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NolR Regulates Diverse Symbiotic Signals of *Sinorhizobium fredii* HH103

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We have investigated in *Sinorhizobium fredii* HH103-1 (=HH103 Str^r) the influence of the *nolR* gene on the production of three different bacterial symbiotic signals: Nod factors, signal responsive (SR) proteins, and exopolysaccharide (EPS). The presence of multiple copies of *nolR* (in plasmid pMUS675) repressed the transcription of all the flavonoid-inducible genes analyzed: *nodA*, *nodD1*, *nolO*, *nolX*, *noeL*, *rhcJ*, *hesB*, and y4pF. Inactivation of *nolR* (mutant SVQ517) or its overexpression (presence of pMUS675) altered the amount of Nod factors detected. Mutant SVQ517 produced Nod factors carrying N-methyl residues at the nonreducing N-acetyl-glucosamine, which never have been detected in *S. fredii* HH103. Plasmid pMUS675 increased the amounts of EPS produced by HH103-1 and SVQ517. The flavonoid genistein repressed EPS production of HH103-1 and SVQ517 but the presence of pMUS675 reduced this repression. The presence of plasmid pMUS675 clearly decreased the secretion of SR proteins. Inactivation, or overexpression, of *nolR* decreased the capacity of HH103 to nodulate *Glycine max*. However, HH103-1 and SVQ517 carrying plasmid pMUS675 showed enhanced nodulation capacity with *Vigna unguiculata*. The *nolR* gene was positively identified in all *S. fredii* strains investigated, *S. xinjiangense* CCBAU110, and *S. saheli* USDA4102. Apparently, *S. teranga* USDA4101 does not contain this gene.

Additional keywords: LCO, symbiotic interaction.

Rhizobia are α-proteobacteria belonging to the genera *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Allorhizobium,* and *Azorhizobium*. These soil bacteria establish specific symbiotic relationships with leguminous plants resulting in the formation of nitrogen-fixing nodules (Cullimore et al. 2001; Geurts and Bisseling 2002; Perret et al. 2000). The nodulation process is a complex event that requires a molecular dialogue between the partners. In response to flavonoids exuded by the roots, rhizobia secrete specific signal molecules called Nod factors or lipochitooligosaccharides (LCO) that induce in the plant the development of root nodules. Putative receptors for the rhizobial Nod factors recently have been identified in several leguminous plants (Limpens et al. 2003; Madsen et al. 2003; Spaink 2002).

Rhizobial nodulation genes (*nod*, *nol,* and *noe*) are involved in Nod factor production and constitute the *nod* regulon, whose expression is finely regulated by both positive and negative elements (Schlaman et al. 1998). In the presence of flavonoid inducers, the transcription of most of the nodulation genes is activated by the constitutively expressed NodD protein. The NodD protein belongs to the bacterial LysR regulatory family, and it binds to conserved nucleotidic sequences, called *nod* boxes, which are present in the promoter regions of the *nod* operons. On the other side, some *Sinorhizobium meliloti* strains produce a 13-kDa protein, NolR, that represses the expression of the nodulation genes, and it is necessary for optimal nodulation on alfalfa (Kondorosi et al. 1991). DNA hybridization experiments have shown that *nolR* homologous sequences are present in species belonging to the *Rhizobium* and *Sinorhizobium* genera, but not in species from *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium,* and *Agrobacterium* genera (Kiss et al. 1998).

The *S. meliloti* NolR protein binds to a specific DNA target sequence that is present in the *nolR* promoter as well as in the promoter regions of *nod* genes involved in the synthesis of the Nod factor core (Cren et al. 1995). However, *nod* operons required for the decoration of the Nod factors are not repressed by NolR. Because of this, it has been suggested that the activity of NolR results in the preferential synthesis of fully decorated *S. meliloti* Nod factors that, in low amounts, appear to be optimal for nodulation. NolR appears to belong to the AsrR regulatory family, a group of proteins that comprises both positive and negative regulators (Kiss et al. 1998). Proteomic analyses of *S. meliloti* have revealed that NolR is a global regulatory protein that increases, or decreases, the levels of numerous proteins participating in different cellular processes (Chen et al. 2000).

In addition to Nod factors, other rhizobial molecules are required for an efficient nodulation process (Fraysse et al. 2003; Perret et al. 2000). Thus, several rhizobia secrete symbiotically active proteins. *Rhizobium leguminosarum* bv. *viciae* secretes a Ca^{2+}-binding protein, NodO, that probably forms cation-specific channels in cell membranes of leguminous plants and that allows nodulation of *Vicia hirsuta* by *nod-*

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FELMNT mutants of this bacterium (Economou et al. 1990). By a type-III secretion system (TTSS), *Sinorhizobium* sp. NGR234 and *S. fredii* strains secrete several signal responsive (SR) proteins in response to inducing flavonoids in a NodD1-dependent manner (Krishnan et al. 1995; Viprey et al. 1998). In *S. fredii* USDA257, the locus *nolXWBTUV*, involved in this secretion process, regulates the nodulation of *Glycine max* in a cultivar-specific manner (Meinhardt et al. 1993). This locus also determines *S. fredii* nodulation capacity with other legumes, such as *Erythrina* spp. (Bellato et al. 1997). Genes encoding a TTSS recently have been found in some other rhizobia, such as *Bradyrhizobium japonicum* (Krause et al. 2002). Rhizobial exopolysaccharides (EPSs), lipopolysaccharides (LPSs), K-antigen capsular polysaccharides (KPSs), and the cyclic glucans also appear to play important roles in different stages of the nodulation process (Fraysse et al. 2003).

In this study, we have isolated and studied the *nolR* gene of *S. fredii* HH103. Our results indicate that *S. fredii* HH103 NolR regulates not only the production of Nod factors but also other rhizobial symbiotic signals, such as EPS and SR proteins. The absence of NolR, or its overexpression, also affects the symbiotic interaction of *S. fredii* HH103 with *G. max* cv. Williams and *Vigna unguiculata* in a host-plant-dependent manner. In addition, we show that the *nolR* nucleotidic sequence is well conserved among several *Sinorhizobium* spp. and in *Rhizobium* sp. NGR234.

RESULTS

Isolation of the *S. fredii nolR* gene.

Two primers, *nolR*int-f and *nolR*int-r, were designed from the homologous regions of the *nolR* coding sequences of *S. meliloti* AK631 (X59050) and *R. leguminosarum* bv. *viciae* TOM (AJ001934), and used for polymerase chain reaction (PCR) amplification of a 227-bp fragment of *S. fredii* HH103. DNA sequencing of the HH103-amplified fragment showed a high degree of identity to the *nolR* gene of AK631 (89%); therefore, it was used as a hybridization probe to isolate cosmid pMUS671 from a HH103 genomic library. A 2,547-bp *Eco*RI fragment from cosmid pMUS671 containing the *S. fredii* HH103 *nolR* gene was subcloned into plasmid pBluescript (generating plasmid pMUS672) and sequenced (accession number AY186253). This was the unique *Eco*RI fragment present in the HH103-1 genomic DNA showing hybridization to the probe (data not shown), indicating that there is only one copy of the *nolR* gene in the genome of *S. fredii* HH103-1.

The 2,547-bp *Eco*RI fragment contains two complete and one partial open reading frame (ORF) showing the same polarity. The *nolR* gene extends between positions 307 and 663, encoding for a 118-amino acid (aa) polypeptide that shows 89 and 75% of identity to the NolR proteins of *S. meliloti* AK631 and *R. leguminosarum* bv. *viciae* TOM, respectively. The NolR-binding site (atgcatcacg**G**ctaat, nucleotide positions 897 to 912 of X59050) that appears upstream of the *S. meliloti nolR* gene is also present with only one mismatch in *S. fredii* HH103 (atgcatcacg**T**ctaat, nucleotide positions 247 to 262 of AY186253).

The second ORF, called ORF2, is located between positions 795 and 2,195 and encodes for a putative polypeptide of 466 aa that is homologous to a putative omega amino acid pyruvate aminotransferase of *S. meliloti* 1021 (97% of identity, AL591790.1) and to a beta-alanine-pyruvate transferase of *Mesorhizobium loti* (83% of identity, AP002997.2). The HH103 sequenced fragment also contains the 5′ terminus of a putative ORF (starting at position 2,284) whose encoded product (87 aa) shows 94 and 50% of identity with a putative adenine deaminase of *S. meliloti* (AL591790.1) and with an adenine deaminase of *Bacillus subtilis* (X83795), respectively.

The nucleotide homology (80.3% of identity) of the *S. fredii* fragment to that of *S. meliloti* AK631 extends between positions 1 and 744 in the former and 651 and 1,402 in the latter. In addition, the HH103-sequenced fragment shows homology (88% of identity) to several DNA segments located between nucleotides 212,557 and 215,482 of a chromosomal region of *S. meliloti* 1021 (AL591790.1). This region contains the nonfunctional *nolR* gene of this strain (Cren et al. 1994), as well as genes that putatively encode for an omega amino acid pyruvate aminotransferase and an adenine deaminase mentioned above.

S. fredii NolR represses *nod* genes.

The 2,547-bp *Eco*RI fragment containing the HH103 *nolR* gene was subcloned into plasmid pMP92 (generating plasmid pMUS675) and transferred to different *S. fredii* HH103 mutants carrying a Tn*5-lacZ* insertion into different flavonoid-inducible genes that are responsible for the production of Nod factors (*nodA*, *nolO*, *noeL*, and *nodD1*), for the secretion of SR proteins (*nolX*, *rhcJ*, and *rhcQ*), or for other nondetermined functions (*hesB* and a locus homologous to the *Rhizobium* sp. NGR234 y4pF). Plasmid pMUS675 decreased the level of β-galactosidase activity exhibited by all the HH103 Tn*5-lacZ* derivatives assayed in the presence of the flavonoid genistein (Table 1). In contrast, transfer of plasmid pMUS810 (similar to pMUS675 but carrying the Ω interposon inserted into the *nolR* gene) to *S. fredii* SVQ116 (HH103 Rif^r *nodA*::Tn*5-lacZ*) had no effect on the bacterial β-galactosidase activity. Plasmid pMUS675 also was transferred to strain SVQ296, a derivative of HH103-1 carrying a Tn*5-lacZ* insertion elsewhere in the bacterial genome, in which its β-galactosidase activity is not influenced by the exogenous addition of flavonoids. Plasmid pMUS675 had no effect on the level of β-galactosidase activity of strain SVQ296 in either the presence or absence of genistein. These results indicate that the capacity of plasmid pMUS675 to repress the expression of nodulation genes and other genes showing transcriptional activation by flavonoids is provided by the HH103 *nolR* gene.

Computer analysis of the *S. fredii* sequences available in the databases revealed the presence of putative NolR-binding sites in the upstream regions of several genes whose expression has been shown to be influenced by this protein, such as *nodD1*, *hesB,* and the *nodABCIJnolOnoeI* and *nodZnoeLnolK* operons (Fig. 1). In addition, the intergenic region between the divergently transcribed *nodD2* and *ttsI* genes contains a *nod* box (in the direction of *ttsI*) and a well-conserved NolR-binding site. The *ttsI*-encoded product appears to act as a transcriptional activator of genes involved in the TTSS of *Rhizobium* sp. NGR234 and *B. japonicum* (Krause et al. 2002; Viprey et al. 1998). In *S. fredii* HH103, the putative NolR-binding sites of the *nodD1*, *nodD2,* and *nolR* genes are located in the complementary strand, as has been described for the *nolR*, *nodD1*, *nodD2*, and *nodD3* genes of *S. meliloti* (Cren et al. 1995).

Mutation of the *S. fredii* HH103 *nolR* gene alters the production of Nod factors.

An *nolR* mutant derivative (SVQ517) of strain HH103 was generated (discussed below). In the absence of flavonoids, the β-galactosidase activity of HH103 carrying plasmid pMP240 (it contains a transcriptional fusion between the *R. leguminosarum* bv. *viciae nodA* promoter and the *lacZ* gene) was 57% of that shown by its *nolR* derivative SVQ517 carrying this plasmid (550 ± 31 and 960 ± 69 Miller units, respectively). In the presence of genistein, β-galactosidase activities of HH103 (pMP240) and SVQ517 (pMP240) were 11,071 ± 678 and 13,689 ± 1,506, respectively.

S. fredii strains HH103-1, SVQ517, and their respective derivatives carrying plasmid pMUS675 were cultured in the presence and absence of the *S. fredii nod*-inducer flavonoid genistein. Nod factors isolated from these bacterial cultures were analyzed by reverse-phase thin-layer chromatography (RPTLC) (data not shown). In the absence of flavonoids, none of the tested strains produced detectable amounts of Nod factors. When genistein was present, subtle differences were observable. Nod factors production by the HH103-1 *nolR* mutant appeared to be slightly higher than that of its parental strain HH103-1. The presence of plasmid pMUS675 did not show any detectable effect on the amount of Nod factors produced by HH103-1, but it slightly reduced the level of LCO secreted by the *nolR* mutant.

Experiments using high-performance liquid chromatography (HPLC) also indicated that *nolR* inactivation increased Nod factor production. Strains HH103-1 and SVQ517 secreted approximately 3.9 and 17.7 mg of LCO, respectively, when they were grown in 3 liters of B^- medium (Spaink et al. 1992) supplemented with genistein. The presence of plasmid pMUS675 did not significantly affect the level of Nod factors secreted by HH103-1 (approximately 4.6 mg) but clearly reduced the amount of Nod factors produced by SVQ517 (approximately 6.8 mg).

Fast-atom-bombardment mass spectrometry (FABMS) analyses revealed differences on the set of LCO produced by strains HH103-1 and SVQ517 (Table 2). Twenty-two different LCO could be identified in SVQ517. Some of them, such as those carrying N-methyl residues at the nonreducing N-acetyl-glucosamine, have never been found before in the parental strain *S. fredii* HH103-1. The presence of plasmid pMUS675 in SVQ517 abolished the production of these N-methylated Nod factors. Overexpression of *nolR* also exerted an effect on the specific set of Nod factors detected, increasing the amount of fucosylated LCO with respect to the methyl-fucosylated ones. These results indicate that the *nolR* gene not only affects the total amount of LCO secreted by *S. fredii* HH103 but also the relative quantities of the different molecular species produced.

S. fredii HH103 *nolR* influences SR proteins secretion and EPS production.

The *nolR* gene affects the expression level of several HH103 genes involved in the TTSS (such as *nolX*, *rhcQ*, or *rhcJ*);

Table 1. β-Galactosidase activity of HH103-1 derivatives carrying *lacZ* insertions into different flavonoid-inducible genes in the presence or absence of plasmid pMUS675

Strain	Gene carrying *lacZ*	β-Galactosidase activity (Miller units)		Expression (%)[z]
		– Genistein	+ Genistein	
SVQ116	*nodA*	117 ± 20	887 ± 189	
SVQ116(pMUS675)		117 ± 20	413 ± 58	47
SVQ116(pMUS810)		107 ± 7	871 ± 127	98
SVQ118	*nolX*	162 ± 5	678 ± 81	…
SVQ118(pMUS675)		175 ± 13	237 ± 12	35
SVQ120	*rhcQ*	38 ± 13	135 ± 24	…
SVQ120(pMUS675)		47 ± 20	56 ± 11	43
SVQ121	*nolO*	54 ± 10	212 ± 36	…
SVQ121(pMUS675)		52 ± 12	124 ± 26	58
SVQ123	Homologous to y4pF of *Rhizobium* sp. NGR234	117 ± 0	870 ± 59	…
SVQ123(pMUS675)		145 ± 5	138 ± 30	16
SVQ124	*hesB*	179 ± 35	509 ± 52	…
SVQ124(pMUS675)		158 ± 42	174 ± 3	34
SVQ287	*noeL*	64 ± 16	286 ± 16	…
SVQ287(pMUS675)		73 ± 1	155 ± 1	54
SVQ288	*rhcJ*	247 ± 60	1,255 ± 260	…
SVQ288(pMUS675)		298 ± 67	524 ± 167	42
SVQ502	*nodD1*	99 ± 6	109 ± 4	…
SVQ502(pMUS675)		42 ± 10	46 ± 7	42
SVQ296	Not characterized (not flavonoid-inducible)	277 ± 14	292 ± 35	…
SVQ296(pMUS675)		299 ± 43	276 ± 31	95

[z] Numbers refer to the percentage of β-galactosidase activity of genistein-induced cultures of each mutant carrying plasmid pMUS675, or pMUS810, compared with that observed in the absence of this plasmid.

Gene or operon	Sequence	Accession number
nodABCIJnolOnoeI	ATTAGAAGATGCTCAC	M73699
nodD1[a]	GTTAGAAAGCGCTTAA	Y08938
ttsI-nodD2[a]	TTTAGGATTGGGTAAT	AY184383
nolR[a]	ATTAGACGTGATGCAT	AY186253
hesB	TTTAGTTTCCTTCAAT	AY009937
nodZnoeLnolK	ATTAGGAAGCTCTGAA	AF072888
***S. meliloti* consensus sequence**	(A/T)TTAG-N(9)-A(T/A)	Cren *et al.* (1995)

[a] Reverse complementary sequence.

Fig. 1. Putative NolR-binding sites in the promoter regions of different *Sinorhizobium fredii nod* genes. For each sequence, nucleotides matching the consensus sequence are underlined.

therefore, we investigated the effect of *nolR* inactivation or *nolR* overexpression on the secretion of SR proteins. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) experiments showed that *S. fredii* HH103-1 secretes at least five proteins in response to the presence of genistein (Lyra 2001). Two of these proteins show an apparent molecular weight that is lower than 6.5 kDa. The others are of approximately 60, 40, and 20 kDa (Fig. 2, lane 2, bands marked with black asterisks). Inactivation of the *S. fredii* HH103 *nolR* gene did not produce any clear alteration on the SR-protein profile. However, the presence of pMUS675 in strains HH103-1 and SVQ517 exerted a dramatically negative effect on the secretion of SR proteins because only small amounts of the two largest proteins were detected in the supernatants of bacterial cultures grown in the presence of genistein (Fig. 2, lanes 7 and 9). The presence of the empty vector plasmid pMP92 or plasmid pMUS810 (*nolR*::Ω) affected the amounts of SR proteins secreted by neither HH103-1 nor SVQ517 (data not shown). These results indicated that, in *S. fredii* HH103, *nolR* also represses the secretion of SR proteins.

The procedure to isolate extracellular proteins includes one precipitation step, in which acetone is added to cultures supernatants. This treatment with acetone also provokes the aggregation of the EPS that is present in the medium. In the course of the isolation of the SR proteins, it was serendipitously found that the amount of EPS recovered from *S. fredii* HH103 cultures was influenced by the absence, or the overexpression, of the *nolR* gene.

In the absence of genistein, the amount of EPS recovered (7.7 mg) from SVQ517 cultures was approximately nine times lower than that (69.0 mg) from its parental strain HH103-1. Although plasmid pMUS675 increased the amount of EPS recovered (93.8 mg) from HH103-1 cultures, this positive effect on bacterial polysaccharide production was even clearer in SVQ517 (98.5 mg). The presence of genistein reduced the amount of EPS recovered from all of the bacterial cultures investigated, although this reduction was attenuated when bacteria carried plasmid pMUS675 (10.4 and 69.4 mg for HH103-1 and HH103-1 pMUS675, respectively). Moreover, in the presence of this *nod* inducer, EPS was recovered from SVQ517 cultures only if this mutant also was carrying plasmid pMUS675 (42.3 mg). The presence of the empty vector pMP92 did not affect the amount of EPS recovered from

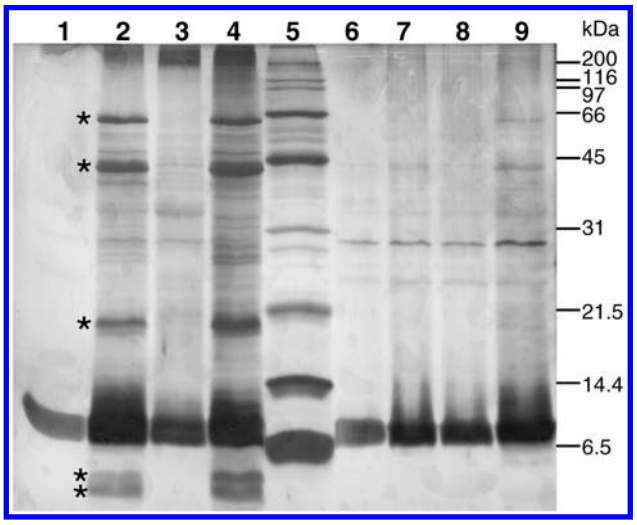

Fig. 2. Effect of NolR on the secretion of signal responsive (SR) proteins by *Sinorhizobium fredii* HH103. Lanes 1 and 2: HH103-1; lanes 3 and 4: SVQ517; lane 5: molecular weight marker (sodium dodecyl sulfate polyacrylamide gel electrophoresis standard broad range from Bio-Rad, CA); lanes 6 and 7: HH103-1(pMUS675); lanes 8 and 9: SVQ517(pMUS675). Lanes 2, 4, 7, and 9: extracellular proteins from cultures grown in the presence of genistein 3.7 μM. Molecular weights of the marker are indicated on the left. SR proteins of *S. fredii* HH103 are indicated with an asterisk.

Table 2. Summary of the lipochitooligosaccharide (LCO) structures identified as being produced by *Sinorhizobium fredii* strains HH103-1 and SVQ517, with and without plasmid pMUS675, in the presence of genistein (3.7 μM)[x]

		Strain			
$[M+Na]^+$ *m/z*	LCO structure	HH103-1	HH103-1 (pMUS675)	SVQ517	SVQ517 (pMUS675)
990	III($C_{16:1}$, Fuc)	–	–	+	–
1004	III($C_{16:1}$, MeFuc)	–[y]	–	+	–
1018	III($C_{18:1}$, Fuc)	–	–	+	–
1032	III($C_{18:1}$, MeFuc)	–[y]	–	+	–
1046	III($C_{18:1}$, NMe,MeFuc)	–	–	+	–
1048	III($C_{18:0}$, NMe,MeFuc)	–	–	+	–
1193	IV($C_{16:1}$, Fuc)	–[y]	+[z]	+	+[z]
1195	IV($C_{16:0}$, Fuc)	–	+[z]	–	+[z]
1207	IV($C_{16:1}$, MeFuc)	–[y]	–	+	+
1209	IV($C_{16:0}$, MeFuc)	+	+	+	+
1221	IV($C_{18:1}$, Fuc)	+	+[z]	+	+[z]
1223	IV($C_{18:0}$, Fuc)	–	+[z]	–	+[z]
1235	IV($C_{18:1}$, MeFuc)	+	+	+	+
1237	IV($C_{18:0}$, MeFuc)	+	+	+	+
1249	IV($C_{18:1}$, NMe,MeFuc)	–	–	+	–
1251	IV($C_{18:0}$, NMe,MeFuc)	–	–	+	–
1396	V($C_{16:1}$, Fuc)	–[y]	–	+	+
1398	V($C_{16:0}$, Fuc)	–[y]	+	+	+
1410	V($C_{16:1}$, MeFuc)	+	+	+	+
1412	V($C_{16:0}$, MeFuc)	+	+	+	+
1424	V($C_{18:1}$, Fuc)	+	+[z]	–	+
1438	V($C_{18:1}$, MeFuc)	+	+	+	+
1440	V($C_{18:0}$, MeFuc)	+	+	+	+
1452	V($C_{18:1}$, NMe,MeFuc)	–	–	+	–
1454	V($C_{18:0}$, NMe,MeFuc)	–	–	+	–

[x] LCOs were extracted from 3 liters of culture.
[y] These LCOs have been detected previously when 10 liters of culture were used (Gil-Serrano et al. 1997).
[z] The molecular ratio of these fucosylated LCOs with respect to their methyl-fucosylated homologues is higher than in HH103-1, according to the intensity of the pseudomolecular ion in mass spectrometry analyses.

HH103-1 cultures (66.9 and 13.3 mg in the absence or presence of genistein, respectively).

Similar effects of the *nolR* gene on EPS production were observed when bacteria were grown on solid B^- medium (data not shown). In the absence of genistein, strain HH103-1 was more mucous than SVQ517. However, conjugal transfer of plasmid pMUS675 to HH103-1 and SVQ517 produced transconjugants that were equally mucous. On the other hand, in the presence of genistein, the production of EPS was evident only when plasmid pMUS675 was present. Neither the presence of the empty vector pMP92 in HH103-1 and SVQ517 nor that of plasmid pMUS810 (*nolR*::Ω) in HH103-1 affected the amount of EPS produced.

The *nolR* gene increases the amount of EPS produced by *S. fredii* HH103; therefore, we investigated whether the absence, or overexpression, of *nolR* produces any alteration of the bacterial LPS profile in SDS-polyacrylamide gels. The LPS profiles of strains HH103-1, SVQ517, and their derivatives carrying plasmid pMUS675 apparently were identical (data not shown).

NolR affects the symbiotic behavior of *S. fredii* HH103.

Plant tests were carried out to investigate the role of the *nolR* gene on the symbiotic interaction of *S. fredii* HH103 with two host plants, *G. max* cv. Williams and *V. unguiculata* (cowpea). Three different parameters were analyzed: plant-top dry weight, nodule number, and dry weight of nodules.

Number and dry weight of nodules formed by *G. max* plants inoculated with mutant SVQ517 were significantly lower than those formed by soybean plants inoculated with the parental strain HH103-1 (Table 3). This reduction in the nodulation capacity exhibited by SVQ517 resulted in a significant decrease of the plant-top dry weight compared with that of soybean plants inoculated with HH103-1. The presence of plasmid pMUS675 in mutant SVQ517 slightly increased all the parameters analyzed, although significant differences were not scored. Surprisingly, the presence of plasmid pMUS675 in strain HH103-1 reduced its capacity to nodulate soybeans. These results indicate that not only the absence of a functional *nolR* gene, but also its overexpression, is detrimental for nodulation of *S. fredii* HH103 on soybean Williams.

Additional plant tests showed that the nodulation rate of soybean plants inoculated with SVQ517 was significantly delayed with respect to its parental strain HH103-1. This is reflected by the mean number of nodules per plant for HH103-1 and SVQ517: 0.6 versus 0.5 at day 10, 5.1 versus 1.0 at day 12, 11.8 versus 2.3 at day 17, 13.1 versus 7.1 at day 21, and 20.0 versus 11.9 at day 25. The number of nodules formed by HH103-1 and SVQ517 became significantly different 12 days after inoculation (at the significance level of $\alpha = 5\%$, Mann-Whitney nonparametric test). Competition experiments between strains HH103-1 and SVQ517 also were carried out with soybean Williams. When competitors were mixed at a 1:1 inoculate ratio (3×10^8 cells per competitor), nodule occupancy by SVQ517 was only 19.3%.

Plant tests with *V. unguiculata* showed different results (Table 3). Number and dry weight of nodules as well as plant-top dry weight of cowpea plants inoculated with SVQ517 were not significantly different ($\alpha = 5\%$) from those scored by inoculation with the parental strain HH103-1. The presence of plasmid pMUS675 in both strains produced a general increase in the three parameters analyzed. Plant-top dry weight of cowpea plants inoculated with SVQ517(pMUS675) was significantly higher ($\alpha = 5\%$) than that of plants inoculated with SVQ517. The presence of pMUS675 in strain HH103-1 also significantly increased the number of nodules formed by cowpea plants.

NolR is well conserved among *Sinorhizobium* and *Rhizobium* spp.

In order to investigate the presence of the *nolR* gene in other *S. fredii* strains and related rhizobia, we designed several pairs of primers from the sequence of the *nolR* region of *S. fredii* HH103-1 that allow amplification of either the complete *nolR* coding sequence or an internal fragment of this gene.

By using the pair of primers *nolR*upst/*nolR*dwst (discussed below), we could amplify a 479-bp fragment containing the complete *nolR* gene from *S. fredii* strains USDA205, USDA257, SMH12, and 042B(s) (accession numbers AY194594, AY194595, AY194596, and AY194597, respectively). The primer pair *nolR*upst/*nolR*-r led us to amplify a fragment of 367 bp containing the *nolR* gene from *Rhizobium* sp. NGR234 (AY194598). None of these primers amplified *nolR*-homologous sequences from *S. xinjiangense* CCBAU110, *S. saheli* USDA4102, or *S. teranga* USDA4101. However, primers *nolR*int-f and *nolR*int-r allowed the amplification of an

Table 3. Plant responses to inoculation of *Glycine max* cv. Williams and *Vigna unguiculata* with *Sinorhizobium fredii* strains HH103-1, SVQ517, and their derivatives carrying plasmid pMUS675[v]

Legume tested, inoculant[w]	Plant-top dry weight (g)[x]	Number of nodules	Nodule dry weight (mg)
G. max[y]			
HH103-1	5.04 ± 0.88 a	268.4 ± 23.4 a	501.5 ± 44.3 a
SVQ517	2.98 ± 0.68 b	115.0 ± 32.9 b	258.0 ± 69.3 b
HH103-1(pMUS675)	2.93 ± 0.89 b	161.8 ± 63.5 ab	264.8 ± 75.0 b
SVQ517(pMUS675)	3.11 ± 0.82 b	158.3 ± 37.3 b	297.5 ± 52.3 b
V. unguiculata[z]			
HH103-1	2.49 ± 0.88 ab	234.3 ± 62.9 a	258.3 ± 75.2 a
SVQ517	1.88 ± 0.59 a	282.6 ± 110.0 ab	255.6 ± 85.4 ab
HH103-1(pMUS675)	3.45 ± 1.21 ab	593.0 ± 149.0 b	403.4 ± 136.0 ab
SVQ517(pMUS675)	3.76 ± 0.88 b	401.0 ± 186.2 ab	451.3 ± 103.7 b

[v] Data represent averages of seven or eight plants for soybean and of five or six plants for cowpea.
[w] Bacteria isolated from 12 nodules formed by each inoculant showed the expected resistance markers.
[x] Plan-top dry weight of noninoculated plants was 0.45 ± 0.25 g for soybean and 0.12 ± 0.02 g for cowpea.
[y] For soybean plants, numbers in the same column flanked by the same letter are not significantly different at the significance level $\alpha = 5\%$ following the nonparametric test of Kruskal-Wallis. The numbers of nodules formed by HH103-1 and HH103-1(pMUS675) were significantly different at $\alpha = 10\%$ (and at the $\alpha = 1\%$ following the nonparametric test of Mann-Whitney).
[z] For cowpea plants, numbers in the same column flanked by the same letter are not significantly different at the significance level $\alpha = 5\%$ following the nonparametric test of Kruskal-Wallis. The dry weight of nodules formed by SVQ517 and SVQ517(pMUS675) were significantly different at $\alpha = 10\%$. Pairwise comparisons using the nonparametric test of Mann-Whitney also allowed the detection of other significant differences. The number of nodules formed by SVQ517 was significantly lower ($\alpha = 2\%$) than that formed by HH103-1(pMUS675). Plant-top dry weight of cowpea plants inoculated with SVQ517 was significantly lower ($\alpha = 2\%$) than that of plants inoculated with HH103-1(pMUS675). Nodule dry weight of plants inoculated with SVQ517 was significantly lower ($\alpha = 3\%$) than that of plants inoculated with SVQ517(pMUS675).

internal fragment (188 bp) of the *nolR* gene of *S. xinjiangense* CCBAU110 (AY194599) and *S. saheli* USDA4102 (AY194600).

Sequence analysis of the different PCR products obtained revealed that i) the *nolR* gene of all *S. fredii* strains and *Rhizobium* sp. NGR234 encodes a deduced polypeptide of 118 aa and ii) the *nolR* locus is well conserved among the different (sino)rhizobia included in this study. In fact, the NolR-deduced sequences of *S. fredii* strains HH103, USDA205, SMH12, and 042B(s) were identical. The partial polypeptide sequences (62 residues) deduced from the internal *nolR* sequences of *S. xinjiangense* CCBAU110 and *S. saheli* USDA4102 also were 100% identical to the corresponding region of the *S. fredii* HH103-1 NolR protein. The NolR-deduced sequences from *S. fredii* USDA257 and *Rhizobium* sp. NGR234 were 98.3% identical, and the same percentage of identity was found between each of these sequences and that of the *S. fredii* HH103 NolR polypeptide.

DISCUSSION

Participation of the bacterial NodD transcriptional activator in conjunction with inducer flavonoids is required for *nod* gene expression in most rhizobia. In addition to *nodD*, which can be present in one or in several copies, there are others positive regulatory elements of *nod* gene expression, such as *syrM* in *S. meliloti* and *Rhizobium* sp. NGR234 (Schlaman et al. 1998). Several repressors of *nod* genes also have been reported. Fellay and associates (1998) have reported that NodD2 of *Rhizobium* sp. NGR234 is involved in the repression of the *nodABC* operon. In addition, the presence of Nod factors in cultures of *B. japonicum* USDA110 induces the expression of *nolA*, which in turns activates *nodD2* and, thus, the transcription of nodulation genes is repressed (Loh and Stacey 2001).

The *nolR* gene initially was found in *S meliloti* AK631 (Kondorosi et al. 1991), in which its relevance for an optimal nodulation on alfalfa was demonstrated. Some *S. meliloti* strains, such as 1021, do not contain a functional *nolR* gene (Cren et al. 1994). More recently, Kiss and associates (1998) reported the presence of a functional *nolR* gene in *R. leguminosarum* bv. *viciae* strain TOM and also demonstrated by hybridization the presence of *nolR* homologous sequences in several *Rhizobium* and *Sinorhizobium* spp., including *S. fredii* and *Rhizobium* sp. NGR234. By using PCR and hybridization techniques, Fellay and associates (1998) demonstrated that NGR234 harbors a single, chromosomal copy of *nolR*. In this article, we report the isolation and sequence of the *nolR* gene of five different *S. fredii* strains and also provide the sequence of the *nolR* gene of *Rhizobium* sp. NGR234. Internal fragments of the *nolR* genes of *S. xinjiangense* and *S. saheli* also have been isolated and sequenced. Our results presented here indicate that the sequence of the *nolR* gene is well conserved among all the (sino)rhizobia strains investigated. *S. teranga* was the only exception because it appears that *nolR* is not present in this bacterium. The fact that all attempts to amplify the complete *S. saheli* and *S. xinjiangense nolR* genes failed indicates that the *S. fredii* sequences used as primers (external to the *nolR* coding sequence) are poorly conserved, or even absent, in these two bacteria.

In the presence of genistein, the introduction of multiple copies of *nolR* in *S. fredii* HH103 clearly produced a decrease of the expression of all the flavonoid-inducible genes tested in this work (Table 1). In addition, multiple copies of *nolR* also repressed the expression of *nodD1*, this gene being the only one that also is downregulated in the absence of flavonoids. The absence of NolR also had an effect on the expression of *nod* genes. In the absence of flavonoids, the β-galactosidase activity of the *nolR* mutant SVQ517 carrying plasmid pMP240 (it contains a transcriptional fusion between the *R. leguminosarum* bv. *viciae nodA* promoter and the *lacZ* gene) was nearly double that shown by the parental strain HH103 (pMP240). In the presence of genistein, such differences were not clearly observed.

Studies carried out by Cren and associates (1995) showed that, in *S. meliloti*, NolR represses both *nodD1* and *nodD2* genes, causing a general decrease of *nod* gene expression. In addition, the *S. meliloti* NolR protein specifically binds to the promoter regions of *nod* operons involved in the synthesis of the Nod factor core, but not to those of *nod* operons related to the decoration of LCO because these operons lack NolR-binding boxes in their promoters. This differential regulation results in the preferential synthesis of fully decorated LCO. The situation appears to be different in *S. fredii* because putative NolR-binding sites are found in the promoter regions of two different operons, one comprising both common and specific *nod* genes (*nodABCIJnolOnoeI*) and the other one containing only genes involved in Nod factor decoration (*nodZnoeLnolK*). The repression caused in the expression of *nod* genes of *S. fredii* HH103 by multiple copies of *nolR* varies from 40 to 66%, if mutant SVQ123 is not included (Table 1). This mutant carries a Tn*5*-*lacZ* insertion into a putative ORF that is homologous to the y4pF gene of *Rhizobium* sp. NGR234 that encodes a putative transposase whose symbiotic relevance, if any, is unknown.

Our results show that inactivation of the *S. fredii nolR* gene not only led to an increase of the amount of LCO detected but also to the synthesis of specific Nod factors that apparently are not produced by the parental strain HH103, such as those bearing N-methylations (Table 2). The possibility that the wild type strain HH103 also produces these new Nod factors, albeit at very low quantities, cannot be totally discarded. However, N-methylated Nod factors have not been identified among those produced by *S. fredii* HH103 when larger volumes (10 instead of 3 liters) were used (Gil-Serrano et al. 1997). Moreover, mutant SVQ517 carrying plasmid pMUS675 did not produce detectable amounts of N-methylated Nod factors. Hence, although *S. fredii* HH103-1 has the intrinsic capacity to produce N-methylated LCO, these Nod factors are produced only in the absence of a functional *nolR* gene. Krishnan and associates (1992) demonstrated that the inability of *S. fredii* USDA257 to nodulate *Leucaena* spp. was due to the lack of expression of the *nodS* gene, which is implied in the N-methylation of Nod factors in *Rhizobium* sp. NGR234 (Jabbouri et al. 1995). However, the production of N-methylated LCO by *S. fredii* strain SVQ517 did not habilitate this strain to nodulate *Leucaena leucocephala* (data not shown). The presence in *S. fredii* HH103 of a functional *nodS* gene, or another gene involved in N-methylation of LCO, has not been investigated.

The fact that a wider range of Nod factors are detected in the HH103 *nolR* mutant (compared with its wild type, HH103) could be due to differences in the levels of repression of the different nodulation genes (Table 1). Variations in the relative amounts of the different LCO detected (Table 2) clearly are observed when multiple copies of *nolR* are present, which again suggest different levels of repression of the different *nod* genes involved in LCO synthesis. Inactivation of the *noeL* gene (which is involved in LCO fucosylation) also provokes the appearance of new LCO that were not detected in the wild-type strain (Lamrabet et al. 1999), although they were different from those observed in the *nolR* mutant. In addition, Tn*5*-*lacZ* insertions upstream the *noeI* gene (which codes for a methyl-transferase) generated mutants in which the ratio of fucosylated/methyl-fucosylated Nod factors is increased

(Madinabeitia et al. 2002), as is observed when HH103 carries multiple copies of *nolR*.

The NolR protein belongs to a family of small bacterial regulatory proteins that comprises both positive and negative elements (Kiss et al. 1998). Proteomic analyses in *S. meliloti* have shown that *nolR* regulates proteins involved in different cellular processes such as the tricarboxilic acid cycle, heat- and cold-shock responses, and protein synthesis (Chen et al. 2000). Although these results indicate that NolR acts as a global regulator, LCO production is the only symbiotic signal in which the role of NolR has been studied (Kiss et al. 1998; Kondorosi et al. 1991). In the present study, we provide evidence that *S. fredii* HH103 NolR not only regulates the production of LCO but also two other symbiotic signals: SR proteins and EPS.

In *S. fredii* and *Rhizobium* sp. NGR234, SR proteins are involved in host range determination (Bellato et al. 1997; Viprey et al. 1998). Some *S. fredii* strains, such as USDA257, form nitrogen-fixing nodules with Asiatic soybean cultivars but fail to nodulate American cultivars (Buendía-Clavería and Ruiz-Sainz 1985; Keyser et al. 1982). This cultivar specificity somehow also is conditioned by SR proteins. In fact, some mutations into the *nolXWBTUV* locus (it encodes part of the TTSS and also some of the SR proteins) of *S. fredii* USDA257 abolish SR protein production and enable the bacteria to establish effective nodulation with American soybean cultivars (Krishnan et al. 1995; Meinhardt et al. 1993). Paradoxically, *S. fredii* strains, such as HH103, that naturally form nitrogen-fixing nodules with American soybean cultivars also secrete SR proteins (Lyra 2001).

In the presence of flavonoids, transcriptional activation of *Rhizobium* sp. NGR234 *nod* genes involved in LCO production occurs earlier than that of the *nolXWBTUV* locus, suggesting a possible role of rhizobial SR proteins in advanced stages of root infection (Viprey et al. 1998). This hypothesis is supported by the fact that NolX, one of the *S. fredii* USDA257 SR proteins, has been localized in the infection threads of developing soybean and cowpea nodules (Krishnan 2002). In *Rhizobium* sp. NGR234, inactivation of *nolX* (also called *nopX*, for nodulation outer protein) has little effect on NopL and NopA secretion but greatly affected the interaction of this strain with many plant host tested (Marie et al. 2003).

SR proteins from *S. fredii* and *Rhizobium* sp. NGR234 are produced in a flavonoid and *nodD1* dependent manner. Genes involved in the TTSS of *Rhizobium* sp. NGR234 and *B. japonicum* lack *nod* boxes but are positively regulated by the product of *ttsI* (previously named y4xI) which is under the control of a *nod* box (Krause et al. 2002; Viprey et al. 1998). In *S. fredii* HH103, NolR represses different genes involved in SR protein production, such as *nolX* (encoding for an SR protein), and *rhcQ* and *rhcJ* (encoding for proteins of the TTSS) (Table 1). The promoter region of HH103 *ttsI* contains an NolR-binding site, which probably contributes to the dramatic reduction observed in the amount of SR proteins secreted by this strain in the presence of plasmid pMUS675. Only one *S. fredii* HH103 gene (*nolX*) coding for SR proteins has been identified (Bellato et al. 1997). Whether NolR affects the transcription of other genes coding for SR proteins remains to be investigated.

Rhizobial surface polysaccharides are of crucial importance in the infection process of legume roots (Fraysse et al. 2003; Perret et al. 2000). Although EPS$^-$ mutants of *S. fredii* are fully effective on *G. max* (Kim et al. 1989), some evidence indicates that it could be of biological significance: i) the presence of flavonoids reduces the amount of EPS produced by *S. fredii* USDA193 (Dunn et al. 1992); ii) the presence of multiple copies of *nodD2* reduces EPS production by *S. fredii* USDA191 (Appelbaum et al. 1988; Machado and Krishnan 2003); and iii) USDA191 *nodD1* or *nodD2* mutants produced significantly more EPS than the wild-type strain, this effect being stronger in the *nodD2* mutant. Results presented in this work add two new evidences that link EPS production with elements of the *nod* gene regulatory circuit. First, the amount of EPS recovered from *S. fredii* HH103 cultures is reduced in a NolR$^-$ background (mutant SVQ517), but increased if *nolR* is present in a multicopy vector (HH103-1 or SVQ517 carrying pMUS675); and second, the negative effect that genistein exerts on bacterial EPS production is attenuated by the presence of *nolR*.

We also have shown that the *hesB* gene of *S. fredii* is repressed by NolR. This gene contains both *nod* and *nif* boxes in its promoter region, suggesting that *hesB* might act in late steps of the symbiotic interaction.

Previous reports have described a negative effect of *nolR* mutations on the symbiotic properties of *S. meliloti* and *R. leguminosarum* bv. *viciae* with their leguminous host plants (Kiss et al. 1998; Kondorosi et al. 1989). In this article, we show that either inactivation or overexpression of *nolR* impairs the symbiotic interaction of *S. fredii* HH103 with soybean plants. Surprisingly, the symbiotic performance of mutant SVQ517 with *V. unguiculata* was similar to that shown by strain HH103 and the presence of plasmid pMUS675 in HH103-1 and SVQ517 produced a general improvement of the symbiotic parameters analyzed.

In conclusion, our studies demonstrate the presence of *nolR* in all the *S. fredii* strains so far investigated and also in other *Sinorhizobium* spp. (such as *S. saheli* and *S. xinjiangense*). NolR in *S. fredii* HH103 clearly influences the production of at least three different symbiotic signals (Nod factors, SR proteins, and EPS) and also exerts a significant impact on the bacterial symbiotic capacity that varies with the host legume tested. Because the symbiotic signals affected by NolR act at different steps of the nodulation process, *nolR* appears to exert its influence at different stages of nodule formation. The regulating effect of NolR appears to be the result of the repression of genes that are directly involved in the synthesis of signals (such as *nodA*) or through the repression of other regulating genes that act as activators or repressors. The reduction in the production of LCO and the increase in EPS production would be carried out by repressing *nodD1*, and possibly *nodD2*, whereas the repression of the secretion of SR proteins in the presence of multiple copies of *nolR* could be achieved by repressing *nodD1*, and probably *ttsI*. This overall alteration of symbiotic signals, together with the relative importance of each signal alteration for the nodulation process with *G. max* and *V. unguiculata,* would account for the differences in symbiotic performance observed for the different bacterial strains tested. All these results indicate that it would be worthwhile to investigate whether *nolR* influences other symbiotic signals, such as KPS.

MATERIALS AND METHODS

Microbiological techniques.

The bacterial strains and plasmids used in this work are described in Table 4. *Sinorhizobium* strains were grown at 28°C on tryptone yeast medium (Beringer 1974), yeast extract/mannitol (YM) medium (Vincent 1970), or B$^-$ medium (Spaink et al. 1992). *Escherichia coli* was cultured on Luria-Bertani (LB) medium (Sambrook et al. 1989) at 37°C. When required, the media were supplemented with the appropriate antibiotics (μg ml^{-1}) as described by Lamrabet and associates (1999). Genistein was dissolved in ethanol and used at 1 μg ml^{-1} (3.7 μM). Plasmids were transferred from *E. coli* to rhizobia by conjugation as described by Simon (1984).

DNA manipulations.

Recombinant DNA techniques were performed according to the general protocols of Sambrook and associates (1989). DNA sequencing was done by using the dideoxy-chain termination method (Sanger et al. 1977). PCR amplifications were performed according to protocols described by Saiki (1990). Primers used were: *nolR*int-f (5′ GAGGAAGCCGAAATAGCAG), *nolR*int-r (5′ GAGTCGGACGAGCTCGAATA), *nolR*upst (5′ TATGCTACCCCCAATTCTTGC), *nolR*dwst (5′ GAAAAAGC CCCGCGATTGCT), and *nolR*-r (5′ CCTGCGGCTTTTCGCT TCTCA). DNA and deduced protein sequences were analyzed with the UWGCG program (Devereux et al. 1984).

The collection of HH103-1 derivatives carrying Tn*5*-*lacZ* insertions into flavonoid-inducible genes was generated as previously described (Lamrabet et al. 1999; Madinabeitia et al. 2002). Construction of strain SVQ502 (=HH103 Rif^r *nodD1*::*lacZ*Δp-Gm^r) is described by Vinardell and associates (2004).

The HH103 *nolR* derivative was generated as follows: plasmid pMUS672 was digested with *Nco*I, whose unique site is located in the middle of the *nolR* gene, filled with the Klenow enzyme, and fused to a 2-kb *Sma*I fragment carrying the Ω interposon (Spc^r Str^r) (Prentki and Krisch 1984). The plasmid generated, pMUS728, was digested with *Eco*RI and the 4.5-kb fragment carrying the *nolR*::Ω fusion was subcloned into the unique *Eco*RI site of pK18mob. The resulting plasmid, called pMUS735, was transferred to HH103-1 and Spc^r Km^s transconjugants were selected in order to identify putative double recombinants in which the wild-type *nolR* gene had been substituted by the mutated copy of this gene. Homogenization of the *nolR*::Ω in several candidates was confirmed by hybridization, and strain SVQ517 was selected for further studies. The 4.5-kb *Eco*RI fragment from pMUS728 carrying the *nolR*::Ω fusion also was subcloned into the broad-host-range vector pMP92, rendering plasmid pMUS810.

Measurement of β-galactosidase activity.

Assays for bacterial β-galactosidase activity in liquid YM medium were as described by Miller (1972). At least three independent experiments were performed, in duplicate, for each strain.

Analysis of Nod factors.

LCO from *S. fredii* strains grown in B$^-$ minimal medium were analyzed by RPTLC as described by Spaink and associates (1992).

For chemical analyses, LCO were extracted and purified from 3 litters of bacterial cultures grown in B$^-$ minimal medium supplemented with genistein, as described by Lamrabet and associates (1999). Glycosyl composition and FABMS analyses were carried out as described by Madinabeitia and associates (2002).

Analysis of extracellular proteins.

Extracellular proteins from *S. fredii* strains were recovered from 50 ml of YM bacterial cultures grown on a orbital shaker (180 rpm) for 48 h (approximately 10^9 bacteria ml^{-1}). Cultures

Table 4. Bacterial strains and plasmids

Strain or plasmid	Derivation and relevant properties	Source or reference
Sinorhizobium fredii		
HH103-1	HH103 Str^R	Buendía-Clavería et al. (1989)
SVQ269	HH103 Rif^R	Madinabeitia et al. (2002)
SVQ116	SVQ269 *nodA*::Tn*5*-*lacZ*	Buendía-Clavería et al. (2003)
SVQ118	HH103-1 *nolX*::Tn*5*-*lacZ*	Bellato et al. (1997)
SVQ120	HH103-1 *rhcQ*::Tn*5*-*lacZ*	Madinabeitia et al. (2000)
SVQ121	HH103-1 *nolO*::Tn*5*-*lacZ*	Madinabeitia et al. (2002)
SVQ123	HH103-1 carrying a Tn*5*-*lacZ* insertion into a locus homologous to y4pF of *Rhizobium* sp. NGR234	N. Madinabeitia
SVQ124	HH103-1 *hesB*::Tn*5*-*lacZ*	Espuny et al. (2000)
SVQ287	HH103-1 *noeL*::Tn*5*-*lacZ*	Lamrabet et al. (1999)
SVQ288	HH103-1 *rhcJ*::Tn*5*-*lacZ*	Lyra (2001)
SVQ296	HH103-1 carrying a Tn*5*::*lacZ* insertion elsewhere	M. T. Cubo
SVQ502	SVQ269 *nodD1*::*lacZ*Δp-Gm^R	Vinardell et al. (2004)
SVQ517	HH103-1 *nolR*::Ω	This work
042B(s)^R	042B(s) Rif^R	A. Buendía-Clavería
USDA205	Wild-type strain	Keyser et al. (1982)
USDA257	Wild-type strain	Keyser et al. (1982)
SMH12	Wild-type strain	Rodríguez-Navarro et al. (1996)
S. saheli		
USDA4102	Wild-type strain	de Lajudie et al. (1994)
S. teranga		
USDA4101	Wild-type strain	de Lajudie et al. (1994)
S. xinjiangense		
CCBAU110	Wild-type strain	Chen et al. (1988)
Rhizobium sp.		
NGR234	Wild-type strain	Trinick (1980)
Escherichia coli		
DH5α	*supE44*, Δ*lacU169*, *hsdR17*, *recA1*, *endA1*, *gyrA96* , *thi-1*, *relA1,* Nx^R	Stratagene
Plasmids		
pBluescript II SK+	Cloning and sequencing vector, Ap^R	Stratagene
pHP45Ω	Ap^R vector containing the Ω interposon (Spc^{R}Str^R)	Prentki and Krisch (1984)
pK18mob	Cloning vector, Km^R	Schafer et al. (1994)
pMP92	Broad-host-range cloning vector; IncP; Tc^R	Spaink et al. (1987)
pRK2013	Helper plasmid, Km^R	Figurski and Helinski (1979)
pMUS671	Cosmid pLAFR1 carrying the *nolR* gene of *S. fredii* HH103	This work
pMUS672	pBluescript carrying a 2,547-bp *Eco*RI fragment from pMUS671 containing HH103 *nolR*	This work
pMUS675	pMP92 carrying a 2,547-bp *Eco*RI fragment from pMUS671 containing HH103 *nolR*	This work
pMUS728	pMUS672 carrying the Ω interposon subcloned into the *Nco*I site of *nolR*.	This work
pMUS735	pK18mob carrying a 4.5-kb *Eco*RI fragment containing HH103 *nolR*::Ω derived from pMUS728	This work
pMUS810	pMP92 carrying a 4.5-kb *Eco*RI fragment containing HH103 *nolR*::Ω derived from pMUS728	This work

were centrifuged at 10,000 × *g* for 10 min and three volumes of acetone were added to the supernatants. The resultant mixtures were stored at –20°C for 24 h and centrifuged at 22,000 × *g* for 20 min. Dried pellets were resuspended in 500 µl of sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS [wt/vol], 10% glycerol [wt/vol], 5% β-mercaptoethanol [wt/vol], 0.001% bromophenol blue [wt/vol]). Extracellular proteins were separated by SDS-PAGE using the discontinuous buffer system of Laemmli (1970). Electrophoresis were performed in 0.75-mm-thick gels (15% acrylamide for the separation gel; 4% acrylamide for the stacking gel) in a Protean electrophoresis system (Bio-Rad, Hercules, CA, U.S.A.) at 25°C and 150 V. The amount of each sample loaded into the wells varied (25 to 50 µl) according to the bacterial density estimated for each culture. Gels were silver stained as described by Switzer and associates (1979).

Studies of external polysaccharides.

For extraction of EPS from liquid cultures, *S. fredii* strains were grown in 50 ml of liquid B^- minimal medium on an orbital shaker (180 rpm) for 96 h (approximately 5 to 7 x 10^9 bacteria ml^{-1}). When required, the media were supplemented with tetracycline (1 µg/ml), genistein (1 µg/ml), or both. After centrifugation at 10,000 × *g* for 10 min, supernatants were mixed with three volumes of acetone and the EPS was recovered with a glass-rod, dried at 60°C for 3 h, and weighed. This analysis was repeated twice, giving similar results. To investigate EPS production on solid B^- medium, rhizobial strains were grown for 120 h at 28°C followed by 48 h at room temperature. LPS extraction, separation on SDS-PAGE, and silver staining were performed as previously described (Gil-Serrano et al. 1999).

Plant assays.

S. fredii strains were tested on *G. max* cv. Williams and *V. unguiculata* (cowpea) as described by Buendía-Clavería and associates (1989). Each Leonard jar contained two soybean plants or one cowpea plant. Each plant was inoculated with approximately 5 × 10^8 bacteria. Plants were grown for 52 days with a 16-h photoperiod at 25°C in the light and 18°C in the dark. Plant tops and nodules were dried at 70°C for 48 h and weighed. Bacteria were isolated from surface-sterilized nodules as previously reported (Buendía-Clavería et al. 1989). For competition studies on *G. max* cv. Williams, each plant was inoculated with approximately 3 × 10^8 bacteria of each coinoculant, HH103-1 (Str^r) and SVQ517 (Str^rSpc^r). Nodule occupancy by strain SVQ517 was determined in 125 nodules by assessing the presence of the Ω interposon (presence of the Spc^r marker). Experiments to determine the nodulation rate of soybean plants inoculated with strains HH103-1 or SVQ517 were carried out in mini-Leonard jars (approximately 200 ml for the upper part containing vermiculite and 170 ml for the reservoir containing the plant nutritive solution). At least six plants inoculated with each strain were analyzed for nodulation at each time point.

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