103  GATGTCCATTCGGCGCTCAACAACACGACGGCTCCTGCGC  160

mucR_USDA257  CCGTGGTCGTGCCGGTCGAAAAGCCGAAGCCGGCCGTCTC  200
  mucR_HH103  CGGTGATCGTGCCGGTCGAAAAGCCGAAGCCGGCGGTCTC  200

mucR_USDA257  GGTTCGCAAGTCGGTGCAGGACGATCAGATCACCTGCCTC  240
  mucR_HH103  CGTCCGCAAGTCCGTGCAGGATGACCAGATCACCTGCCTC  240

mucR_USDA257  GAATGCGGGGGCACGTTCAAATCGCTGAAGCGCCACTTGA  280
  mucR_HH103  GAGTGCGGTGGCACGTTCAAATCGCTGAAGCGCCATTTGA  280
```

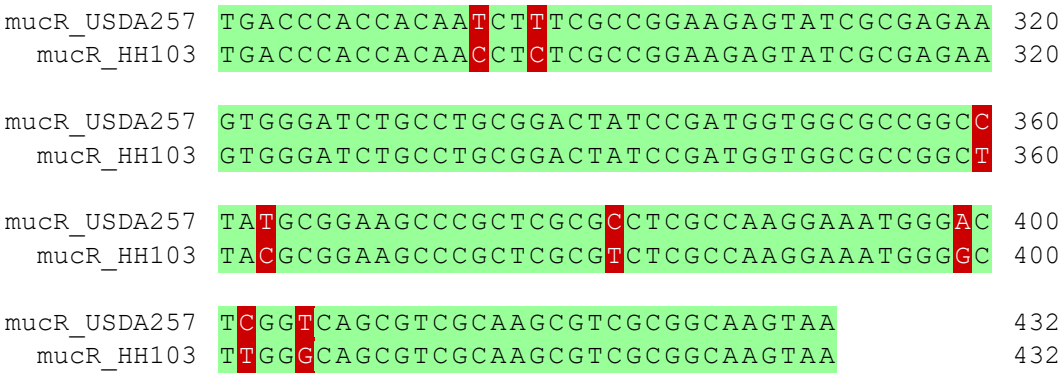
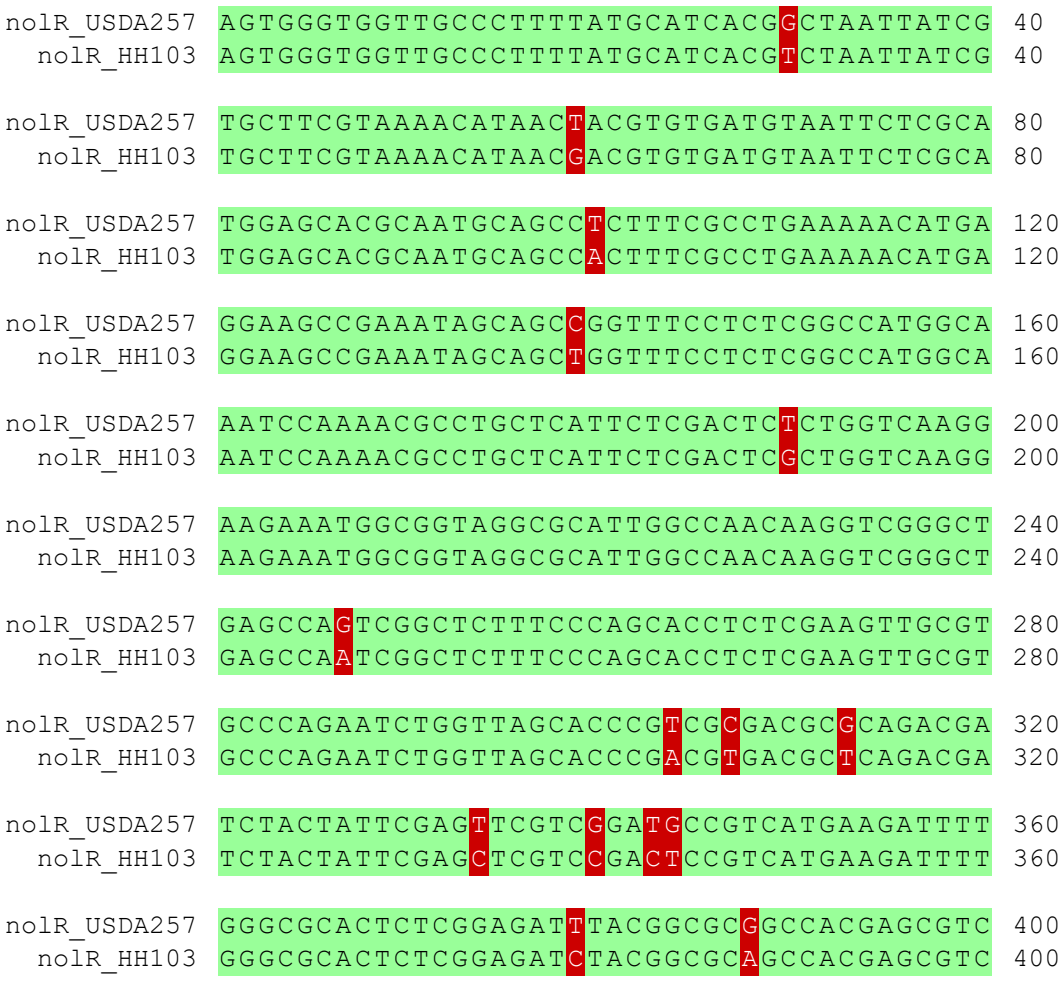


Figure 5. Alignment between the *mucR* gene of *S. fredii* USDA257 and *S. fredii* HH103. Identical nucleotides are coloured in green, while different nucleotides are coloured in red.

nodD2 sequences were identical, therefore being 100% the identity and coverage between them.

nolR sequences shared 96,45% identity between them, without gaps and being the coverage 100%. The sequences differ in 17 out of 479 nucleotides.



```
nolR_USDA257  GTGATAGAAAAGCCCCTCGTGCGCAAGTCGGCCTGACTCC  440
  nolR_HH103  GTGATAGAAAAGCCTTTCGTGCGCAAGTCGGCCTGACTCC  440

nolR_USDA257  CAAGACGCCGACAAGAGCTGAATGCCCAAAGCCCCGACG   479
  nolR_HH103  CAAGACGCCGACAAGAGCTGAATGCCCAAAGCCCCGACG   479
```

Figure 6. Alignment between the *mucR* gene of *S. fredii* USDA257 and *S. fredii* HH103. Identical nucleotides are coloured in green, while different nucleotides are coloured in red.

Considering these similarities and the comparative analysis carried out between genes of interest in both strains, constructs created for HH103 in previous works by our group (Acosta-Jurado et al., 2020, 2019, 2016; Vinardell et al., 2004), were used to mutate the homologous genes in USDA257 in order to study the impact of these regulatory symbiotic genes on the regulation and assembly of T6SS in this rhizobium.

4.2. Simple recombinant mutants of *syrM* and *mucR* genes were obtained

Regarding *syrM*, five simple recombinant candidates (Km^R), harbouring both wild-type (1020 bp) and deleted (400 bp) versions of *syrM* resulting from the triparental conjugation, were confirmed by colony PCR using the external primers SyrM-F and SyrM-R previously described in Table 6 (Fig. 7).

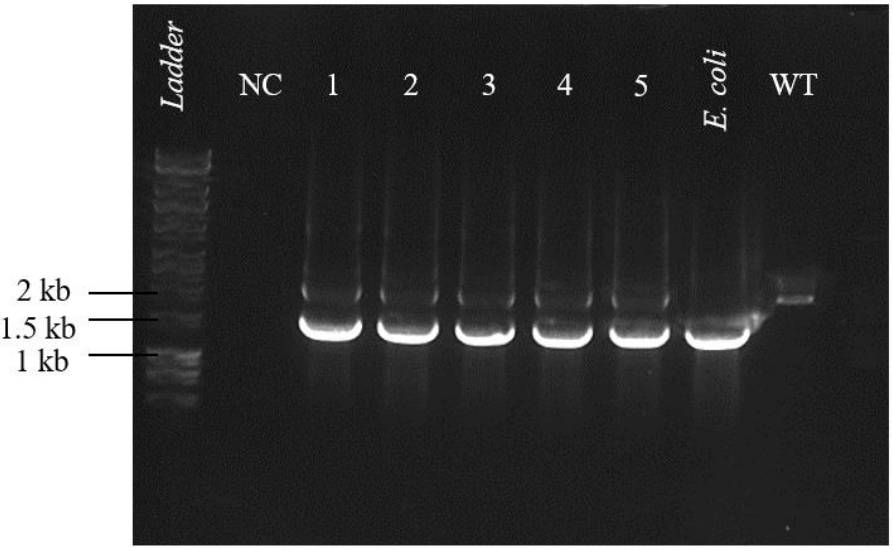


Figure 7. Colony PCR of the potential *syrM* simple recombinant mutants. NC: Negative control. 1, 2, 3, 4, 5 wells correspond to each of the potential simple recombinant mutants. *E. coli: E. coli* strain containing the pMUS1232 construct harbouring only the deleted copy of *syrM*. WT: WT USDA257 containing the original *syrM* gene.

All five candidate colonies presented two DNA bands, one around 2 kb corresponding to the original copy of the *syrM* gene (1020 bp) plus approximately 500 bp upstream and 500 bp downstream, and the other around 1.4 kb corresponding to the deleted version (600-pb less) of the gene.

As positive controls, we used the *E. coli* strain containing the pMUS1232 construct harbouring only the deleted copy of *syrM* that presented only the 1.4 kb band, and the WT strain of USDA257 that contains only the original copy of the *syrM* gene and therefore presented only the 2 kb band. The negative control confirmed the absence of non-specific amplification.

Respecting *mucR*, five simple recombinant candidates (Km^R), harbouring both wild-type (429 pb) and deleted (600-pb less, including deletion of upstream and downstream sequences) versions of *mucR* were obtained by triparental conjugation. Confirmation of the candidates was performed using colony PCR with the specific primers MucR-F and MucR-R described in Table 6 (Fig. 8).

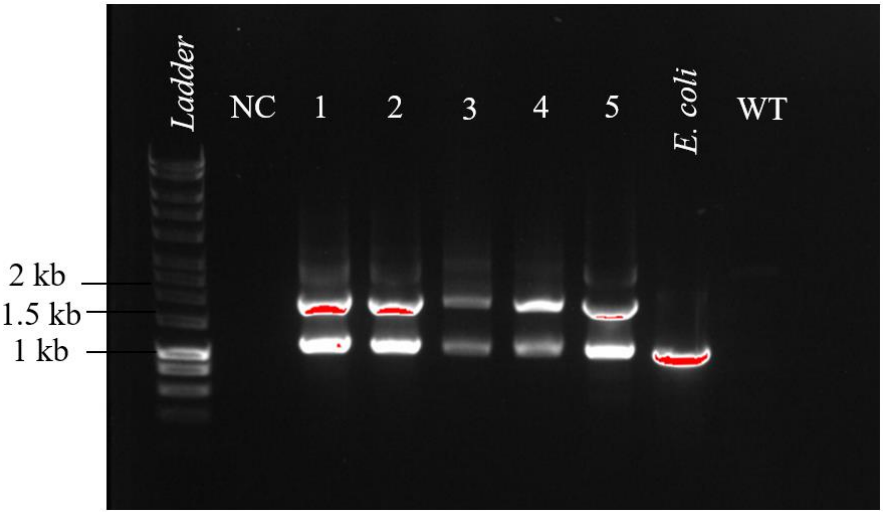


Figure 8. Colony PCR of the potential *mucR* simple recombinant mutants. NC: Negative control. 1, 2, 3, 4, 5 wells correspond to each of the potential simple recombinant mutants. *E. coli: E. coli* strain containing the pMUS1176 construct harbouring only the deleted copy of *mucR*. WT: WT USDA257 containing the original *mucR* gene.

All five candidate colonies exhibited two DNA bands, one band at approximately 1.6 kb, representing the original copy of the *mucR* gene plus approximately 500 bp

upstream and 500 bp downstream, and another band at around 1 kb, identifying as the deleted version of the gene (600-pb shorter).

For positive controls, two samples were utilized: an *E. coli* strain carrying the pMUS1176 construct with the deleted copy of *mucR*, which exhibited a single DNA band at approximately 1 kb; and the WT strain of USDA257, which contained only the original copy of the *mucR* gene, resulting in a single DNA band at around 1.6 kb. The negative control confirmed the absence of non-specific amplification.

In the case of the obtention of *nodD2* and *nolR* mutants, the triparental conjugation yielded potential candidates that resulted Spc^R Km^S. However, the candidates were dismissed after the confirmation PCR since their genotype was that of the WT (data not shown), meaning that a spontaneous spectinomycin resistance had occurred instead of the desired double recombination event.

4.3. The T6SS expression is induced especially in MM, and to a lesser extent in YM

PpkA is essentially the key regulatory protein of the T6SS cluster and its cognate gene is one of most transcribed when the system is activated (Bernal et al., 2018). Therefore, the *ppkA* promoter (P_{ppkA}) was cloned upstream *lacZ* and selected to perform expression assays by β-galactosidase activity under different liquid culture conditions to test the T6SS activity. For such purpose, pMP220 and pMP220 P_{ppkA}::*lacZ* plasmids were introduced into the WT strain of USDA257.

The expression was significantly greater in both MM (MM3 and MM10) compared to TY Medium. The TY medium is the medium where the system was induced the least, leaving both YM (YM3 and YM10) in an intermediate but not statistically significant position. However, there were no significant differences between the MM or YM supplemented with 3 g L^{-1} mannitol or 10 g L^{-1} mannitol, meaning that the supply of carbon source does not have an influence on the expression of T6SS genes (Fig. 9).

The expression of the samples harbouring the empty pMP220 vector was considered as the basal gene expression in non-activating conditions and was subtracted from the

total expression values obtained in the samples harbouring the pMP220 P_{ppkA}::*lacZ* construct (data not shown).

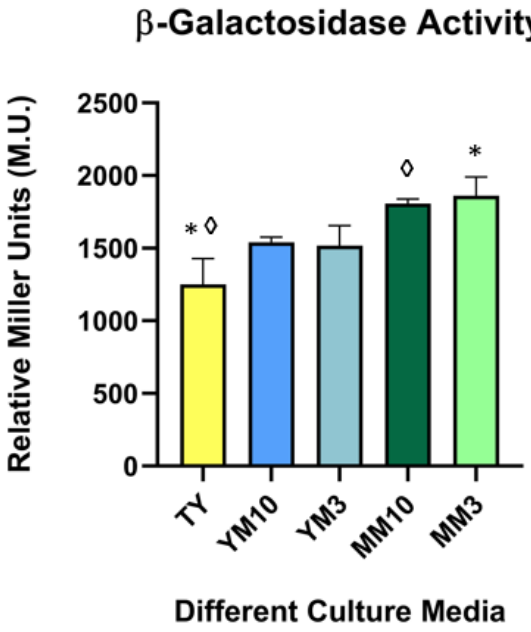


Figure 9. Barplot representation of the expression of the *lacZ* gene under the USDA257 *ppkA* gene promoter. An ANOVA assay was carried out using a p-value of 0.05. Values tagged by asterisks (*) or diamonds (◊) are significantly different.

4.4. Hcp is only secreted in YM and MM media, especially in MM

Salinero-Lanzarote et al. (2019) used the Hcp protein as a secretion marker to test the correct performance and functioning of the T6SS, which ended in satisfactory results for the research. Therefore, we decided to use the same marker in this study to assess the level of T6SS activity in different media.

For this assay, we first obtained a WT strain of USDA257 harbouring the pMP92::*hcp*-HA, which allows us to detect the presence of Hcp indirectly by means of antibodies for the HA epitope, since we did not have specific antibodies against Hcp.

Interestingly, the amount of both intracellular and extracellular Hcp protein was greater in the MM medium compared to the YM and specially TY, where there was no presence of secreted (extracellular) Hcp protein (Fig. 10). These data are in agreement with expression results obtained by β-galactosidase assays.

The β-RNA polymerase protein was used as a cell integrity control for the extracellular samples and a positive control for the intracellular samples, besides indicating the relative amount of protein present in each media. As expected, there was no β-RNA polymerase protein in any of the extracellular samples, meaning that there is no contamination due to cell lysis in any analysed sample. On the other side, this protein was also detected in all whole cell samples, with a lower concentration in samples obtained from YM medium (Fig. 10).

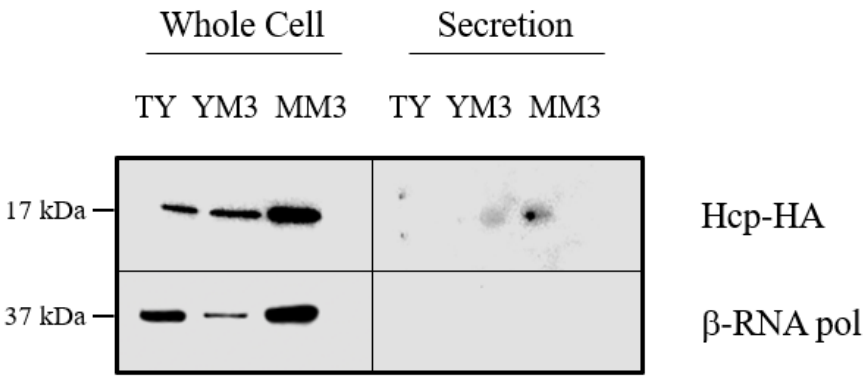


Figure 10. Western Blot assay targeting β-RNA pol protein as a control and T6SS Hcp protein as a secretion marker. Both whole cell samples and extracellular samples were used to assay protein secretion.

5. Discussion and Conclusions

The focus of this work is to study T6SS regulation in USDA257 and determine whether it is affected by general regulators of nodulation genes. For this purpose, we followed two approaches: identifying the culture conditions in which the system is activated or repressed and obtaining mutants of the regulators to test changes in regulation in the selected media.

This study shows that the expression of the T6SS genes and the secretion of T6SS proteins is activated to a greater extent in YM and MM media, especially in MM. Both β-galactosidase activity and protein secretion assays corroborate that the MM is the medium where the T6SS is activated the most. Therefore, MM is a T6SS-inducing medium. The gene expression in TY is the lowest and no secreted Hcp was detected in

this medium, hence it can be resolved that the TY medium is a repressing medium. We can also assume that there would be a greater amount of Hcp protein in the YM medium if it had the same amount of general protein according to the β-RNA polymerase control, since the amount of this protein remains constant in cells independently of culture conditions. Thus, the β-galactosidase activity and protein secretion assays set a precedent about the media where the T6SS is the most and the least induced for future research on the T6SS in rhizobia.

To continue with the research, the simple recombinant mutants of *syrM* and *mucR* should be subjected to the process of counterselection by sucrose addition again, while *nodD2* and *nolR* double recombinant mutants should be obtained by repetitive conjugation. Once mutants defected in these regulator genes are obtained, the next step would be to introduce the pMP220 and pMP220 P_{ppkA}::*lacZ* plasmids into the mutants and carry out β-galactosidase activity assays to quantify the expression of the T6SS in the different mutant strains and therefore discern the effect of each regulator protein on the system. The expression in each mutant should be tested in the media that we have determined as T6SS-inducing or repressing in this study. If a mutant strain shows greater activity in repressing media, its function is that of a repressor; if the activity is lower in inducing media, its role is that of an activator. Along with expression assays, protein secretion assays such as the presented in this work should be carried out to assess T6SS functionality in the mutant strains and confirm the data obtained in expression assays. In any case, it would be convenient to create Hcp-specific antibodies to implement in future secretion assays. Regarding future perspectives, these mutant strains are expected to be inoculated into compatible legume hosts, such as *Glycine max* cv Pekin, to undertake nodulation assays and test their effect on the plant's phenotype.

Studies on the T6SS will help discern the role of this system in rhizobia, its implication in symbiosis or competition, and its interrelationship with the T3SS, thus revealing potential applications that may cause a positive impact on the agriculture field.

In conclusion, the highlights of this study are:

- Mutant strains of *syrM, mucR, nodD2* and *nolR* regulator genes *in S. fredii* USDA257 are still to be obtained.
- The T6SS expression and protein secretion are greatly induced in Minimum Medium and to a lesser extent in YM medium in *S. fredii* USDA257.
- The T6SS expression and protein secretion are not induced in TY medium in *S. fredii* USDA257.

6. References

Acosta-Jurado, Sebastián, Alias-Villegas, C., Navarro-Gómez, P., Almozara, A., Rodríguez-Carvajal, M. A., Medina, C., & Vinardell, J. M. (2020). Sinorhizobium fredii HH103 syrM inactivation affects the expression of a large number of genes, impairs nodulation with soybean and extends the host-range to Lotus japonicus. *Environmental Microbiology*, *22*(3), 1104–1124. https://doi.org/10.1111/1462-2920.14897

Acosta-Jurado, Sebastián, Rodríguez-Navarro, D. N., Kawaharada, Y., Rodríguez-Carvajal, M. A., Gil-Serrano, A., Soria-Díaz, M. E., … Vinardell, J. M. (2019). Sinorhizobium fredii HH103 nolR and nodD2 mutants gain capacity for infection thread invasion of Lotus japonicus Gifu and Lotus burttii. *Environmental Microbiology*, *21*(5), 1718–1739. https://doi.org/10.1111/1462-2920.14584

Acosta-Jurado, Sebastian, Alias-Villegas, C., Navarro-Gomez, P., Zehner, S., Del Socorro Murdoch, P., Rodríguez-Carvajal, M. A., … Vinardell, J. M. (2016). The Sinorhizobium fredii HH103 MucR1 global regulator is connected with the nod regulon and is required for efficient symbiosis with Lotus burttii and Glycine max cv. Williams. *Molecular Plant-Microbe Interactions*, *29*(9), 700–712. https://doi.org/10.1094/MPMI-06-16-0116-R

Bernal, P., Llamas, M. A., & Filloux, A. (2018). Type VI secretion systems in plant-associated bacteria. *Environmental Microbiology*, *20*(1), 1–15. https://doi.org/10.1111/1462-2920.13956

Boyer, F., Fichant, G., Berthod, J., Vandenbrouck, Y., & Attree, I. (2009). Dissecting the bacterial type VI secretion system by a genome wide in silico analysis: What can be learned from available microbial genomic resources? *BMC Genomics*, *10*(1), 104. https://doi.org/10.1186/1471-2164-10-104

Cascales, E. (2008). The type VI secretion toolkit. *EMBO Reports*, *9*(8), 735–741. https://doi.org/10.1038/embor.2008.131

Figurski, D. H., & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans (plasmid replication/replication origin/trans-complementation/broad host range/gene cloning). *Proc. Nati. Acad. Sc*, *76*(4), 1648–1652.

Frey, J., & Krisch, H. M. (1985). Ω Mutagenesis in Gram-Negative Bacteria: a Selectable Interposon Which Is Strongly Polar in a Wide Range of Bacterial Species. *Gene*, *36*(1–2), 143–150. https://doi.org/10.1016/0378-1119(85)90078-2

Gage, D. J. (2004). Infection and Invasion of Roots by Symbiotic, Nitrogen-Fixing Rhizobia during Nodulation of Temperate Legumes. *Microbiology and Molecular Biology Reviews*, *68*(2), 280–300. https://doi.org/10.1128/mmbr.68.2.280-300.2004

Gallique, M., Bouteiller, M., & Merieau, A. (2017). The type VI secretion system: A dynamic system for bacterial communication? *Frontiers in Microbiology*, *8*, 1–10. https://doi.org/10.3389/fmicb.2017.01454

Goyal, R. K., Mattoo, A. K., & Schmidt, M. A. (2021). Rhizobial–Host Interactions and Symbiotic Nitrogen Fixation in Legume Crops Toward Agriculture Sustainability. *Frontiers in Microbiology*, *12*, 1290. https://doi.org/10.3389/fmicb.2021.669404

Hachani, A., Lossi, N. S., Hamilton, A., Jones, C., Bleves, S., Albesa-Jové, D., & Filloux, A. (2011). Type VI secretion system in Pseudomonas aeruginosa: Secretion and multimerization of VgrG proteins. *Journal of Biological Chemistry*, *286*(14), 12317–12327. https://doi.org/10.1074/jbc.M110.193045

Jiménez-Guerrero, I., Acosta-Jurado, S., del Cerro, P., Navarro-Gómez, P., López-Baena, F. J., Ollero, F. J., … Pérez-Montaño, F. (2018). Transcriptomic studies of the effect of nod gene-inducing molecules in rhizobia: Different weapons, one purpose. *Genes*, *9*(1), 1–27. https://doi.org/10.3390/genes9010001

Kassaw, T. & Frugoli, J. (2013). Journey to Nodule Formation: From Molecular Dialogue to Nitrogen Fixation. In R. Aroca (Ed.), *Symbiotic Endophytes* (Vol. 37). Springer

Lamrabet, Y., Bellogín, R. A., Cubo, T., Espuny, R., Gil, A., Krishnan, H. B., Megias, M., Ollero, F. J., Pueppke, S. G., Ruiz-Sainz, J. E., Spaink, H. P., Tejero-Mateo, P., Thomas-Oates, J., & Vinardell, J. M. (1999). Mutation in GDP-fucose synthesis genes of Sinorhizobium fredii alters Nod factors and significantly decreases competitiveness to nodulate soybeans. *Molecular plant-microbe interactions : MPMI*, *12*(3), 207–217. https://doi.org/10.1094/MPMI.1999.12.3.207.

Lea, P. J., & Miflin, B. J. (2010). Nitrogen Assimilation and its Relevance to Crop Improvement. In Foyer, H. & Zhang, H. (Eds.) *Annual Plant Reviews* (Vol. 42). Blackwell Publishing Ltd https://doi.org/https://doi.org/10.1002/9781444328608.ch1

Liu, C.-W., & Murray, J. (2016). The Role of Flavonoids in Nodulation Host-Range Specificity: An Update. *Plants*, *5*(3), 33. https://doi.org/10.3390/plants5030033

López-Baena, F. J., Ruiz-Sainz, J. E., Rodríguez-Carvajal, M. A., Vinardell, J. M., Gresshoff, P. M., & Ferguson, B. (2016). Bacterial Molecular Signals in the Sinorhizobium fredii-Soybean Symbiosis. *International Journal of Molecular Sciences Article*, *17*(5), 755. https://doi.org/10.3390/ijms17050755

Maitra, S., Brestic, M., Bhadra, P., Shankar, T., Praharaj, S., Palai, J. B., … Hossain, A. (2021). Bioinoculants-Natural Biological Resources for Sustainable Plant Production. *Miroorganisms*, *10*(1), 51. https://doi.org/10.3390/microorganisms10010051

Maróti, G., & Kondorosi, É. (2014). Nitrogen-fixing Rhizobium-legume symbiosis: Are polyploidy and host peptide-governed symbiont differentiation general principles of endosymbiosis? *Frontiers in Microbiology*, *5*, 326. https://doi.org/10.3389/fmicb.2014.00326

Mougous, J. D., Gifford, C. A., Ramsdell, T. L., & Mekalanos, J. J. (2007). Threonine phosphorylation post-translationally regulates protein secretion in Pseudomonas aeruginosa. *Nature Cell Biology*, *9*(7), 797–803. https://doi.org/10.1038/ncb1605

Navarro-Gómez, P. (2020). *Identificación y estudio de genes simbióticos de Sinorhizobium fredii HH103 previamente no caracterizados* [Doctoral thesis, University of Seville]. Seville.

Noel, K. D. (2009). Rhizobia. In M. Schaechter (Ed.), *Encyclopedia of Microbiology*, 676–685. Elsevier. San Diego, CA: Academic Press. https://doi.org/10.1016/b978-012373944-5.00043-2

Oldroyd G. E. (2013). Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nature reviews. Microbiology*, *11*(4), 252–263. https://doi.org/10.1038/nrmicro2990

Pérez-Montaño, F., Jiménez-Guerrero, I., Acosta-Jurado, S., Navarro-Gómez, P., Ollero, F. J., Ruiz-Sainz, J. E., … Vinardell, J. M. (2016). A transcriptomic analysis of the effect of genistein on Sinorhizobium fredii HH103 reveals novel rhizobial genes putatively involved in symbiosis. *Scientific Reports*, *6*, 1–12. https://doi.org/10.1038/srep31592

Pueppke, S. G., & Broughton, W. J. (1999). Rhizobium sp. strain NGR234 and R. fredii USDA257 share exceptionally broad, nested host ranges. *Molecular plant-microbe interactions : MPMI*, *12*(4), 293–318. https://doi.org/10.1094/MPMI.1999.12.4.293

Rajkumari, J., Katiyar, P., Dheeman, S., Pandey, P., & Maheshwari, D. K. (2022). The changing paradigm of rhizobial taxonomy and its systematic growth upto postgenomic technologies. *World Journal of Microbiology and Biotechnology*, *38*(11), 1–23. https://doi.org/10.1007/s11274-022-03370-w

Rodríguez-Navarro, D. N., Dardanelli, M. S., & Ruíz-Saínz, J. E. (2007). Attachment of bacteria to the roots of higher plants. *FEMS Microbiology Letters*, *272*(2), 127–136. https://doi.org/10.1111/j.1574-6968.2007.00761.x

Salinero-Lanzarote, A., Pacheco-Moreno, A., Domingo-Serrano, L., Durán, D., Durán, D., Ormeño, E., … Jos´, J. (2019). The Type VI secretion system of Rhizobium etli Mim1 has a positive effect in symbiosis. *FEMS Microbiology Ecology*, *95*, 54. https://doi.org/10.1093/femsec/fiz054

Sambrook, Joseph. & Russell, David W. (1989). Molecular cloning: a laboratory manual. Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory

Schuldes, J., Orbegoso, M. R., Schmeisser, C., Krishnan, H. B., Daniel, R., & Streit, W. R. (2012). Complete genome sequence of the broad-host-range strain Sinorhizobium fredii USDA257. *Journal of Bacteriology*, *194*(16), 4483–4483. https://doi.org/10.1128/JB.00966-12

Silverman, J. M., Brunet, Y. R., Cascales, E., & Mougous, J. D. (2012). Structure and regulation of the type VI secretion system. *Annual review of microbiology*, *66*, 453–472. https://doi.org/10.1146/annurev-micro-121809-151619

Spaink, H. P., Okker, R. J. H., Wijffelman, C. A., Pees, E., & Lugtenberg, B. J. J. (1987). Promoters in the nodulation region of the Rhizobium leguminosarum Sym plasmid pRL1JI. *Plant Molecular Biology*, *9*(1), 27–39. https://doi.org/10.1007/BF00017984

Staehelin, C., & Krishnan, H. B. (2015). Review Article: Nodulation outer proteins: Double-edged swords of symbiotic Rhizobia. *Biochemical Journal*, *470*(3), 263–274. https://doi.org/10.1042/BJ20150518

Sugawara, M., Epstein, B., Badgley, B. D., Unno, T., Xu, L., Reese, J., Gyaneshwar, P., Denny, R., Mudge, J., Bharti, A. K., Farmer, A. D., May, G. D., Woodward, J. E., Médigue, C., Vallenet, D., Lajus, A., Rouy, Z., Martinez-Vaz, B., Tiffin, P., Young, N. D., … Sadowsky, M. J. (2013). Comparative genomics of the core and accessory genomes of 48 Sinorhizobium strains comprising five genospecies. *Genome biology*, *14*(2), R17. https://doi.org/10.1186/gb-2013-14-2-r17

Tighilt, L., Boulila, F., De Sousa, B. F. S., Giraud, E., Ruiz-Argüeso, T., Palacios, J. M., Imperial, J., & Rey, L. (2022). The Bradyrhizobium Sp. LmicA16 Type VI Secretion System Is Required for Efficient Nodulation of Lupinus Spp. *Microbial ecology*, *84*(3), 844–855. https://doi.org/10.1007/s00248-021-01892-8

Vinardell, J. M., Acosta-Jurado, S., Zehner, S., Göttfert, M., Becker, A., Baena, I., … Weidner, S. (2015). The Sinorhizobium fredii HH103 genome: A comparative analysis with S. fredii strains differing in their symbiotic behavior with soybean. *Molecular Plant-Microbe Interactions*, *28*(7), 811–824. https://doi.org/10.1094/MPMI-12-14-0397-FI

Vinardell, J. M., Ollero, F. J., Hidalgo, Á., López-Baena, F. J., Medina, C., Ivanov-Vangelov, K., … Ruiz-Sainz, J. E. (2004). NolR regulates diverse symbiotic signals of Sinorhizobium fredii HH103. *Molecular Plant-Microbe Interactions*, *17*(6), 676–685. https://doi.org/10.1094/MPMI.2004.17.6.676

Wang, Q., Liu, J., & Zhu, H. (2018). Genetic and Molecular Mechanisms Underlying Symbiotic Specificity in Legume-Rhizobium Interactions. *Frontiers in plant science*, *9*, 313. https://doi.org/10.3389/fpls.2018.00313

World Food and Agriculture – Statistical Yearbook 2021. (2021). In *World Food and Agriculture – Statistical Yearbook 2021*. https://doi.org/10.4060/cb4477en