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Regulation and symbiotic significance of nodulation outer proteins secretion in *Sinorhizobium fredii* HH103

Francisco Javier López-Baena, José María Vinardell, Francisco Pérez-Montaño, Juan Carlos Crespo-Rivas, Ramón A. Bellogín, M[a] del Rosario Espuny and Francisco Javier Ollero

Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, Sevilla, Spain

Correspondence
Francisco Javier Ollero
fjom@us.es

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In this work we show that the *Sinorhizobium fredii* HH103 *ttsI* gene is essential for the expression of the *tts* genes and secretion of nodulation outer proteins (Nops). Moreover, we demonstrate for the first time, to our knowledge, that the *nod* box preceding *ttsI* is necessary for Nops secretion. TtsI is responsible for the transcriptional activation of *nopX*, *nopA*, *rhcJ* and *rhcQ*. We confirm that the *S. fredii* HH103 *ttsI* gene is activated by NodD1 and repressed by NolR. In contrast, NodD2 is not involved in the regulation of *ttsI* expression. Despite the dependence of expression of both *ttsI* and *nodA* on NodD1 and flavonoids, clear differences in the capacity of some flavonoids to activate these genes were found. The expression of the *ttsI* and *nodA* genes was also sensitive to differences in the pH of the media. Secretion of Nops in the *ttsI* mutant could not be complemented with a DNA fragment containing the *ttsI* gene and its *nod* box, but it was restored when a plasmid harbouring the *ttsI*, *rhcC2* and y4xK genes was transferred to the mutant strain. The symbiotic effect of Nops secretion was host-dependent but independent of the type of nodule formed by the host legume. Nops are beneficial in the symbiosis with *Glycine max* and *Glycyrrhiza uralensis*, and detrimental in the case of the tropical legume *Erythrina variegata*.

INTRODUCTION

Rhizobia are soil alphaproteobacteria able to establish symbiotic associations with many legumes. This symbiosis leads to the formation of specialized structures called nodules on the roots, and in several cases also in the stems of the host plant. Within these nodules, rhizobia differentiate into nitrogen-fixing bacteroids that are able to reduce atmospheric nitrogen to ammonia. This ammonia can be assimilated by the plant and used for growth and development. In exchange, the host plant provides the bacteria with a carbon source and an appropriate environment that stimulates their growth.

The nodule organogenesis process requires the exchange of symbiotic signals between both members of the symbiosis. Flavonoids exuded by legume roots are recognized by the rhizobial protein NodD, which in turn binds to specific and conserved sequences, called *nod* boxes, and activates the transcription of the *nod* genes. These genes encode enzymes responsible for the biosynthesis and secretion of the lipochitin oligosaccharides (LCOs), also called Nod factors. These molecules are recognized by the plant and play an important role in triggering the initiation of nodule organogenesis.

Bacterial surface structures such as exopolysaccharides, lipopolysaccharides and capsular polysaccharides, or type III-secreted proteins, are also important for nodulation and host-range determination (Broughton *et al.*, 2000; Perret *et al.*, 2000).

Some Gram-negative bacterial strains possess a specialized apparatus for protein secretion called the type III secretion system (T3SS). The type III secretion apparatus is formed by about 20 proteins, many of them homologous to proteins involved in the biosynthesis of the flagellum. Symbiotic and pathogenic bacteria use the T3SS to deliver proteins into the eukaryotic host cell (Pallen *et al.*, 2003).

Plant-pathogenic bacteria such as *Pseudomonas* and *Xanthomonas* secrete harpins and avirulence proteins through the T3SS. Harpins are probably translocated to the extracellular space, inducing disease in susceptible

Abbreviations: α, Statistical significance of the differences observed using the Mann–Whitney non-parametric test; LCO, lipochitin oligosaccharide; Nops, nodulation outer proteins; T3SS, type III secretion system.

The GenBank/EMBL/DDBJ accession number for the *ttsI* sequence of *Sinorhizobium fredii* HH103 is AY184383.

plants. Avirulence proteins are translocated into the host cytoplasm and are involved in the hypersensitive response (HR) in resistant plants (Galan & Collmer, 1999). Some of the genes involved in the biosynthesis of the machinery of the type III secretion apparatus are well conserved among plant-pathogenic bacteria, and have been named *hrp* for hypersensitive response and pathogenicity. Genes homologous to *hrp* genes have been found in some rhizobia, such as *Rhizobium* sp. NGR234 (Viprey *et al.*, 1998), *Sinorhizobium fredii* HH103 (de Lyra *et al.*, 2006), *S. fredii* USDA257 (Krishnan *et al.*, 2003), *Mesorhizobium loti* MAFF303999 (Kaneko *et al.*, 2000) and *Bradyrhizobium japonicum* USDA110 (Göttfert *et al.*, 2001). Genes responsible for the biosynthesis of the rhizobial T3SS are organized in the *tts* region, which also contains genes that encode secreted proteins collectively known as nodulation outer proteins (Nops). To date, eight secreted proteins have been identified: NopA, NopB, NopC, NopD, NopL, NopM, NopP and NopX (Ausmees *et al.*, 2004; Bartsev *et al.*, 2003; Deakin *et al.*, 2005; Krishnan, 2002; Lorio *et al.*, 2004; Rodrigues *et al.*, 2007; Saad *et al.*, 2005; Skorpil *et al.*, 2005). Recently, eight proteins have been found to be type III-secreted in *B. japonicum*; one of them, GunA2, has been identified as an endoglucanase (Süss *et al.*, 2006).

Despite type III-dependent secretion requiring the presence of flavonoids and the NodD protein, only one gene of the *tts* region, the *ttsI* gene, is preceded by a *nod* box. In *Rhizobium* sp. NGR234, the expression of *ttsI* is induced by flavonoids and depends on the transcriptional activator NodD1 (Marie *et al.*, 2004). In the case of *B. japonicum*, the presence of NodW is also needed (Krause *et al.*, 2002). Amino acid sequence analysis shows that TtsI shares characteristics of two-component response regulators (Marie *et al.*, 2004), and it has been proposed as an intermediary in the regulatory cascade between NodD1 and T3SS-related genes (Viprey *et al.*, 1998). TtsI seems to regulate the expression of some genes by binding to specific promoter sequences called *tts* boxes (Krause *et al.*, 2002).

Inactivation of *ttsI* in *Rhizobium* sp. NGR234 abolishes Nops secretion and affects the symbiosis in a host-dependent manner (Marie *et al.*, 2004). A similar phenotype has been reported in mutant strains of *S. fredii* USDA257 and HH103 that contain a non-functional secretion machinery (Bellato *et al.*, 1997; de Lyra *et al.*, 2006; Meinhardt *et al.*, 1993).

In this work, we report a complete analysis of the transcriptional regulation of *ttsI* and other *S. fredii* HH103 genes that belong to the *tts* region. We also show how the inactivation of the *ttsI* gene and its *nod* box blocks Nops secretion and alters, positively or negatively, the capacity of *S. fredii* HH103 to nodulate some of its host legumes.

METHODS

Microbial and molecular techniques. Bacterial strains and plasmids used in this work are listed in Table 1. *Sinorhizobium* strains were grown at 28 °C on tryptone yeast (TY) medium (Beringer, 1974) or yeast extract mannitol (YM) medium (Vincent, 1970). *Escherichia coli* strains were cultured on Luria–Bertani (LB) medium (Sambrook *et al.* 1989) at 37 °C. When required, the media were supplemented with the appropriate antibiotics as described by Lamrabet *et al.* (1999). Flavonoids were dissolved in ethanol and used at 1 μg ml^{-1}, which gave final concentrations between 3.0 μM (quercetin) and 6.2 μM (umbelliferone). Plasmids were transferred from *E. coli* to *Sinorhizobium* strains by conjugation, as described by Simon (1984), using plasmid pRK2013 as helper.

Assays for β-galactosidase activity in liquid bacterial cultures on YM media were carried out as described by Miller (1972). Bacterial cultures at OD_{660} 0.8–1.0 were diluted 100-fold before the addition of flavonoids to ensure that bacterial cultures had an OD_{660} in the range 0.15–0.30 when β-galactosidase activity was measured (16 h after induction). When necessary, the pH of the YM medium was buffered to pH 6 with MES (20 mM), or to pH 7 or 8 with HEPES (20 mM).

Recombinant DNA techniques were performed according to the general protocols of Sambrook *et al.* (1989). For hybridization, DNA was blotted to Hybond-N nylon membranes (Amersham), and the DigDNA method of Roche was employed according to the manufacturer's instructions. PCR amplifications were performed as described previously (Vinardell *et al.*, 2004a). Primer pairs used for amplification of the *S. fredii* HH103 *ttsI*, *nodD2* and *nopA* genes were, respectively: y4xiF (5′-TAATCAGCCTGGCTGACA) and y4xiR (5′-AACAGAACGAGCGCGTAGA); D2d (5′-CTAACCAAGCCGGAGGA) and D2r (5′-CCGAAGCCGTGTACCA); fy1secF (5′-CCAGGGAGTCCAGATCGTGCA) and fy1secR (5′-GAGGCGTGGTTTACCGATCGA). The NCBI BLAST program was used for homology searches. Plasmids pMUS741 and pMUS746 were obtained, respectively, by cloning a 1.4 kb PCR fragment containing the *ttsI* gene and its *nod* box and a 1.4 kb PCR fragment containing the *nodD2* gene into the broad-host-range vector pMP92.

The strategy used to generate the different mutant strains described in Table 1 is shown in Fig. 1. Plasmid pK18mob, which is a suicide plasmid in rhizobia, was used for the homogenotization of the mutated versions of the *ttsI nod* box and the *ttsI*, *nodD2* and *nopA* genes in *S. fredii* HH103 Rif^R.

RT-PCR analysis. *S. fredii* strains HH103 Rif^R, SVQ533 and SVQ318 were incubated with shaking at 28 °C in YM medium supplemented with genistein (3.7 μM) when necessary. When the cultures reached an OD_{660} of 0.5, cells were harvested and RNA was extracted using the RNAeasy mini kit (Qiagen) following the manufacturer's instructions. Retrotranscription of the RNA was carried out using the Quantitect kit (Qiagen). Primers used for amplification were: rhcQintF (5′-CGGATGCCGATCTCGATGACA) and rhcQintR (5′-CCAACCTTCCACGGAGTCTGA); nopAintF (5′-TGTCACGAGTGCAGTTGGA) and nopAintR (5′-TGTCTGGAGCTCGGTCGTAA); HH16S-F (5′-GGATCGGAGACAGGTGCTGCA) and HH16S-R (5′-CGTGTGTAGCCCAGCCCGTA).

Purification and analysis of secreted proteins. Extracellular proteins from *S. fredii* strains were recovered from 50 ml of YM bacterial cultures grown on an orbital shaker (180 r.p.m.) for 40 h (~10^9 bacteria ml^{-1}). Cultures were centrifuged for 20 min at 10 000 ***g*** at 4 °C. The supernatants were mixed with three volumes of cold acetone and maintained at −20 °C for 24 h. The mixtures were centrifuged for 45 min at 22 000 ***g*** at 4 °C. Dried pellets were resuspended in 300 μl sample buffer [62.5 mM Tris/HCl, pH 6.8, 2 % (w/v) SDS, 10 % (w/v) glycerol, 5 % (w/v) β-mercaptoethanol and 0.001 % (w/v) Bromophenol Blue]. Extracellular proteins were separated by SDS-PAGE using the discontinuous buffer system of Laemmli (1970). Electrophoresis was performed on 15 % (w/v) SDS-polyacrylamide gels and proteins were visualized by silver staining.

Table 1. Bacterial strains and plasmids

Resistance phenotypes: Str^R, Rif^R, Gm^R, Km^R, Nx^R, Ap^R and Spc^R, streptomycin, rifampicin, gentamicin, kanamycin, nalidixic acid, ampicillin and spectinomycin, respectively.

Strain or plasmid	Relevant properties	Source or reference
***S. fredii* strains**		
SVQ269	HH103 Rif^R	Madinabeitia *et al.* (2002)
SVQ116	SVQ269 *nodA*::Tn*5-lacZ*	Buendía-Clavería *et al.* (2003)
SVQ118	SVQ269 *nopX*::Tn*5-lacZ*	Bellato *et al.* (1997)
SVQ288	SVQ269 *rhcJ*::Tn*5-lacZ*	de Lyra *et al.* (2006)
SVQ318	SVQ269 *nodD1*::Ω	Lamrabet *et al.* (1999)
SVQ513	SVQ269 *nolR*::Ω	This work
SVQ515	SVQ269 *nodD2*::Ω	This work
SVQ519	SVQ269 *nod* box *ttsI*::Ω	This work
SVQ533	SVQ269 *ttsI*::Ω	This work
SVQ534	SVQ269 *ttsI*::*lacZ*-Gm^R	This work
SVQ542	SVQ269 *ttsI*::Ω *nopA*::*lacZ*-Gm^R	This work
SVQ543	SVQ269 *nopA*::*lacZ*-Gm^R	This work
SVQ544	SVQ269 *nodD1*::Ω *ttsI*::*lacZ*-Gm^R	This work
SVQ545	SVQ269 *nodD2*::Ω *ttsI*::*lacZ*-Gm^R	This work
SVQ553	SVQ269 *nolR*::Ω *ttsI*::*lacZ*-Gm^R	This work
***E. coli* strain**		
DH5α	*supE44*, Δ*lacU169*, *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*, Nx^R	Stratagene
Plasmids		
pAB2001	Ap^R vector containing the *lacZ*Δp-Gm^R cassette	Becker *et al.* (1995)
pHP45Ω	Ap^R vector containing the Ω interposon (Spc^R, Str^R)	Prentki & Krisch (1984)
pK18mob	Cloning vector, Km^R	Schäfer *et al.* (1994)
pMP92	Broad-host-range cloning vector; IncP; Tc^R	Spaink *et al.* (1987)
pRK2013	Helper plasmid, Km^R	Figurski & Helinski (1979)
pMUS296	pMP92 carrying the HH103 *nodD1* gene	Vinardell *et al.* (2004b)
pMUS659	pK18mob derivative containing *ttsI*::*lacZ*-Gm^R	This work
pMUS661	pK18mob derivative containing *ttsI*::Ω	This work
pMUS675	pMP92 carrying the HH103 *nolR* gene	Vinardell *et al.* (2004a)
pMUS689	pK18mob derivative containing *nodD2*::Ω	This work
pMUS741	pMP92 carrying a HH103 1.4 kb DNA fragment containing *ttsI* and its *nod* box	This work
pMUS746	pMP92 carrying a HH103 1.4 kb DNA fragment containing *nodD2*	This work
pMUS760	pK18mob derivative containing *nod* box *ttsI*::Ω	This work
pMUS822	pK18mob derivative containing *nopA*::*lacZ*-Gm^R	This work
pMUS984	pMP92 carrying a HH103 3.8 kb DNA fragment containing *ttsI*, *rhcC2* and y4xK	This work

For immunostaining, extracellular proteins were separated on 15 % (w/v) SDS-polyacrylamide gels and electroblotted to Immun-Blot PVDF membranes (Bio-Rad) using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). Membranes were blocked with Tris-buffered saline (Sambrook *et al.*, 1989) containing 2 % (w/v) BSA and then incubated with antibodies raised against NopA (Bartsev *et al.*, 2003; Deakin *et al.*, 2005) diluted 1 : 3000 in the same solution. Anti-rabbit immunoglobulin antibodies (alkaline phosphatase conjugate) were used as secondary antibodies. Reaction results were visualized using nitro-blue tetrazolium chloride–5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt (NBT–BCIP) [45 µl NBT solution (75 mg ml^{-1} in 70 %, v/v, *N,N*-dimethylformamide) and 35 µl BCIP solution (50 mg ml^{-1} in *N,N*-dimethylformamide) were added to 10 ml developing buffer (80 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$)].

Plant assays. Nodulation assays on *Glycine max* (L.) Merrill cvs Williams, Peking, Heinong 33, Kochi and Tribune, and on *Erythrina variegata* (L.) were performed as described by de Lyra *et al.* (2006). Each Leonard jar contained two plants (one plant in the case of *E. variegata*). For *Glycyrrhiza uralensis*, nodulation assays were carried out in mini-Leonard jars (~200 ml for the upper part containing vermiculite and ~170 ml for the reservoir containing the plant nutritive solution) according to Vinardell *et al.* (2004a). Each plant was inoculated with $\sim 5 \times 10^8$ bacteria. Plants were grown for 42 days (90 days in the case of *E. variegata*) with a 16 h photoperiod at 25 °C in the light and 18 °C in the dark. Plant tops were dried at 70 °C for 48 h and weighed.

RESULTS

Isolation of the *S. fredii* HH103 *ttsI* gene

In order to isolate the *S. fredii* HH103 *ttsI* gene, primers y4xiF and y4xiR were designed from the coding sequence of the *ttsI* gene of *Rhizobium* sp. NGR234 (GenBank accession no. AE000106). With these primers and using *S. fredii* HH103 genomic DNA as template, a 1405 bp PCR

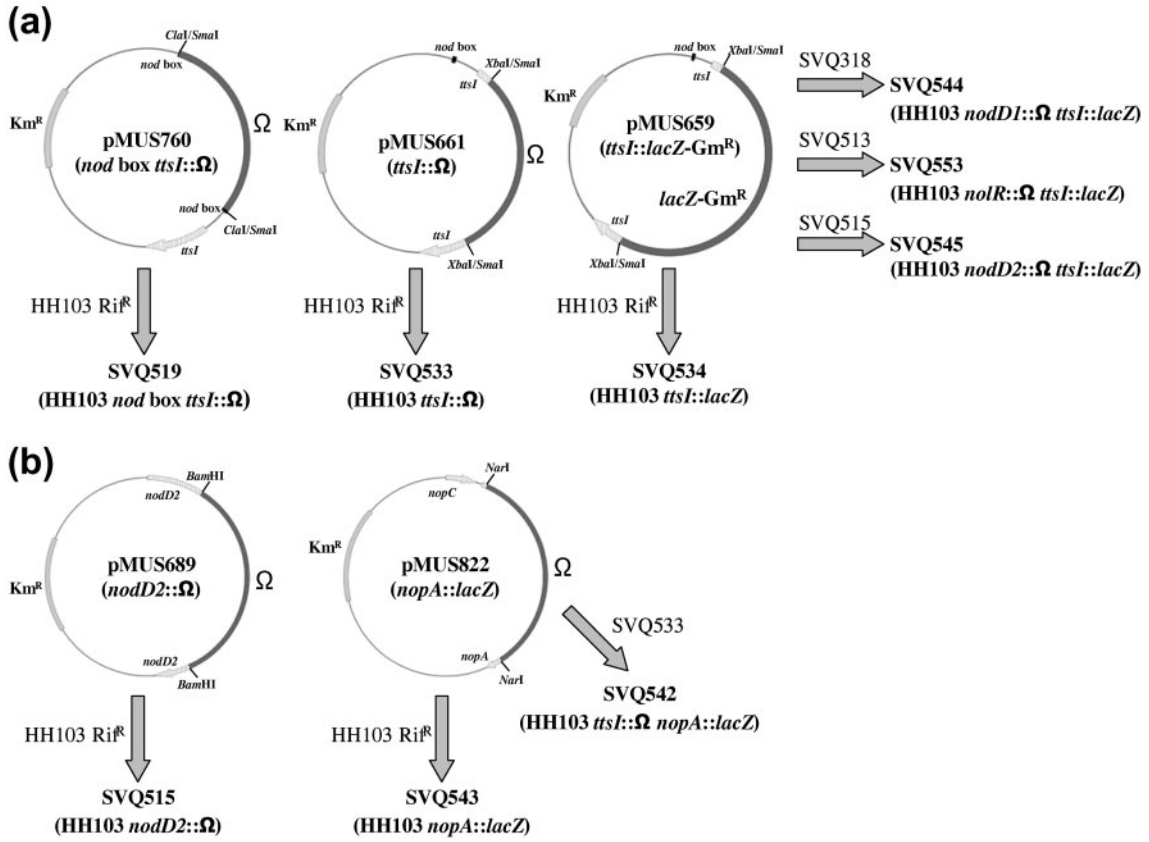

Fig. 1. (a) Derivatives of plasmid pK18mob carrying the Ω interposon to the *S. fredii* HH103 Rif^R *ttsI nod* box (pMUS760), and the *ttsI* coding sequence interrupted by the Ω interposon (pMUS661) or by the *lacZ*-Gm^R cassette (pMUS659). Plasmids were individually transferred to HH103 Rif^R. Transconjugants were selected in which the wild-type sequences had been substituted with their mutated versions by double recombination. In addition, the *ttsI*::*lacZ*-Gm^R mutation was homogenotized in strains SVQ318 (HH103 Rif^R *nodD1*::Ω), SVQ513 (HH103 Rif^R *nolR*::Ω) and SVQ515 (HH103 Rif^R *nodD2*::Ω) in order to obtain double mutants. (b) Derivatives of plasmid pK18mob carrying the Ω interposon to the *nodD2* (pMUS689) and *nopA* (pMUS822) genes. Both *in vitro*-mutated genes were homogenotized in the wild-type strain HH103 Rif^R. The *nopA*::*lacZ*-Gm^R mutation was also homogenotized in strain SVQ533 (HH103 Rif^R *ttsI*::Ω) to generate a *ttsI*, *nopA* double mutant. Restriction endonucleases used for *in vitro* mutagenesis are indicated. Homogenotizations were confirmed by hybridization in all cases.

fragment was amplified. This fragment was sequenced (AY184383) and cloned into the broad-host-range vector pMP92, generating plasmid pMUS741.

The 1405 bp sequenced fragment contains one complete ORF, the *ttsI* gene, which extends between positions 589 and 1269 and shows 100, 99, 79 and 69 % identity to the *ttsI* genes of *S. fredii* USDA257 (AF229441), *Rhizobium* sp. NGR234 (U00090), *M. loti* MAFF303099 (BA000012) and *B. japonicum* USDA110 (AF322012), respectively. The deduced protein sequence of the *S. fredii* HH103 *ttsI* gene (226 aa, AAO25539) was 100, 99, 80 and 67 % identical to those of strains *S. fredii* USDA257 (226 aa, AAL98682), *Rhizobium* sp. NGR234 (226 aa, AAB91932), *M. loti* MAFF303099 (223 aa, BAB52643) and *B. japonicum* USDA110 (229 aa, BAC47108).

The *nod* box located upstream of the *Rhizobium* sp. NGR234 *ttsI* gene was also present in *S. fredii* HH103 (positions 251–295), with only one mismatch (position 254: T instead of C). Interestingly, a well-conserved NolR-binding site (TTTAGGATTGGGTAAT), extending between positions 83 and 98 (Vinardell *et al.*, 2004a), was also found upstream of the *nod* box.

As described in Methods, three *ttsI* mutants were generated. Mutants SVQ533 and SVQ534 harbour the Ω interposon and the *lacZ*-Gm^R cassette, respectively, in the *ttsI* coding sequence. Strain SVQ519 harbours the Ω interposon in the *nod* box upstream of *ttsI*. These mutants showed no alteration in LCO, LPS and plasmid profiles when compared with the parental strain HH103 Rif^R (data not shown).

Regulation of *ttsI* by NodD1, NodD2 and NolR

As previously mentioned, type III secretion of proteins is a flavonoid-dependent process that requires the presence of NodD (Krause *et al.*, 2002; Marie *et al.*, 2004). The role of

other regulatory genes, such as *nodD2* and *nolR*, in Nops secretion has also been investigated (Krishnan *et al.*, 1995; Marie *et al.*, 2003; Vinardell *et al.*, 2004a).

To elucidate the effects of the *S. fredii* HH103 NodD1, NodD2 and NolR proteins on Nops secretion, we first individually analysed their effect on the transcriptional regulation of *ttsI*. For this purpose, the *ttsI*::*lacZ*-Gm^R mutation was homogenotized into *nodD1*, *nodD2* and *nolR* mutant backgrounds. Thus, three double mutants were obtained: SVQ544 (HH103 Rif^R *nodD1*::Ω *ttsI*::*lacZ*-Gm^R), SVQ553 (HH103 Rif^R *nolR*::Ω *ttsI*::*lacZ*-Gm^R), and SVQ545 (HH103 Rif^R *nodD2*::Ω *ttsI*::*lacZ*-Gm^R). In addition, plasmids pMUS296, pMUS746 and pMUS675, which harbour the *nodD1*, *nodD2*,and *nolR* genes, respectively, subcloned into plasmid pMP92, were transferred by conjugation to strain SVQ534 (HH103 Rif^R *ttsI*::*lacZ*-Gm^R).

The results are summarized in Table 2. As expected, the activity of *ttsI* in the absence of NodD1 could only reach basal levels when induced with genistein, confirming that the expression of *ttsI* depends on NodD1. The presence of multiple copies of *nodD1* significantly increased the expression of the *ttsI*::*lacZ*-Gm^R fusion when compared with the control without plasmid. In marked contrast, expression of *ttsI* in the presence of genistein and multiple copies of *nolR* showed a statistically significant decrease, whereas the inactivation of the *nolR* gene caused a twofold increase in the expression of *ttsI*. Although NodD2 has been described as being necessary for Nops secretion in other rhizobial strains, the inactivation or overexpression of the *nodD2* gene did not exert a clear effect on the transcription of *ttsI*.

Regulation of *ttsI* by flavonoids and pH

A comparative study of the effect of 20 different flavonoids on the activities of *ttsI* and *nodA*, involved in the biosynthesis of Nod factors, was carried out. The results, shown in Table 3, indicate that the best flavonoid inducers for *ttsI* were coumestrol, genistein and daidzein (all released by roots of soybeans McCall and Peking; Pueppke *et al.*, 1998), apigenin and morin. Coumestrol was the most potent inducer among these five flavonoids. Curiously, 7-hydroxyflavone, naringenin, hesperetin and isoliquiritigenin, which induce the expression of *nodA* at least sixfold in comparison with the control without flavonoids, could only induce the expression of *ttsI* less than twofold. Other good inducer flavonoids for *nodA*, such as 7,4′-dihydroxyflavone, quercetin, fisetin and chrysin, only induced the expression of *ttsI* twofold.

Environmental acidity is a host-associated signal of importance in host detection. The soil adjacent to plant roots is generally acidic, with pH values between 5 and 6.5 (Marschner, 1995). As expression of T3SS genes occurs in the early stages of the infection process (Perret *et al.*, 1999), the environmental pH could be a factor affecting the activity of these genes. To elucidate how the pH could affect the expression of *ttsI* and *nodA*, β-galactosidase assays were carried out, adjusting the pH of the YM medium to the range 6–8. Results obtained indicated that the expression of both *ttsI* and *nodA*, when induced with genistein, showed its maximum value at pH 6 and then decreased progressively to pH 8 (Table 4).

Table 2. β-Galactosidase activity (Miller units) of the *ttsI*::*lacZ*-Gm^R fusion of *S. fredii* HH103 in the presence of extra copies of *nodD1* (pMUS296), *nodD2* (pMUS746) and *nolR* (pMUS675), and activity in *nodD1*, *nodD2* and *nolR* mutant backgrounds

Data are the mean ± SD of at least two independent experiments performed in triplicate.

Strain	Without genistein	With genistein*
ttsI::*lacZ*	62 ± 6	232 ± 30
ttsI::*lacZ* (pMUS296)	71 ± 3	491 ± 53
ttsI::*lacZ* (pMUS746)	88 ± 4	272 ± 13
ttsI::*lacZ* (pMUS675)	70 ± 2	132 ± 3
nodD1::Ω *ttsI*::*lacZ*	43 ± 1	46 ± 3
nodD2::Ω *ttsI*::*lacZ*	42 ± 1	224 ± 9
nolR::Ω *ttsI*::*lacZ*	75 ± 4	473 ± 24

*Genistein concentration was 3.7 μM.

ttsI is the transcriptional regulator of T3SS genes in *S. fredii* HH103

To study the transcriptional regulation of the *S. fredii* HH103 *tts* region, plasmid pMUS741, which harbours the *S. fredii* HH103 *ttsI* gene and its *nod* box cloned into broad-host-range plasmid pMP92, was transferred to two *S. fredii* HH103 mutant strains carrying a Tn*5-lacZ* insertion into *nopX* and *rhcJ*, and also to a *nopA*::*lacZ*-Gm^R mutant derivative. Analysis of the promoter sequences of these genes showed that they are preceded by a *tts* box, the putative promoter sequence located upstream of genes controlled by *ttsI* (TTGTCAGCTT-TTCGAAAGCTGGAGCTCATA, 43 bp upstream of *nopCA*; TAGTCAGCGTGTCGTCAGCTCGCCTCGCTA, 40 bp upstream of *nopBrhcJUV*; TCGTCAGTTTCT-CGAAAGCTAAACCGCTCA, 189 bp upstream of *nopX*).

As shown in Table 5, overexpression of *ttsI* significantly increased the activity of all genes tested upon induction with genistein. Interestingly, their activity was also enhanced in the presence of extra copies of *ttsI* in the absence of an inducer, almost reaching the values obtained when induced with genistein.

To confirm the results obtained by the addition of extra copies of *ttsI*, a double mutant *ttsI*::Ω *nopA*::*lacZ*-Gm^R was constructed. As shown in Table 5, addition of genistein strongly induced the transcription of *nopA*, but this activation dropped to basal levels in the *ttsI* mutant background, suggesting that expression of *nopA* is strictly TtsI-dependent.

Table 3. Responsiveness of the *ttsI* and *nodA* genes to different flavonoids

Data (expressed as Miller units) are the mean of at least two independent experiments performed in triplicate. *n*, Fold induction with respect to the control without flavonoids.

Flavonoid	Concentration (μM)	Hydroxylation pattern	*ttsI*::*lacZ*		*nodA*::*lacZ*	
			Mean ± SD	*n*	Mean ± SD	*n*
None			57.8 ± 10	1	68.5 ± 3	1
Flavones						
Flavone	4.5		46.7 ± 1.15	0.8	70.73 ± 5.9	1
5-Hydroxyflavone	4.2	5	65.3 ± 3.2	1.1	75.97 ± 2.2	1.1
7-Hydroxyflavone	4.2	7	94 ± 13	1.6	439.1 ± 12.2	6.4
7,4′-Dihydroxyflavone	3.9	7, 4′	121.7 ± 19.4	2.1	520 ± 19.6	7.6
Chrysin	3.9	5, 7	149.3 ± 9.3	2.6	462.3 ± 5.7	6.7
Apigenin	3.7	4′, 5, 7	208.7 ± 15.3	3.6	561.3 ± 14.2	8.2
Luteolin	3.5	3′, 4′, 5, 7	127.3 ± 2.3	2.2	359.7 ± 8.1	5.2
Flavonols						
Kaempferol	3.5	4′, 3, 5, 7	163.3 ± 4.5	2.8	472 ± 7.8	6.9
Quercetin	3.0	3′, 4′, 3, 5, 7	122.7 ± 10.3	2.1	426 ± 28.8	6.2
Fisetin	3.5	3′, 4′, 3, 7	130.3 ± 13.6	2.2	437.7 ± 26.6	6.4
Morin	3.3	2′, 4′, 3, 5, 7	216.7 ± 12.6	3.7	456.8 ± 53.3	6.7
Flavanones						
Flavanone	4.5		57.3 ± 2.5	1	74.7 ± 3.8	1.1
6-Hydroxyflavanone	4.2	6	54.3 ± 2.5	0.9	70.5 ± 9.1	1
Naringenin	3.7	4′, 5, 7	99 ± 9.8	1.7	446.7 ± 15.2	6.5
Hesperetin	3.3	3′, 4′-CH_3, 5, 7	98.3 ± 5.8	1.7	467.2 ± 14.8	6.8
Isoflavones						
Daidzein	3.9	7, 4′	214.3 ± 7.1	3.7	472 ± 17.6	6.9
Genistein	3.7	5, 7, 4’	220.7 ± 11.6	3.8	462 ± 34.0	6.7
Others						
Coumestrol	3.9	7, 12	252.9 ± 30.6	4.4	359 ± 9.85	5.2
Isoliquiritigenin	3.7	4, 2′, 4′	105.7 ± 10.3	1.8	561 ± 5.9	8.2
Umbelliferone	6.2	7	77.8 ± 2.7	1.3	83.7 ± 18	1.2

Semiquantitative RT-PCR assays were also carried out to study the expression of the *nopA* and *rhcQ* genes in *ttsI* and *nodD1* mutant backgrounds and in the absence or presence of genistein. An internal fragment of the HH103 16S rDNA was used as a control. As shown in Fig. 2, inactivation of *nodD1* or *ttsI* led to a basal level of transcription of the *rhcQ* and *nopA* genes. However, addition of genistein to the growth media clearly enhanced both *nopA* and *rhcQ* transcript levels.

Table 4. Effect of pH on the β-galactosidase activity (Miller units) of the *ttsI*::*lacZ* and *nodA*::*lacZ* fusions

Data are the mean ± SD of at least two independent experiments performed in triplicate.

Strain	pH 6.0	pH 7.0	pH 8.0
ttsI::*lacZ*	58 ± 5	69 ± 4	70 ± 6
ttsI::*lacZ* + genistein*	256 ± 9	216 ± 5	163 ± 6
nodA::*lacZ*	123 ± 18	125 ± 7	145 ± 14
nodA::*lacZ* + genistein*	1294 ± 121	631 ± 40	424 ± 34

*Genistein concentration was 3.7 μM.

Inactivation of *ttsI* abolishes Nops secretion

The involvement of the *S. fredii* HH103 *ttsI* gene in Nops secretion was investigated. Rodrigues *et al.* (2007) have shown previously that HH103 secretes, in response to flavonoids, at least eight Nops with approximate molecular masses of 180 kDa (NopD), 60 kDa (NopX and NopM), 37 or 34 kDa (NopL), 31 kDa (NopP), 21 kDa (NopB), 12 kDa (NopC) and 6 kDa (NopA).

SDS-PAGE experiments showed that inactivation of *ttsI* or its *nod* box completely abolished secretion of Nops to the extracellular medium upon induction with flavonoids, indicating that the *nod* box preceding *ttsI* is essential for Nops secretion (Fig. 3). Protein secretion could not be restored when a broad-host-range plasmid carrying *ttsI* (pMUS741) was transferred to any of the mutant strains (data not shown). However, transfer of plasmid pMUS984, a derivative of plasmid pMP92 that carries the *ttsI*, *rhcC2* and y4xK genes, to SVQ533 restored its capacity to secrete Nops to the extracellular medium in response to flavonoids (Fig. 3).

As described previously, NodD1 and flavonoids are necessary for the transcriptional activation of *ttsI*, NolR

Table 5. β-Galactosidase activity (Miller units) of different mutants that harbour *lacZ* insertions in genes belonging to the *tts* region in the presence and absence of plasmid pMUS741

Data are the mean ± SD of at least two independent experiments performed in triplicate.

Strain	Without genistein	With genistein*
HH103-1 *nopX*::*lacZ*	113 ± 26	1240 ± 48
HH103-1 *nopX*::*lacZ* (pMUS741)	1240 ± 66	1510 ± 17
HH103-1 *rhcJ*::*lacZ*	179 ± 5	1214 ± 69
HH103-1 *rhcJ*::*lacZ* (pMUS741)	1167 ± 48	1416 ± 13
HH103 Rif^R *nopA*::*lacZ*	326 ± 31	2146 ± 46
HH103 Rif^R *nopA*::*lacZ* (pMUS741)	1617 ± 63	3022 ± 29
HH103 Rif^R *ttsI*::Ω *nopA*::*lacZ*	361 ± 25	392 ± 23

*Genistein concentration was 3.7 μM.

functions as a repressor, and NodD2 is not involved in its transcriptional regulation. The role of NolR in Nops secretion has been studied previously (Vinardell *et al.*, 2004a). In this work, the effect of the mutation of *nodD1* or *nodD2* on Nops secretion was analysed. As shown in Fig. 3, inactivation of *nodD1* completely abolished Nops secretion, whereas the mutation of *nodD2* had no effect on secretion of proteins to the extracellular medium upon induction with genistein. These results were confirmed by using specific antibodies raised against NopA. This protein was not detected in the supernatants of the *ttsI* or *nodD1* mutant strains when induced with genistein (Fig. 3b).

Secretion of Nops did not become constitutive in the presence of multiple copies of *ttsI* (Fig. 4a, b) or a DNA fragment that contained the *ttsI*, *rhcC2* and y4xK genes (data not shown). However, the *nopX*, *rhcJ* and *nopA* genes were expressed in the presence of multiple copies of *ttsI* but in the absence of flavonoids (Table 5). The absence of Nops secretion in the presence of flavonoids when multiple copies of the *nolR* gene were present (Vinardell *et al.*, 2004a) was confirmed by using anti-NopA antiserum (Fig. 4b). In contrast to the results obtained by Krishnan *et al.* (1995), constitutive secretion of Nops could not be observed in the presence of extra copies of *nodD2* (Fig. 4).

The absence of Nops affects the symbiosis between *S. fredii* HH103 and its host legumes in a host-dependent manner

To elucidate the role of Nops in the interaction between *S. fredii* HH103 and *Glycine max*, which forms determinate nodules, the symbiotic properties of the mutant strain SVQ519 (HH103 Rif^R *nod* box *ttsI*::Ω) were determined in plant infection tests with five different soybean cultivars:

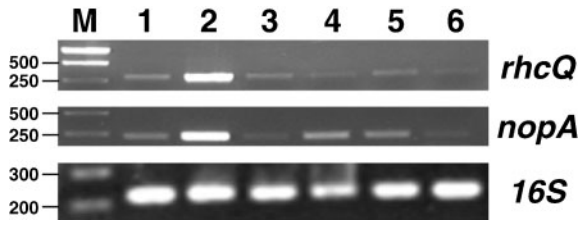

Fig. 2. Semiquantitative RT-PCR analysis of *S. fredii* HH103 mRNA. Total RNA isolated from HH103 grown in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of genistein (3.7 μM) was used as a template for RT-PCR. The products obtained using primers designed to amplify an internal fragment of the coding region of *rhcQ* and *nopA* are shown. The 16S RNA gene was used as a control. Sizes of the molecular mass markers (M) are shown on the left. Lanes: 1 and 2, HH103 Rif^R; 3 and 4, HH103 Rif^R *ttsI*::Ω; 5 and 6, HH103 Rif^R *nodD1*::Ω.

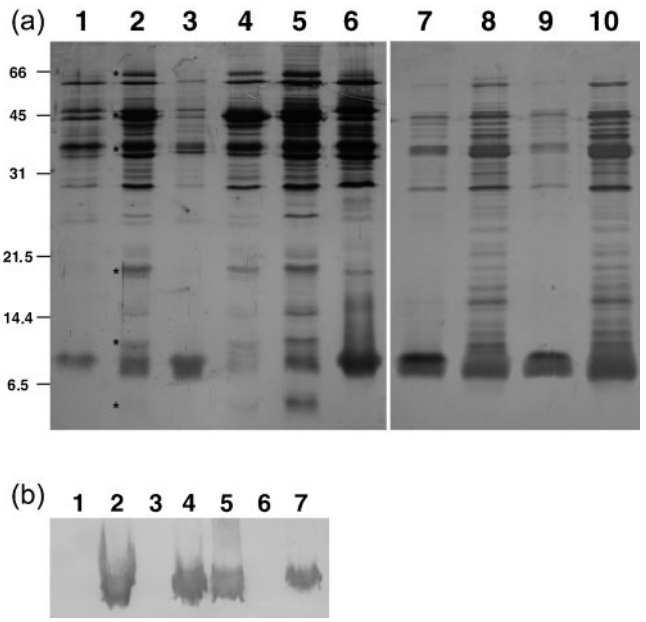

Fig. 3. Extracellular protein profiles of various *S. fredii* HH103 derivatives. (a) Silver-stained gel of secreted proteins of non-induced cultures of HH103 Rif^R (lane 1), HH103 Rif^R *nod* box *ttsI*::Ω (lane 7), HH103 Rif^R *ttsI*::Ω (lane 9), and secreted proteins of induced cultures (genistein, 3.7 μM) of HH103 Rif^R (lane 2), HH103 Rif^R *nodD1*::Ω (lane 3), HH103 Rif^R *nodD2*::Ω (lane 4), HH103 Rif^R *nolR*::Ω (lane 5), HH103 Rif^R *ttsI*::Ω (pMUS984) (lane 6), HH103 Rif^R *nod* box *ttsI*::Ω (lane 8) and HH103 Rif^R *ttsI*::Ω (lane 10). Proteins whose secretion depends on genistein are marked with an asterisk. Molecular masses (kDa) of the marker are shown on the left. (b) Immunodetection of NopA in extracellular-protein extracts of non-induced cultures of *S. fredii* HH103 Rif^R (lane 1) and induced cultures (genistein, 3.7 μM) of HH103 Rif^R (lane 2), HH103 Rif^R *nodD1*::Ω (lane 3), HH103 Rif^R *nodD2*::Ω (lane 4), HH103 Rif^R *nolR*::Ω (lane 5), HH103 Rif^R *ttsI*::Ω (lane 6), and HH103 Rif^R *ttsI*::Ω (pMUS984) (lane 7). Samples were separated by 15% SDS-PAGE.

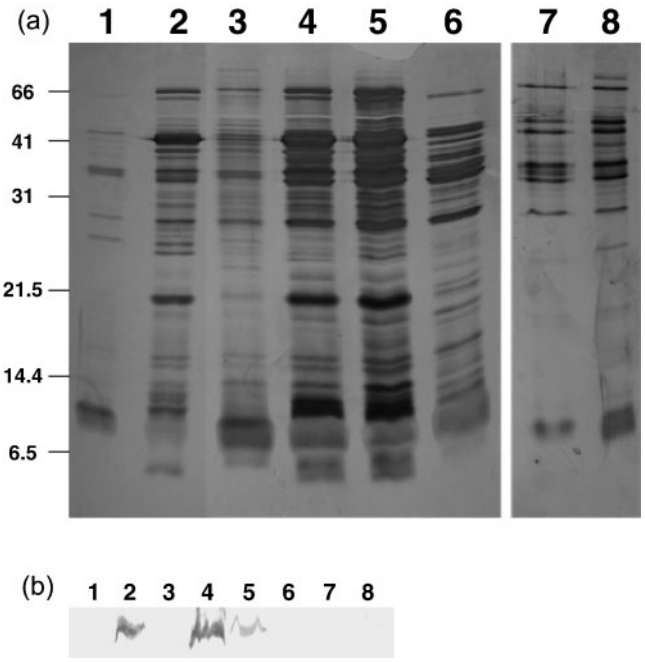

Fig. 4. (a) Effect of the transfer of a plasmid carrying extra copies of *ttsI* (pMUS741), *nodD2* (pMUS746) or *nolR* (pMUS675) on the extracellular protein profiles of *S. fredii* HH103. Lanes: 1 and 2, HH103 Rif^R; 3 and 4, HH103 Rif^R (pMUS741); 5 and 6, HH103 Rif^R (pMUS746); 7 and 8, HH103 Rif^R (pMUS675). Lanes 2, 4, 5 and 8: extracellular proteins obtained from cultures grown in the presence of genistein (3.7 μM). Molecular masses (kDa) of the marker are shown on the left. Samples were separated by 15 % SDS-PAGE. (b) Immunodetection of NopA in the corresponding extracellular-protein extracts of the *S. fredii* HH103 derivatives shown in (a). Samples were separated by 15 % SDS-PAGE.

Williams, Peking, Kochi, Tribune and Heinong 33, all of which are effectively nodulated by the wild-type strain *S. fredii* HH103. Effectors secreted by the T3SSs are usually recognized by specific plant receptors. Therefore, distinct soybean varieties could show differences in these receptors that would change their symbiotic behaviour with HH103. Two other hosts, *E. variegata*, a tropical legume that is poorly nodulated by HH103, and *Glycyrrhiza uralensis*, which forms indeterminate nodules, were also tested.

In *Glycine max* cultivars Williams and Peking, the number and fresh weight of nodules formed, as well as the plant-top dry weight, were significantly lower (α=5 %, where α is the statistical significance of the differences observed using the Mann–Whitney non-parametric test) in plants inoculated with the mutant strain SVQ519 than in those inoculated with the parental strain HH103 Rif^R. In soybean cultivars Kochi and Heinong 33, only the number of nodules was significantly reduced (α=5 and 10 %, respectively). No differences were detected between mutant SVQ519 and the parental strain HH103 Rif^R in 'Tribune' soybean (Table 6). Complementation of the mutant SVQ533 with plasmid pMUS984 restored its capacity to nodulate soybean Williams to that of the parental strain HH103 Rif^R (data not shown).

This symbiotic behaviour was also observed in *Glycyrrhiza uralensis*. Although the symbiotic performance of *S. fredii* HH103 Rif^R on this plant was highly variable (nine plants nodulated of 10 inoculated, with a nodule number per plant that varied between 22 and 95), it was clearly superior to that of mutant SVQ519 (six plants nodulated of 10 inoculated, with the number of nodules per plant between two and 35). Despite this variability, which is reflected in the high SDs observed, all the parameters analysed were significantly lower (α=5 %) in plants inoculated with SVQ519 than in those inoculated with the parental strain (Table 6).

In marked contrast, in *E. variegata* there was a significant increase (α=5 %) in nodule number, fresh weight and plant-top dry weight when plants were inoculated with mutant SVQ519 in comparison with those inoculated with HH103 Rif^R (Table 6). These data suggest that Nops may be acting as a positive factor for a successful nodulation of *S. fredii* HH103 on some *Glycine max* cultivars and *Glycyrrhiza uralensis*, but could be interfering with nodulation in *E. variegata*.

Strains SVQ533 (HH103 Rif^R *ttsI*::Ω) and SVQ534 (HH103 Rif^R *ttsI*::*lacZ*-Gm^R) exhibited the same symbiotic phenotype shown by SV519 in *Glycine max*, *E. variegata* and *Glycyrrhiza uralensis* plants (data not shown).

DISCUSSION

Rhizobial genes involved in type III secretion are grouped in the *tts* region, and secretion of nodulation outer proteins requires the presence of flavonoids and NodD. However, only one gene of the *tts* region, the *ttsI* gene, is preceded by a *nod* box (Marie *et al.*, 2003; Viprey *et al.*, 1998). TtsI has been proposed to be an intermediary in the regulatory cascade between NodD1 and T3SS-related genes, and seems to regulate the expression of the *tts* genes by binding to specific promoter sequences called *tts* boxes (Krause *et al.*, 2002; Viprey *et al.*, 1998). We have shown that the *S. fredii* HH103 *ttsI* gene is also activated by NodD1 and flavonoids (Table 2). As expected, inactivation of *nodD1* completely abolished Nops secretion (Fig. 3). In contrast, the absence of the repressor protein NolR enhanced the expression of *ttsI* (Table 2) but did not cause an apparent change in the profiles of secreted proteins upon induction with genistein (Vinardell *et al.*, 2004a; Fig. 3). However, the presence of multiple copies of *nolR* clearly reduced the activity of *ttsI* (Table 2) and repressed secretion of Nops (Vinardell *et al.*, 2004a; Fig. 4). In *S. meliloti*, NolR repressed both *nodD1* and *nodD2*, causing a general decrease in *nod* gene expression (Cren *et al.*, 1995). In *S. fredii* HH103 the presence of multiple copies of *nolR* repressed the transcription not only of *nod* genes but also of several *tts* genes, including *nopX* and *rhcJ* (Vinardell *et al.*, 2004a). In this strain, the *nodD1* and *ttsI* coding

Table 6. Plant responses to inoculation of *Glycine max*, *E. variegata* and *Glycyrrhiza uralensis* with *S. fredii* HH103 Rif^R and SVQ519 (=HH103 Rif^R *nod* box *ttsI*::Ω)

Data represent mean ± SD of six jars for each soybean cultivar. Each jar contained two soybean plants. Determinations were made 6 weeks after inoculation for soybean. For *E. variegata* plants, data represent mean ± SD of five plants. Determinations were made 90 days after inoculation. For *Glycyrrhiza uralensis*, data represent mean ± SD values obtained with nodulated plants. Determinations were made 6 weeks after inoculation. For each legume tested, bacteria isolated from 20 nodules formed by each inoculant showed the expected resistance markers. Mutant SVQ519 was individually compared with its parental strain HH103 Rif^R by using the Mann–Whitney non-parametric test.

Legume tested and inoculant	Number of nodules	Fresh weight of nodules (g)	Plant-top dry weight (g)
***Glycine max* cv. Williams**			
None	0	0	0.848 ± 0.312
HH103 Rif^R	163.4 ± 49.8	2.409 ± 1.016	7.307 ± 2.378
SVQ519	51.4 ± 22.8*	0.734 ± 0.325*	1.594 ± 0.412*
***Glycine max* cv. Peking**			
None	0	0	0.2 ± 0.028
HH103 Rif^R	220 ± 56.3	2.252 ± 0.452	6.762 ± 2.827
SVQ519	40.8 ± 19.2*	0.818 ± 0.22*	1.645 ± 0.406*
***Glycine max* cv. Heinong 33**			
None	0	0	0.66 ± 0.155
HH103 Rif^R	196.2 ± 53.4	1.796 ± 0.831	2.81 ± 1.222
SVQ519	99 ± 36.3**	1.071 ± 0.369	1.75 ± 0.625
***Glycine max* cv. Tribune**			
None	0	0	0.357 ± 0.225
HH103 Rif^R	72 ± 36.6	0.491 ± 0.225	0.908 ± 0.362
SVQ519	30.7 ± 17.1	0.299 ± 0.192	0.68 ± 0.311
***Glycine max* cv. Kochi**			
None	0	0	0.208 ± 0.057
HH103 Rif^R	82.3 ± 25.7	0.519 ± 0.248	0.755 ± 0.324
SVQ519	36.5 ± 22.1*	0.36 ± 0.177	0.738 ± 0.274
E. variegata			
None	0	0	0.895 ± 0.035
HH103 Rif^R	12.7 ± 4.7	0.89 ± 0.26	1.043 ± 0.087
SVQ519	60 ± 22.6*	6.558 ± 1.471*	7.973 ± 2.167*
Glycyrrhiza uralensis			
None	0	0	0.028 ± 0.005
HH103 Rif^R	46.7 ± 22	0.286 ± 0.165	0.862 ± 0.587
SVQ519	11.2 ± 12.8*	0.036 ± 0.027*	0.097 ± 0.049*

*Numbers in the same column significantly different at the $\alpha = 5\%$ level.
**Numbers in the same column significantly different at the $\alpha = 10\%$ level.

regions are preceded by a NolR-binding site. Therefore, the negative effect of NolR on the activity of *ttsI* and in Nops secretion could be explained by a repression of *nodD1*, which is necessary for the transcription of *ttsI*, by a repression of *ttsI* mediated by direct binding of NolR to the NolR-binding site upstream of *ttsI*, or by both mechanisms.

The presence of multiple copies of the *nodD2* gene in *S. fredii* USDA257 makes Nops secretion constitutive (Bellato *et al.*, 1997; Krishnan *et al.*, 1995). However, in *Rhizobium* sp. NGR234 the presence of multiple copies of the *nodD2* gene blocks Nops secretion (Marie *et al.*, 2003). In contrast, in *S. fredii* HH103 no changes in the activity of *ttsI* or in Nops secretion were observed, either when the *nodD2* gene was mutated or when it was overexpressed, suggesting that *nodD2* is not involved in the regulation of expression of *ttsI* in *S. fredii* HH103 (Table 2, Fig. 4).

Inactivation of the *S. fredii* HH103 *ttsI* gene completely abolished secretion of Nops to the extracellular medium (Fig. 3). Moreover, inactivation of the *nod* box preceding *ttsI* gave the same protein profile as the *ttsI* mutant strain, confirming for the first time, to our knowledge, that this *nod* box is essential for the expression of the *tts* genes and secretion of nodulation outer proteins (Fig. 3). This is in agreement with the transcriptional regulation function assigned to TtsI. The *S. fredii* HH103 *ttsI* mutant was only complemented by a DNA fragment that, in addition to the *ttsI* gene, carries the *rhcC2* and y4xK genes (Fig. 3a, b). This result suggests that these genes constitute an operon. If this were the case, the mutation of the *ttsI* gene would have a

polar effect on the transcription of downstream genes, which could be required for the biosynthesis of the secretion apparatus. The *rhcC2* gene encodes a secretin that is thought to be required for the biosynthesis of the type III secretion apparatus, while the amino acid sequence of Y4xK indicates that it could be a lipoprotein (Marie *et al.*, 2004; Viprey *et al.*, 1998). To our knowledge, this is the first time that it has been possible to complement a mutation in a rhizobial *ttsI* gene.

Despite the dependence of the expression of both *ttsI* and *nodA* on NodD1, clear differences in the capacity of certain flavonoids to activate these genes were found (Table 3). The mechanism by which different flavonoids can differentially modulate the expression of the *nod* and *tts* genes is currently unknown. Although clear differences in flavonoid-mediated expression were found between *ttsI* and *nodA*, the expression of both genes was much higher at acidic than at alkaline pH (Table 4). The fact that *ttsI* is responsive to differences in pH suggests that the *ttsI* gene could be sensitive to environmental changes that may occur in the rhizosphere in the early steps of the symbiotic interaction.

The HH103 *ttsI* gene is responsible for the transcriptional activation of at least four genes of the *tts* region, *nopX*, *nopA*, *rhcJ* and *rhcQ* (Table 5). Proteins NopX and NopA have been described as being associated with pilus-like surface appendages (Deakin *et al.*, 2005; Krishnan *et al.*, 2003), whereas RhcJ and RhcQ are components of the type III secretion machinery (Viprey *et al.*, 1998). Nucleotide sequence analysis revealed the presence of a *tts* box-like element upstream of the coding region of these genes in HH103. Overexpression of *ttsI* and induction with genistein caused an increase in the activity of these genes when compared with the parental strain (Table 5). In addition, inactivation of *ttsI* completely blocked the expression of *nopA* and *rhcQ* (Table 5, Fig. 2). These results suggest that TtsI acts as a transcriptional activator of the *tts* genes and are in agreement with those reported by Krause *et al.* (2002) and Marie *et al.* (2004) that show that TtsI is responsible for the activation of *nolU*, *nopP* and *rhcV* in *B. japonicum* USDA110 and of the *nopB* operon in *Rhizobium* sp. NGR234. Interestingly, addition of extra copies of *ttsI* induced the expression of *nopX*, *rhcJ* and *nopA* in the absence of flavonoids, making their expression constitutive (Table 5). However, *ttsI* was not constitutively expressed in the presence of extra copies of *nodD1*, although in this case the expression of the *ttsI* gene upon induction with genistein was double that obtained without extra copies of *nodD1* (Table 2). It is also remarkable that the constitutive expression of the *tts* genes due to the presence of extra copies of *ttsI* (Table 5) did not result in constitutive Nops secretion (Fig. 4), suggesting that extra regulatory controls could prevent the translation of the constitutively expressed *tts* mRNAs.

The symbiotic role of nodulation outer proteins has been mainly investigated in *Rhizobium* sp. NGR234, *S. fredii* strains HH103 and USDA257, and *B. japonicum* USDA110. In *Rhizobium* sp. NGR234, *tts* mutants show different symbiotic phenotypes depending on the host plant (Viprey *et al.*, 1998). Abolition of Nops secretion results in delayed nodulation of *B. japonicum* USDA110 on soybean Williams (Krause *et al.*, 2002). In *S. fredii*, different strain-specific phenotypes have been observed. In strain USDA257, mutants in genes belonging to the *nolXWBTUV* locus gain the ability to induce nitrogen-fixing nodules on agronomically improved American soybean cultivars that are not naturally nodulated by the wild-type strain (Meinhardt *et al.*, 1993). In contrast, in HH103, which naturally induces the formation of nitrogen-fixing nodules in both American and Asiatic soybeans, an *rhcJ* mutant strain is impaired in its symbiotic capacity with soybean cultivars Williams and Peking. However, it shows an improved symbiotic behaviour with *E. variegata* (de Lyra *et al.*, 2006).

The inactivation of the *S. fredii* HH103 *ttsI* gene provoked similar but more dramatic symbiotic effects on Williams soybean than the inactivation of the *rhcJ* gene (de Lyra *et al.*, 2006). When compared with the parental strain, the reductions in nodule number and plant-top dry weight were, respectively, 68.5 and 78.2 % for the *ttsI* mutant and 40.0 and 24.6 % for the *rhcJ* derivative. Similar results were observed in Peking soybean. In symbiosis with *E. variegata*, the positive effect of the inactivation of *ttsI* was stronger than that caused by the *rhcJ* mutation: increases of 372.4 versus 270.8 % in nodule number and of 664.4 versus 348.2 % in plant-top dry weight when compared with the parental strain.

Although it is difficult to compare results of different nodulation assays, the differences observed between the symbiotic capacities of the *rhcJ* and *ttsI* mutants could suggest a higher symbiotic relevance for the *ttsI* gene. The mutation of the *rhcJ* gene blocks the biosynthesis of the secretion machinery, but the lack of the transcriptional regulator TtsI could be able to affect not only the secretion of Nops but also the biosynthesis of other symbiotic signals in *S. fredii* HH103.

In *Rhizobium* sp. NGR234, *ttsI* activates the transcription of the *rmlBDAwbg* operon that is involved in the synthesis of rhamnose-rich polysaccharides (Marie *et al.*, 2004). However, HH103 did not synthesize this polysaccharide. In fact, hybridization studies showed that the *rmlB* gene was not present in the genome of this *S. fredii* strain (data not shown). The HH103 *ttsI* mutant showed the same LPS and LCO profiles as the parental strain, and its production of exopolysaccharide was also not affected (data not shown). However, we cannot discount the possibility that other important symbiotic signals, such as capsular polysaccharides, cyclic glucans or even molecules still unknown, could be controlled by *ttsI*.

In *S. fredii* HH103, the inactivation of the *ttsI* gene completely abolished Nops secretion, and the symbiotic effect of the mutation was host-dependent. Thus, the absence of Nops was detrimental to the symbiosis with all

the *Glycine max* cultivars tested and with *Glycyrrhiza uralensis*, but beneficial in the case of the tropical legume *E. variegata* (Table 6). *Glycine max* and *E. variegata* plants form determinate nodules, whereas *Glycine uralensis* forms indeterminate nodules. Therefore, the beneficial or detrimental effect exerted by *S. fredii* HH103 Nops does not depend on the type of nodule formed by the host legume.

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