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The *Sinorhizobium* (*Ensifer*) *fredii* HH103 Nodulation Outer Protein NopI Is a Determinant for Efficient Nodulation of Soybean and Cowpea Plants

Irene Jiménez-Guerrero,[a] Francisco Pérez-Montaño,[a] Carlos Medina,[b] Francisco Javier Ollero,[a] Francisco Javier López-Baena[a]

Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, Seville, Spain[a]; Centro Andaluz de Biología del Desarrollo, Universidad Pablo de Olavide, Consejo Superior de Investigaciones Científicas, Junta de Andalucía, Seville, Spain[b]

ABSTRACT The type III secretion system (T3SS) is a specialized secretion apparatus that is commonly used by many plant and animal pathogenic bacteria to deliver proteins, termed effectors, to the interior of the host cells. These effectors suppress host defenses and interfere with signal transduction pathways to promote infection. Some rhizobial strains possess a functional T3SS, which is involved in the suppression of host defense responses, host range determination, and symbiotic efficiency. The analysis of the genome of the broad-host-range rhizobial strain *Sinorhizobium fredii* HH103 identified eight genes that code for putative T3SS effectors. Three of these effectors, NopL, NopP, and NopI, are *Rhizobium* specific. In this work, we demonstrate that NopI, whose amino acid sequence shows a certain similarity with NopP, is secreted through the *S. fredii* HH103 T3SS in response to flavonoids. We also determined that NopL can be considered an effector since it is directly secreted to the interior of the host cell as demonstrated by adenylate cyclase assays. Finally, the symbiotic phenotype of single, double, and triple *nopI*, *nopL*, and *nopP* mutants in soybean and cowpea was assayed, showing that NopI plays an important role in determining the number of nodules formed in both legumes and that the absence of both NopL and NopP is highly detrimental for symbiosis.

IMPORTANCE The paper is focused on three *Rhizobium*-specific T3SS effectors of *Sinorhizobium fredii* HH103, NopL, NopP, and NopI. We demonstrate that *S. fredii* HH103 is able to secrete through the T3SS in response to flavonoids the nodulation outer protein NopI. Additionally, we determined that NopL can be considered an effector since it is secreted to the interior of the host cell as demonstrated by adenylate cyclase assays. Finally, nodulation assays of soybean and cowpea indicated that NopI is important for the determination of the number of nodules formed and that the absence of both NopL and NopP negatively affected nodulation.

KEYWORDS: *Sinorhizobium fredii*, cowpea, effector, nodulation, soybean, symbiosis, type III secretion

Soil bacteria, collectively known as rhizobia, induce the formation of specialized structures, called nodules, on the roots or stems of host legumes. Within these nodules, rhizobia differentiate into bacteroids that are able to reduce atmospheric nitrogen to ammonia, which is assimilated by the plant. In exchange, rhizobia are provided with a carbon source and an appropriate environment to promote bacterial growth (1).

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Address correspondence to Francisco Javier López-Baena, jlopez@us.es.

This symbiotic process needs the interchange of symbiotic signals between both partners (2). Thus, the rhizobial protein NodD is activated by specific flavonoids exuded by legume roots and then binds to conserved promoter sequences, called *nod* boxes, activating the transcription of the *nod* genes. These genes code for the enzymes responsible for the biosynthesis and secretion of the Nod factors, whose recognition by the host plant triggers the initiation of nodule organogenesis (3).

Some Gram-negative plant-pathogenic bacteria use a specialized secretion apparatus, called the type III secretion system (T3SS), to deliver protein effectors directly into the cytoplasm of the host cells. These effectors are involved in the suppression of the host defense responses to promote infection (4). Despite the conservation of the core components of the secretion machinery, the number and sequences of the secreted proteins vary. However, various studies have shown that some type III effectors from plant- and animal-pathogenic bacteria would exert similar functions in eukaryotic host cells (5). These functions would include the manipulation of Rho GTPases and the ubiquitination cascade, proteolysis, and acetylation of host proteins as well as the disruption of the target membrane or phosphorylation/dephosphorylation of cellular targets, among others (6).

The T3SS has also been identified in a reduced number of symbiotic rhizobial strains, and some of the proteins secreted through this secretion system have been identified and characterized. These proteins, collectively known as nodulation outer proteins (Nops), are involved in host range determination and symbiotic efficiency (7). Recently, Okazaki and coworkers (8) have shown that rhizobial Nops can also hijack nodulation signaling induced by Nod factors and directly activate the host symbiosis signaling. Interestingly, other authors have determined that the *Sinorhizobium fredii* HH103 T3SS is responsible for the suppression of early soybean defense responses to effectively nodulate this legume (9). Synthesis and secretion of Nops is controlled by the transcriptional regulator TtsI, which binds to specific promoter sequences called *tts* boxes. TtsI would be an intermediary in the regulatory cascade between NodD, previously activated by flavonoids, and T3SS-related genes (10–12).

S. fredii HH103, referred to here as HH103, is a broad-host-range bacterium that nodulates many legumes including soybean, which is considered to be its natural host plant (13). HH103 secretes at least eight Nops through the T3SS in response to genistein: NopA (~7 kDa), NopB (~21 kDa), NopC (~11 kDa), NopD (~180 kDa), NopL (~37 kDa), NopM (~60 kDa), NopP (~32 kDa), and NopX (~60 kDa) (14). Three of these proteins, NopA, NopB, and NopX, are components of T3SS extracellular appendages or T3SS pili (15–17), and the rest can be considered putative effectors, with the exception of NopP and NopC, whose delivery to the interior of the root cells of *Vigna unguiculata* and *Glycine max*, respectively, has been confirmed (18, 19). In the case of NopL, translocation studies have been performed in *Nicotiana benthamiana* and *Arabidopsis* leaves using heterologous *Pseudomonas* systems (19, 20). However, results obtained with these systems must be interpreted with caution, and more experiments are required to clarify the discrepancies obtained with this type of study to unequivocally describe a nodulation outer protein as a symbiotic effector (21). Interestingly, NopL, NopP, and NopC are specific to rhizobia and have no homologues in plant and animal pathogens. The *S. fredii* NGR234 NopL is phosphorylated by plant extracts from *Lotus japonicus* and tobacco, and it has been proposed that, once delivered into the plant cell, NopL would modulate host mitogen-activated protein kinase (MAPK) signaling or impair the function of MAPK substrates (22, 23). Inactivation of *nopL* induces the formation of necrotic areas in *Phaseolus vulgaris* nodules, suggesting a possible role of this putative effector in the suppression of premature senescence (24). Phosphorylation sites of NopL have been identified, and recent results have shown that this protein is localized to the plant nucleus where it forms a complex with salicylic acid-induced protein kinase (SIPK), a tobacco MAPK (25). NopP is also phosphorylated by plant kinases (26), but little is known about the exact function of this effector (27). With respect to its role in symbiosis, the inactivation of the HH103 *nopP* is beneficial for nodulation with agronomically improved (American) and primitive (Asiatic) soybean

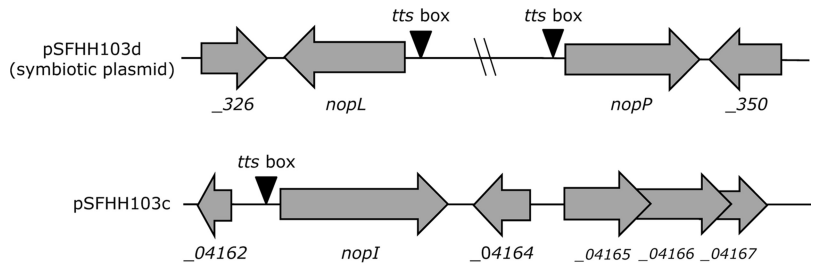

FIG 1 Genome context of the *Sinorhizobium fredii* HH103 *Rhizobium*-specific genes *nopL*, *nopP*, and *nopI*.

varieties and is detrimental for nodulation with the tropical legume *Erythrina variegata* (28). The mutation of *nopC*, however, is detrimental for nodulation with both soybean and *V. unguiculata* (18).

Sequencing of the HH103 genome revealed the presence of genes that could putatively code for other Nops since they are preceded by a *tts* box. This is the case of *nopT*, which codes for a protein with cysteine protease activity (29–31), and *nopI* and *gunA*, two genes that have not been previously described in other *S. fredii* strains (32). GunA, however, has already been described as an endoglucanase in *Bradyrhizobium japonicum*, but its inactivation does not affect symbiosis with soybean, cowpea, and mung bean (33).

In this work, we have studied the role in symbiosis of NopI, NopL, and NopP, three *Rhizobium*-specific nodulation outer proteins present in the genome of *S. fredii* HH103. We showed that NopI was secreted through the HH103 T3SS and that NopL was translocated to the interior of the soybean root cells by means of the T3SS. Finally, single, double, and triple mutants in *nopI*, *nopL*, and *nopP* were generated to analyze the impact of the mutations in the symbiotic association with soybean and cowpea.

RESULTS

Analysis of the *Sinorhizobium fredii* HH103 nodulation outer proteins NopI and NopL. The analysis of the complete genome of HH103 (GenBank accession numbers HE616890 to HE616893, HE616899, CDSA010000001 to CDSA010000004, and LN735562) allowed the identification of several potential effectors that showed no homologies with other proteins from animal- or plant-pathogenic microorganisms, such as NopC, NopI, NopL, and NopP, and hence can be considered *Rhizobium* specific (34).

The *nopI* gene (858 bp), preceded by a conserved *tts* box, is located on plasmid c, which does not contain the T3SS gene cluster (34) (Fig. 1). The presence of this promoter sequence would suggest that this gene codes for a T3SS component or a secreted protein. The *nopL* gene (1,017 bp) is also preceded by a conserved *tts* box, and together with *nopP*, is located on the HH103 T3SS cluster in the symbiotic plasmid (Fig. 1). Comparative analysis of amino acid sequences revealed that the HH103 NopL protein (338 amino acids [aa]; GenBank accession number YP_006575344) showed 100%, 96%, and 50% identity with the corresponding NopL proteins from the *S. fredii* strains USDA257 (338 aa, not annotated) and NGR234 (338 aa; GenBank accession number NP_444148) and from *Bradyrhizobium elkanii* USDA61 (249 aa; GenBank accession number CAQ57553), respectively.

The amino acid sequences of the HH103 nodulation outer proteins NopI (285 aa; sfHH103_04163) and NopP (270 aa; AAY33495) showed 48% identity (Fig. 2A). Although these proteins shared certain similarities, a neighbor-joining similarity tree constructed with the NopI and NopP protein sequences found in several rhizobial strains displayed two clearly separated branches (Fig. 2B). Interestingly, the HH103 NopI protein had homologues in other *S. fredii* strains, such as USDA257 (285 aa, not annotated) and USDA205 (285 aa; GenBank accession number KSV91515) but not in NGR234.

The analysis of the NopI sequence using the NetPhos 2.0 server (35) indicated that this protein may be potentially phosphorylated in tyrosine at position 57, which may be

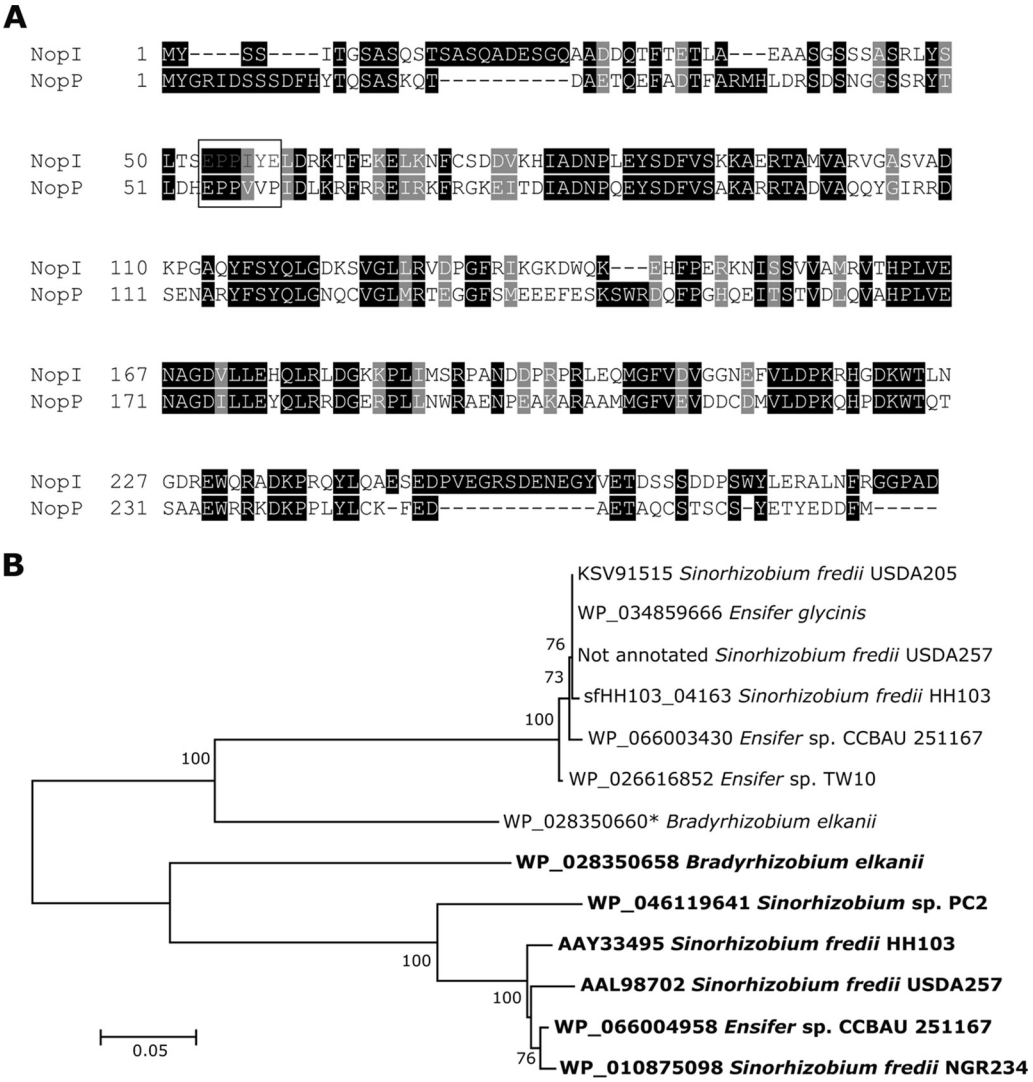

FIG 2 Sequence analysis of the *Sinorhizobium fredii* HH103 nodulation outer proteins NopP and NopI. (A) Alignment of the *S. fredii* HH103 NopI and NopP protein sequences. The putative EPIYA motif (EPPIYE) of NopI is indicated with a square. (B) NopI and NopP neighbor-joining tree of several rhizobial strains. Bootstrap values of ≥60 are indicated for each node. The cluster analysis to group the strains by NopI/NopP sequence similarity was done using the program ClustalW in the MEGA5 software package with the algorithm neighbor-joining method. Tree robustness was assessed by bootstrap resampling (1,000 replicates each). Results shown in bold correspond to NopP.

included in a putative EPIYA domain (EPPIYE in HH103), similar to other EPIYA domains present in animal pathogens, such as *Helicobacter pylori* (36). EPIYA effectors are phosphorylated in the tyrosine (Y) residue by host kinases, and once into the host cell, they interfere with intracellular signaling to enhance bacterial colonization and the effectiveness of the infection (37, 38). Curiously, the *B. elkanii* protein that grouped with NopI (GenBank accession number WP_028350660) separated early in a branch different from the rest of the NopI homologues in sinorhizobia and lacked the putative EPIYA motif. Something similar happened with the *B. elkanii* protein (GenBank accession number WP_028350658) that grouped with NopP. In addition, these proteins were different enough to be separated into two groups (Fig. 2B).

***Sinorhizobium fredii* HH103 NopI is secreted through the T3SS.** As previously published, the expression of *nopP*, *nopL*, and *nopI* is regulated by flavonoids and by the transcriptional regulators NodD1 and TtsI (28, 39). Secretion of several Nops to the extracellular medium through the T3SS has been previously reported in HH103, including NopL and NopP (14). However, NopI has never been detected in the supernatants

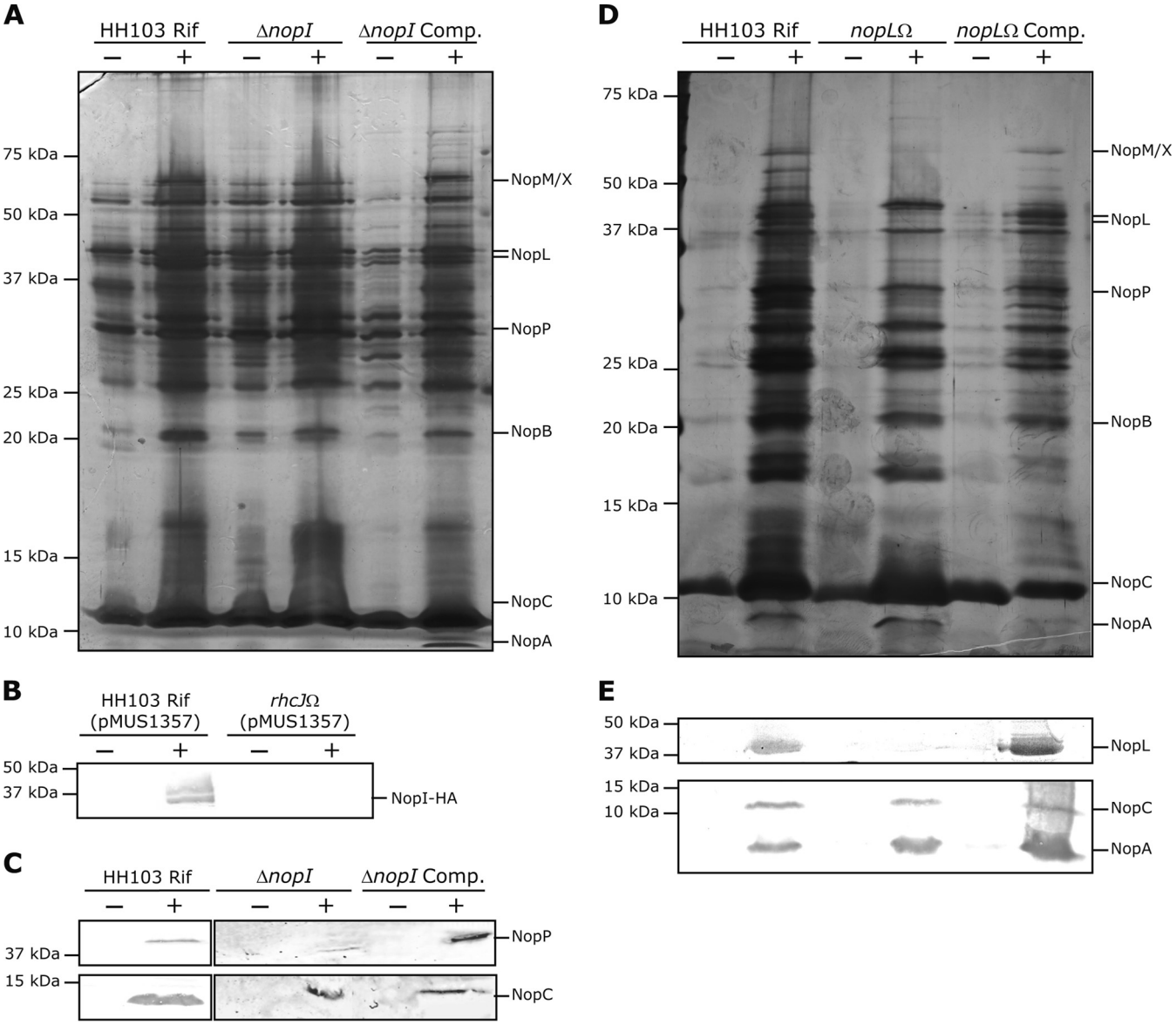

FIG 3 Extracellular protein profiles of the *Sinorhizobium fredii* HH103 *nopI* and *nopL* mutants. (A) Silver-stained gel of extracellular proteins secreted by the *nopI* mutant strain. (B) Immunodetection of the HA epitope fused to *nopI* in extracellular proteins from cultures of HH103 Rif^r (pMUS1357) and HH103 Rif^r *rhcJ*Ω (pMUS1357) in the absence or presence of genistein. (C) Immunodetection of NopC and NopP in extracellular protein extracts of the parental strain HH103 Rif^r, the HH103 Rif^r Δ*nopI* mutant, and the *nopI* mutant complemented in *trans* the absence (−) or presence (+) of genistein. (D) Silver-stained gel of extracellular proteins secreted by the *nopL* mutant strain. (E) Immunodetection of NopA, NopC, and NopL in extracellular protein extracts of the parental strain HH103 Rif^r, the HH103 Rif^r *nopL*Ω mutant, and the *nopL* mutant complemented in the absence (−) or presence (+) of genistein. Samples were separated by 15% SDS-PAGE. Molecular masses (kDa) of the marker are shown in the figure.

of any rhizobia. To determine whether NopI was secreted through the T3SS upon induction with flavonoids, extracellular proteins from HH103 Rif^r, HH103 Rif^r Δ*nopI*, and the mutant HH103 Rif^r Δ*nopI* complemented in *trans* with plasmid pMUS1215 in the presence or absence of genistein were extracted and analyzed by SDS-PAGE (Fig. 3A). Unfortunately, the band corresponding to NopI (~30 kDa), which should be detected in the lane corresponding to the parental strain induced with flavonoids but not in the other lanes, could not be identified. Therefore, to study the secretion of this putative effector, NopI was fused to HA and the construction was transferred to strain HH103 Rif^r and to the mutant strain HH103 Rif^r *rhcJ*Ω, which was unable to secrete Nops. Western blot assays showed that the antibody raised against the HA epitope specifically detected a protein of about 32 kDa in the induced supernatants of HH103 Rif^r but not in the *rhcJ* mutant (Fig. 3B).

In *Sinorhizobium fredii*, mutations in genes coding for components of the T3SS machinery, including the extracellular appendages, completely block the secretion of Nops. However, inactivation of genes coding for potential effectors does not alter the secretion of other nodulation outer proteins (21). It is worth noting that the bands corresponding to the Nops previously identified in HH103, with the exception of NopD, which is not easy to detect in gels due to its high molecular mass, were present in the lanes corresponding to the parental strain HH103 Rif^r and the *nopI* mutant (Fig. 3A). These results were confirmed by Western blotting using specific antibodies against NopC and NopP (Fig. 3C), indicating that the mutation was not affecting the secretion of the rest of the Nops. The mutation of *nopL* only prevented the secretion of NopL (two bands of ~34 and 37 kDa) as shown in the lanes corresponding to the induced supernatants of the HH103 Rif^r *nopL*Ω mutant in comparison to those of the parental strain in the presence of genistein (Fig. 3D). These results were confirmed by western blot assays using an antibody raised against NopL (Fig. 3E). As previously observed with the *nopI* mutant, the secretion of the rest of the Nops was not affected by the inactivation of *nopL* (Fig. 3D and E).

The *Sinorhizobium fredii* HH103 effector NopL is delivered to the interior of soybean nodule cells. To determine whether NopL can be considered a T3SS effector, we analyzed its translocation into plant cells using the adenylate cyclase (Cya) assay. As described by Sory and Cornelis (40), the *Bordetella pertussis* calmodulin-dependent adenylate cyclase toxin is activated only within eukaryotic cells, increasing the cellular cyclic AMP (cAMP) levels. Therefore, when Cya is fused to a possible effector, cAMP production will only be detected if the effector is delivered to the cytoplasm of the host cell.

The previous works of Wenzel and coworkers (41) and Schechter and coworkers (19) demonstrated the translocation of effector-Cya fusions into nodules of *Macroptilium atropurpureum* and *V. unguiculata*, respectively. In the case of soybean, translocation of NopC into soybean nodule cells has been reported recently (18).

In this work, the Cya protein was fused to the carboxy terminus of NopL and integrated into the genome of several HH103 strains by simple homologous recombination. Hence, the transcription of the *nopL-cya* fusion depended on the binding of TtsI to its upstream *tts* box. The gene fusion was expressed in the parental strain HH103 Rif^r and in the HH103 Rif^r *rhcJ*Ω mutant. The parental strain HH103 Rif^r without any chromosome integration was used as a control. The time point chosen for the Cya assay was 18 days after inoculation since previous works indicated that this was the best moment in the symbiotic process to measure the production of cAMP in young nodules (18). The results obtained showed very low levels of cAMP in nodules formed by the parental strain and by the *rhcJ* mutant expressing the NopL-Cya fusion. In contrast, cAMP accumulation was significantly higher in nodules from plants inoculated with the parental strain expressing the NopL-Cya fusion (Fig. 4). These results indicated that NopL was translocated into the host nodule cells via the T3SS of *S. fredii* HH103.

The inactivation of *nopI* negatively affects nodule formation, and the absence of both NopL and NopP is highly detrimental for symbiosis with soybean and cowpea. As previously mentioned, *S. fredii* HH103 NopI, NopL, and NopP can be considered *Rhizobium* specific and may potentially be substrates for plant kinases and hence exert similar or complementary functions within the host cell. Due to these reasons, different single, double, and triple mutants in the *nopI*, *nopL*, and *nopP* genes were generated. These mutations showed no major detrimental effect on bacterial growth (data not shown).

To determine the role of these Nops in symbiosis, the symbiotic properties of the parental strain HH103 Rif^r, the single mutants HH103 Rif^r Δ*nopI*, HH103 Rif^r *nopL*Ω, and HH103 Rif^r *nopP*::*lacZ*-Gm^r, together with their complemented strains, the double mutants *nopL*Ω *nopP*::*lacZ*-Gm^r, *nopI*Δ *nopP*::*lacZ*-Gm^r, and *nopI*Δ *nopL*::Ω and the triple mutant Δ*nopI nopP*::*lacZ*-Gm^r *nopL*Ω were determined in plant infection tests with two plants, soybean and cowpea, in which clear and different effects in nodulation phenotypes associated with the T3SS have been determined (18). Previous results have shown that soybean inoculation with the HH103 Rif^r *nopP*::*lacZ*-Gm^r mutant induces the

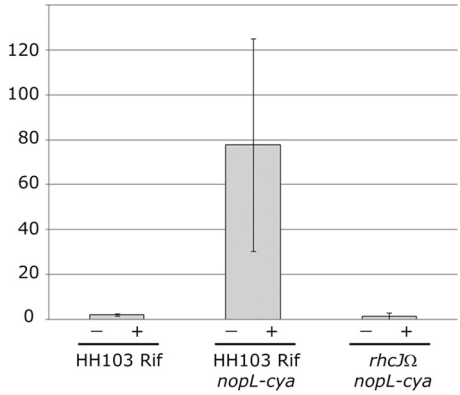

FIG 4 The *Sinorhizobium fredii* HH103 NopL is translocated to the interior of *G. max* cv. Williams 82 root nodule cells. cAMP levels were measured at 18 dpi in soybean nodules. Data shown are the mean (± the standard deviation of the mean) for two biological replicates. Each cAMP value was individually compared to that obtained in plants inoculated with the parental strain carrying the empty vector using the Mann-Whitney nonparametric test. Bars marked with an asterisk are significantly different at an α value of 5%.

formation of a higher number of nodules with respect to that of the parental strain (28). The results shown in this work indicated that the inoculation of soybean plants with strain HH103 Rif^r *nopL*Ω caused an increase in the number and fresh mass of nodules formed compared to that of the parental strain. However, these differences were not statistically significant. In contrast, a significant increase in the plant-top dry mass was observed, indicating that nitrogen fixation was more efficient when plants were inoculated with the mutant (Table 1). The opposite symbiotic phenotype was observed with the *nopI* mutant. Thus, soybean inoculation with the HH103 Rif^r Δ*nopI* strain caused a drastic reduction in the number of nodules formed, which were much bigger than those formed by the parental strain, doubling the averaged mass per nodule. This increase in the size of the nodules was observed with all of the strains in which the *nopI* gene was inactivated (Table 1). However, the plant-top dry masses were similar to those of plants inoculated with the parental strain, indicating that the final nitrogen fixed by the plant was not affected (Table 1). The double mutant affected in the *nopL* and *nopP* genes also caused a reduction in the number of nodules formed, but their size was similar to those formed by the parental strain. All of the double and triple mutant strains provoked a significant reduction in nodule number, with this reduction being stronger when one of the genes affected was *nopI*. In addition, this reduction in the

TABLE 1 Plant responses to inoculation of *Glycine max* cv. Williams 82 with different *Sinorhizobium fredii* HH103 strains[a]

Inoculant	No. of nodules[b]	Nodule fresh mass (g)[b]	Mass per nodule (mg)	Plant-top dry mass (g)[b]
None	0	0	0	0.4 ± 0.07*
HH103 Rif^r	101.8 ± 22.3	1.09 ± 0.35	0.0106 ± 0.002	1.76 ± 0.49
HH103 Rif^r *ttsI*Ω	60.2 ± 12.7*	0.81 ± 0.41	0.0134 ± 0.001	1.107 ± 0.24*
HH103 Rif^r *nopI*	52.5 ± 21.1*	0.99 ± 0.32	0.02 ± 0.005	1.75 ± 0.34
HH103 Rif^r *nopI* complemented in *cis*	124.1 ± 45.5	1.25 ± 0.112	0.01 ± 0.005	2.51 ± 0.97
HH103 Rif^r *nopL*Ω	132.5 ± 13.8	1.32 ± 0.16	0.0128 ± 0.001	2.78 ± 0.43*
HH103 Rif^r *nopL*Ω complemented in *trans*	125 ± 34.5	1.34 ± 0.27	0.0109 ± 0.002	2.39 ± 0.29
HH103 Rif^r Δ*nopI nopL*Ω	43.8 ± 18.7*	1.01 ± 0.49	0.0217 ± 0.005	1.74 ± 0.8
HH103 Rif^r Δ*nopI nopP*::*lacZ*-Gm^r	31.5 ± 7.9*	0.65 ± 0.19**	0.0208 ± 0.005	1.29 ± 0.46
HH103 Rif^r *nopL*Ω *nopP*::*lacZ*-Gm^r	67.2 ± 19.8**	0.81 ± 0.27	0.012 ± 0.001	1.15 ± 0.45**
HH103 Rif^r Δ*nopI nopL*Ω *nopP*::*lacZ*-Gm^r	47.2 ± 15.7*	0.84 ± 0.33	0.0179 ± 0.004	1.22 ± 0.33

[a]Data represent averages of 6 jars. Each jar contained two soybean plants. Determinations were made 6 weeks after inoculation. Bacteria isolated from 20 nodules formed by each inoculant showed the expected resistance markers. Data shown are the mean (± the standard deviation of the mean). *S. fredii* HH103 mutants were individually compared to their parental strain HH103 Rif^r by using the Mann-Whitney nonparametric test. The experiment was performed at least twice obtaining similar results.

[b]Numbers in the same column followed by an asterisk are significantly different at the level of α = 5%. Numbers in the same column followed by a double asterisk are significantly different at the level of α = 10%.

TABLE 2 Plant responses to inoculation of *Vigna unguiculata* with different *Sinorhizobium fredii* strains[a]

Inoculant	No. of nodules[b]	Nodule fresh mass (g)[b]	Plant-top dry mass (g)[b]
None	0	0	0.3 ± 0.14*
HH103 Rif^r	144 ± 9.2	2.43 ± 0.36	4.62 ± 0.26
HH103 Rif^r *ttsI*Ω	60.5 ± 16.7*	1.14 ± 0.22*	2.28 ± 0.63*
HH103 Rif^r Δ*nopI*	60 ± 19.7*	1.22 ± 0.43*	1.67 ± 0.8*
HH103 Rif^r Δ*nopI* complemented in *cis*	159.1 ± 63.1	2.15 ± 0.57	3.96 ± 1.28
HH103 Rif^r *nopL*Ω	271.3 ± 84.5	2.61 ± 0.26	5.94 ± 1.16*
HH103 Rif^r *nopL*Ω complemented in *trans*	119.3 ± 67.1	2.03 ± 0.46	3.63 ± 0.83
HH103 Rif^r *nopP*::*lacZ*-Gm^r	113.3 ± 31.2	1.68 ± 0.12	3.63 ± 0.6
HH103 Rif^r Δ*nopI nopL*Ω	50.5 ± 6.4*	1.58 ± 0.82	2.47 ± 1.94
HH103 Rif^r Δ*nopI nopP*::*lacZ*-Gm^r	48.6 ± 32.8*	2 ± 0.37	2.97 ± 1.2
HH103 Rif^r *nopL*Ω *nopP*::*lacZ*-Gm^r	62.7 ± 7.6*	1.03 ± 0.29*	1.78 ± 0.48*
HH103 Rif^r Δ*nopI nopL*Ω *nopP*::*lacZ*-Gm^r	45 ± 13.2*	1.19 ± 0.38*	2.05 ± 0.87

[a]Data represent averages of 6 jars. Each jar contained two cowpea plants. Determinations were made 6 weeks after inoculation. Bacteria isolated from 20 nodules formed by each inoculant showed the expected resistance markers. Data shown are the mean (± the standard deviation of the mean). *S. fredii* HH103 mutants were individually compared to their parental strain HH103 Rif^r by using the Mann-Whitney nonparametric test. The experiment was performed at least twice obtaining similar results.
[b]Numbers in the same column followed by an asterisk are significantly different at the level of $\alpha = 5\%$.

number of nodules was associated with a reduction in the fresh mass of nodules in plants inoculated with the mutant HH103 Rif^r Δ*nopI nopP*::*lacZ*-Gm^r and plant-top dry mass in the double mutant HH103 Rif^r *nopL*Ω *nopP*::*lacZ* Gm^r (Table 1).

The results obtained in the nodulation assays using *V. unguiculata* as the host plant showed that, as observed in soybean, inoculation with a mutant unable to secrete Nops caused a reduction in the number of nodules formed compared to that in the parental strain (Table 2). Inoculation with the *nopL* mutant caused an increase in the number of nodules formed associated with an increase in the plant-top dry mass. In contrast, inactivation of *nopP* was neutral for symbiosis and caused no significant effects. However, mutation of both *nopL* and *nopP* was highly detrimental, with clear reductions in the number of nodules formed and in the plant-top dry mass. The effect of the absence of NopI was much stronger in cowpea than in soybean and not only caused a decrease in the number of nodules formed but also in nitrogen fixation. Finally, like in soybean, all of the plants inoculated with a mutant in which the *nopI* gene was mutated showed a decrease in the number of nodules formed (Table 2).

DISCUSSION

The analyses of the *S. fredii* HH103 genome and transcriptome have shown that this strain possesses at least eight genes coding for putative effectors: *gunA*, *nopC*, *nopD*, *nopI*, *nopL*, *nopM* (*nopM1* and *nopM2*), *nopP*, and *nopT* (39). Four of them, *nopC*, *nopI*, *nopL*, and *nopP*, have only been described in rhizobia and, therefore, can be considered *Rhizobium* specific (18, 34). In spite of the amino acid sequences of NopI and NopP sharing certain similarities (Fig. 2A), we believe that they should be clearly differentiated since the results obtained in the neighbor-joining analysis grouped them in two different branches that separated early (Fig. 2B).

The NGR234 NopL and NopP are phosphorylated by plant kinases (22, 26), and the searching of putative phosphorylation sites for HH103 NopI, NopL, and NopP identified many potential phosphorylable residues (data not shown). One of the residues identified in NopI was situated in a possible EPIYA motif. These motifs are present in effectors of animal pathogens, like CagA of *H. pylori*. These effectors, once phosphorylated in the EPIYA tyrosine residue, interact with host proteins containing Src homology 2 (SH2) domains (37). Interestingly, legume proteins containing these domains include NSP1 and NSP2, which are involved in the early steps of the signaling cascade necessary for nodule development (42). Future efforts are necessary to (i) determine whether NopI can be phosphorylated by plant kinases, (ii) identify the amino acids phosphorylated, and (iii) determine whether its putative EPIYA motif is functional.

The HH103 *nopI* and *nopL* genes are preceded by *tts* boxes (Fig. 1), and their transcription upon flavonoid induction was blocked when the *nodD1* and *ttsI* genes were inactivated (39), indicating that the expression of both genes was flavonoid, NodD1, and TtsI dependent. Besides, secretion of NopI to the extracellular medium upon flavonoid induction (Fig. 3B) was confirmed. All of these results indicated that NopI can be considered a nodulation outer protein. In *Sinorhizobium fredii*, mutations in genes coding for components of the T3SS machinery, including the extracellular appendages, completely block the secretion of Nops (21). The mutation of *nopI* and *nopL* did not affect secretion to the extracellular medium of Nops other than NopI and NopL, respectively, suggesting that they were not essential components of the T3SS machinery (Fig. 3).

The term T3SS effector groups those proteins secreted through the T3SS that are translocated to the interior of the host cell where they exert their function (43). Analyses of T3SS-dependent translocations of putative effectors from several *Sinorhizobium fredii* (not including HH103) and *Bradyrhizobium japonicum* strains using heterologous *Pseudomonas-Arabidopsis* and *Pseudomonas*-tobacco systems have been performed (19, 20). However, in some cases, positive results were obtained with components of the T3SS machinery, indicating that these results must be interpreted with caution (21). Other authors have confirmed direct delivery of Nops to the cytoplasm of the host cell by fusing to the carboxy terminus of the T3SS effector the calmodulin-dependent adenylate cyclase (Cya) reporter protein. This was the case for HH103 NopC and soybean (18), USDA257 NopP and *V. unguiculata* (19), and *B. japonicum* USDA110 NopE1 and NopE2 and *M. atropurpureum* (41). Thus, if the effector is translocated to the interior of the host cell, the specific bacterial Cya enzyme catalyzes the cAMP production from ATP in the presence of host calmodulin-like proteins, increasing the cAMP levels. In this work, translocation of NopL to the interior of the soybean root nodule cells was also confirmed using this reporter assay (Fig. 4). As expected, the cAMP values induced by the NopL-Cya fusion were similar to those obtained in previous studies (18). The fact that the HH103 T3SS genes are expressed in young but not mature soybean nodules and the detection of both NopC and NopL in these young plant organs (18) support the previous idea that NopL acts to prolong the life span of infected cells by suppressing cell death in *P. vulgaris* nodules (24, 25).

Regarding the HH103-soybean interaction, the inactivation of the *nopP* gene is beneficial for symbiosis but highly detrimental when T3SS-dependent secretion is blocked (11, 28). The results shown in this work indicated that NopL individually, like NopP, plays a negative role in the symbiosis between HH103 and soybean since its inactivation caused a significant increase in the plant-top dry mass and a slight increase in nodule number (Table 1). However, the absence of these two proteins had a negative impact on nodulation properties within this plant, suggesting that they may have an important and related function in the symbiotic process. In *V. unguiculata*, however, NopL but not NopP was detrimental for nodulation. As in the symbiosis with soybean, the absence of both proteins had a very negative impact on nodulation (Table 2).

The symbiotic T3SS is responsible for the nodulation specificity phenotype shown by several *Sinorhizobium fredii* and *Bradyrhizobium elkanii* strains in their symbiosis with certain soybean cultivars (44, 45). In these cases, the inactivation of the T3SS allows nodulation. When the symbiotic relationship is not so specific, the final symbiotic effect of the inactivation of the T3SS can be beneficial, neutral, or detrimental and will always depend on the cocktail of effectors secreted by each strain and the plant host (7). The situation is very complex in legumes that positively react to a functional T3SS. In these cases, more than one effector may be responsible for the positive effect (7). The results shown in this work indicate that NopL and NopP together act positively in the HH103-soybean and cowpea symbiosis since the absence of both proteins is highly detrimental for nodulation. However, the individual inactivation of one of these effectors caused a positive effect on symbiosis. One explanation may be that both proteins function more efficiently alone at the same plant cell compartment or when using related plant cell components. Deciphering the exact role of these proteins in symbiosis may help to answer this question.

Interestingly, the novel T3SS-secreted protein NopI was necessary for an efficient nodulation of both soybean and cowpea since all of the mutants in which the *nopI* gene was affected caused a significant decrease in the number of nodules formed (Tables 1 and 2). These differences, in some cases, did not correlate with a decrease in the plant-top dry mass, possibly because a reduction in the number of nodules is commonly associated with an increase in the nodule size. These results indicate that NopI may be acting previously to NopL and NopP in the symbiotic process or that the plant pathways affected by NopI may be essential for an effective nodulation. Further studies are necessary to elucidate the biochemical activity of NopI and determine the symbiotic processes affected by this nodulation outer protein.

MATERIALS AND METHODS

Microbial and molecular techniques. Bacterial strains and plasmids used in this work are listed in Table S1 in the supplemental material. *Sinorhizobium* strains were grown at 28°C on tryptone yeast (TY) medium (47) or yeast extract mannitol (YM) medium (48). *Escherichia coli* strains were cultured on LB medium (49) at 37°C. When required, the media were supplemented with the antibiotics ampicillin (Ap) (100 μg ml^{-1}), rifampin (Rif) (50 μg ml^{-1}), spectinomycin (Spc) (50 μg ml^{-1} for *Sinorhizobium* and 100 μg ml^{-1} for *E. coli*), kanamycin (Km) (60 μg ml^{-1} for *Sinorhizobium* and 30 μg ml^{-1} for *E. coli*), tetracycline (Tc) (2 μg ml^{-1} for *Sinorhizobium* and 10 μg ml^{-1} for *E. coli*), and gentamicin (Gm) (5 μg ml^{-1} for *Sinorhizobium* and 10 μg ml^{-1} for *E. coli*). Genistein was dissolved in ethanol and used at 1 μg ml^{-1} to give a final concentration of 3.7 μM. Plasmids were transferred from *E. coli* to *Sinorhizobium* strains by conjugation as described by Simon (50) using plasmid pRK2013 as a helper.

Recombinant DNA techniques were performed according to the general protocols of Sambrook and coworkers (49). For hybridization, DNA was blotted onto Hybond-N nylon membranes (Amersham, United Kingdom) and the Dig DNA method of Roche (Switzerland) was employed following the manufacturer's recommendations. PCR amplifications were performed as previously described (51). The primer pairs used for the amplification of the *S. fredii* HH103 genes are summarized in Table S2 in the supplemental material.

The in-frame *nopI* gene deletion was constructed by overlap extension PCR (46) using the pairs of primers nopI-1/nopI-2 and nopI-3/nopI-4. The final DNA fragment obtained, containing the deletion of the *nopI* gene, was cloned into plasmid pK18*mobsacB* (52), obtaining plasmid pMUS1171. This plasmid was then used for the homogenotization of the mutated version of *nopI* in *S. fredii* HH103 Rif^r. The deletion event was confirmed by PCR and hybridization.

The HH103 *nopL* gene and its upstream *tts* box were amplified using primers nopLF and nopLR, and the resulting 1.1-kb DNA fragment was cloned into pGEM-T Easy (Promega, USA) to obtain plasmid pMUS943. This plasmid was digested with EcoRI, and the fragment containing *nopL* was cloned into plasmid pK18*mob* (52), previously digested with the same enzyme, to obtain plasmid pMUS1007. Then, plasmid pHP45Ω was digested with SmaI, and the 2-kb Ω interposon was cloned into a unique EcoRV restriction site in pMUS1007 located in the *nopI* open reading frame (ORF), yielding plasmid pMUS1095. Finally, this plasmid was used for the homogenotization of the mutated version of *nopL* in *S. fredii* HH103 Rif^r and in the *S. fredii* HH103 Rif^r Δ*nopI*, *S. fredii* HH103 Rif^r *nopP*::*lacZ*-Gm^r (28), and *S. fredii* HH103 Rif^r Δ*nopI nopP*::*lacZ*-Gm^r mutant strains.

To obtain the double mutant affected in *nopP* and *nopI*, plasmid pMUS843 (28) was used for the homogenotization of the mutated version of the *nopP* gene in the *S. fredii* HH103 Rif^r Δ*nopI* mutant derivative. All of the double recombination events were confirmed by hybridization.

For complementation in *trans* of the mutations in *nopI* and *nopL*, both genes and their promoter sequences were cloned into the high-copy-number plasmid pMP92. Thus, primers nopI-1 and nopI-4 were used for the amplification of the *nopI* gene and its *tts* box. The resulting 1.7-kb PCR fragment was cloned into pGEM-T Easy, obtaining plasmid pMUS1127. This plasmid was digested with EcoRI, and the DNA fragment containing *nopI* was cloned into plasmid pMP92 (53), previously digested with the same enzyme, to obtain plasmid pMUS1215. Primers nopLext-F and nopLext-R were used for the amplification of the *nopL* gene and its *tts* box. The 2.2-kb PCR fragment obtained was cloned into pMBL-T (Dominion-MBL, Spain) yielding plasmid pMUS1145, which was digested with BamHI and HindIII, and the DNA fragment containing *nopL* was cloned into plasmid pMP92, previously digested with the same enzymes, to obtain plasmid pMUS1191. In addition, the *nopI* deletion was complemented in *cis* by single recombination using plasmid pMUS1171.

For the construction of the *nopL-cya* translational fusion, primers nopLattb1 and nopLattb2ns were used for the amplification of the *nopL* gene without an end codon. The amplified 1.1-kb fragment was then cloned into pDONR207 by Gateway cloning (Invitrogen), obtaining plasmid pMUS1243. This plasmid was used to transfer *nopL* to pLMS150 (19), which possesses recombination sites for Clonase II upstream of the *cya* gene, rendering plasmid pMUS1259. The gene fusion was confirmed by sequencing. The *nopL-cya* fusion was then amplified using primers nopL_HindIII and cya_BamHI, and the fragment obtained was digested with enzymes BamHI/HindIII to clone it into pK18*mob*, which was previously digested with the same pair of enzymes, rendering plasmid pMUS1294. This plasmid was used for the chromosome integration by simple recombination of the *nopL-cya* fusion in *S. fredii* HH103 Rif^r and in an *rhcJ*Ω mutant derivative (54). The integrations resulting from a single recombination event were confirmed by antibiotic resistance and PCR amplification using the primers described in Table S2.

Purification and analysis of nodulation outer proteins. Extracellular proteins were recovered from 50 ml of YM bacterial cultures grown on an orbital shaker (180 rpm) for 40 h (approximately 10^9 bacteria ml^{-1}). Cultures were centrifuged for 20 min at 10,000 × *g* at 4°C. The supernatants were mixed with 3 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 of sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS [mass/vol], 10% glycerol [vol/vol], 5% β-mercaptoethanol [mass/vol], and 0.001% bromophenol blue [mass/vol]). The same volume of extracted extracellular proteins was loaded in each lane, and proteins were separated by SDS-PAGE using the discontinuous buffer system of Laemmli (55). Electrophoresis was performed on SDS 15% (mass/vol) polyacrylamide gels, and proteins were visualized by silver staining.

For immunostaining, extracellular proteins were separated on SDS 15% (mass/vol) polyacrylamide gels and electroblotted to Immun-Blot polyvinylidene difluoride membranes (Bio-Rad, USA) using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). Membranes were blocked with Tris-buffered saline (TBS) containing 2% (mass/vol) bovine serum albumin (BSA) and then incubated with antibodies raised against NopL, NopA, and NopC or the HA epitope (Cell Signaling Technology, USA) diluted 1:1,000 in the same solution. Anti-rabbit immunoglobulin alkaline phosphatase (AP)-conjugated secondary antibody was used, and reaction results were visualized using nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP).

The cluster analysis to group the NopI and NopP aminoacidic sequences of different rhizobial strains was carried out using the program ClustalW in the MEGA5 software package (56) with the neighbor-joining algorithm method (57).

Adenylate cyclase assay. To examine whether the HH103 T3SS translocates the NopL-Cya fusion protein into soybean nodule cells, the protocol described by Jiménez-Guerrero and coworkers (18) was used. Briefly, 18 pregerminated soybean seeds were aseptically transferred to a recipient containing vermiculite and 150 ml of a 1 M Fåhraeus solution (pH 6.8) and grown in a controlled environment chamber with a 16-h day/8-h night cycle and a relative humidity of 70%. Growth temperatures were set to 26°C during the day period and to 18°C during the night. The system was inoculated at the time of transferring the pregerminated seeds with bacterial cultures of about 10^8 CFU ml^{-1} of the HH103 Rif^r or the *rhcJ*Ω mutant strains, both containing a chromosomal integration of the *nopL-cya* fusion. Cyclic AMP (cAMP) accumulation was measured in nodules harvested at 18 days postinfection (dpi). Nodules were frozen in liquid nitrogen, ground to a fine powder, and resuspended in a 0.1 M hydrochloric acid solution. The suspension was centrifuged, and the supernatant was used for cAMP measurement using the cyclic AMP (direct) enzyme immunoassay (EIA) kit (Cayman Chemical Company, USA) according to the manufacturer's recommendations. Each sample was diluted for quantification to measure cAMP concentration in the detection range of the assay. The HH103 Rif^r parental strain was used as a control for quantification.

Plant assays. Nodulation assays on *G. max* (L.) Merrill cv. Williams 82 and *V. unguiculata* were performed as described by de Lyra and coworkers (54). Each Leonard jar contained two plants. Each plant was inoculated with about 5 × 10^8 bacteria. Plants were grown for 42 days 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. Nodulation tests were performed at least twice.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02770-16.

TEXT S1, PDF file, 0.06 MB.

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