RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY (2006) 9:125-133
www.im.microbios.org

INTERNATIONAL MICROBIOLOGY

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Inactivation of the *Sinorhizobium fredii* HH103 *rhcJ* gene abolishes nodulation outer proteins (Nops) secretion and decreases the symbiotic capacity with soybean

Summary. It has been postulated that nodulation outer proteins (Nops) avoid effective nodulation of *Sinorhizobium fredii* USDA257 to nodulate with American soybeans. *S. fredii* HH103 naturally nodulates with both Asiatic (non-commercial) and American (commercial) soybeans. Inactivation of the *S. fredii* HH103 gene *rhcJ*, which belongs to the *tts* (type III secretion) cluster, abolished Nop secretion and decreased its symbiotic capacity with the two varieties of soybeans. *S. fredii* strains HH103 and USDA257, that only nodulates with Asian soybeans, showed different SDS-PAGE Nop profiles, indicating that these strains secrete different sets of Nops. In coinoculation experiments, the presence of strain USDA257 provoked a clear reduction of the nodulation ability of strain HH103 with the American soybean cultivar Williams. These results suggest that *S. fredii* Nops can act as either detrimental or beneficial symbiotic factors in a strain-cultivar-dependent manner. Differences in the flavonoid-mediated expression of *rhcJ* with respect to *nodA* were also detected. In addition, one of the Nops secreted by strain HH103 was identified as NopA. [**Int Microbiol** 2006; 9(2):125-133]

Key words: *Sinorhizobium fredii* HH103 · gene *rhcJ* · type III secretion system · nodulation outer proteins (Nops) · soybean

Received 16 February 2006
Accepted 15 May 2006

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Introduction

Rhizobia are soil α-proteobacteria that have the ability to establish symbiotic associations with many leguminous plants. This symbiosis leads to the formation of nodules on roots of leguminous plants where the bacteria fix nitrogen. Nodule development requires the exchange of symbiotic signals between the partners [13,14,38]. Flavonoids secreted by plant roots interact with the bacterial protein NodD, which activates the transcription of bacterial nodulation genes. In turn, expression of these genes results in the production and secretion of signal molecules, known as Nod-factors or LCOs (lipochitooligosaccharides), that are essential for nodule morphogenesis. In several rhizobia, flavonoids also induce the secretion of proteins into the extracellular environment through a type III secretion system (TTSS). These secreted proteins, called Nops (nodulation outer proteins), have a role in determining the bacterial nodulation host-range in a variety of leguminous plants [36,51].

There is a wide variety of plant and animal symbiotic and pathogenic gram-negative bacteria that export proteins via the TTSS [34,37]. This secretion apparatus is formed by

about twenty proteins in a complex spanning the inner and outer bacterial membranes that is used by the bacteria to secrete proteins to the external environment. The TTSS machinery, but not the proteins exported through this secretion system, is well-conserved among different bacteria [17]. Genes coding for the TTSS have been identified in *Rhizobium* sp. NGR234 [51], *Sinorhizobium fredii* USDA257 [36], *Mesorhizobium loti* MAFF303099 [20], *Bradyrhizobium japonicum* USDA110 [16,25], and *Rhizobium etli* CFN42 (accession number U80928). However, the TTSS is absent in *M. loti* ICMP3153 and in *Sinorhizobium meliloti* 1021 [33,51]. Recently, the presence of a type IV secretion system was reported in *M. loti* strains lacking the TTSS [18]. Genes coding for the TTSS in *S. fredii* USDA257 and *Rhizobium* sp. NGR234 are located on the symbiotic plasmid. They are organized in the *tts* cluster, which contains 29 predicted ORFs, delineated by *ttsI* and y4yS [19,35,51]. Some of the genes of this cluster are necessary for the biosynthesis of the TTSS apparatus and others code for secreted proteins, such as NopB (formerly NolB), NopX (formerly NolX), NopL (formerly y4xL), NopP (formerly y4yP), and NopA [1,3,26,31,34].

The rhizobial *tts* cluster includes the *nolXWBTUV* locus, although *B. japonicum* USDA110 lacks the *nopX* gene. In *S. fredii* USDA257 and *Rhizobium* sp. NGR234, transcription of the *nolXWBTUV* locus and secretion of proteins through the TTSS depend on *nodD1* and flavonoids, in spite of the absence of a conserved nodulation promoter (*nod* box) [28,51]. The only gene of the *tts* cluster that is preceded by a *nod* box is *ttsI* (formerly y4xI). At least in *Rhizobium* sp. NGR234 and *B. japonicum* USDA110, TtsI is involved in the transcriptional regulation of the *tts* cluster [25,33,35]. *Rhizobium* sp. NGR234 *tts* mutants show different symbiotic phenotypes depending on the host plant, such as increased nodule number on *Pachyrrhizus tuberosus*, reduction of the number of nodules induced on *Tephrosia vogelli*, and the absence of any detectable effect on the interaction with *Vigna unguiculata* and *Leucaena leucocephala* [51]. In *B. japonicum* USDA110, abolition of Nop secretion diminishes the number of nodules formed with *Macroptilium atropurpureum* and results in delayed nodulation with the American soybean Williams [25]. In *S. fredii* USDA257 the *nolXWBTUV* locus regulates cultivar-specific nodulation on soybean. USDA257 mutants in this locus gain the ability to induce nitrogen-fixing nodules on agronomically improved American soybean cultivars that are not nodulated by the wild-type strain [36]. In addition, these *tts* mutations extend the host-range of nodulation to different species of *Erythrina* [27].

In this work, the symbiotic role of Nops was analyzed in *S. fredii* HH103, a strain that, although it secretes Nops in response to flavonoids [49], naturally nodulates both American and Asiatic soybeans. For this purpose, a 10264-bp segment of the *tts* cluster of *S. fredii* HH103 was characterized and the effect of inactivation of the *rhcJ* gene, which belongs to this cluster, on Nop secretion and on the symbiotic properties of *S. fredii* HH103 with several host plants was analyzed.

Materials and methods

Molecular and microbiological techniques. The bacterial strains and plasmids used in this work are described in Table 1. *Sinorhizobium* strains were grown at 28ºC on tryptone/yeast extract (TY) medium [6], yeast extract/mannitol (YM) medium [50], or B^- medium [45]. *Escherichia coli* was cultured on Luria-Bertani (LB) medium [42] at 37ºC. When required, the media were supplemented with the appropriate antibiotics as described by Lamrabet et al. [29]. Plasmids were transferred from *E. coli* to *Sinorhizobium* strains by conjugation, as described by Simon [44], using plasmid pRK2013 as helper. Plasmid profiles were visualized by agarose gel electrophoresis using a modified Eckhardt analysis as described by Espuny et al. [10]. Recombinant DNA techniques were carried out according to the general protocols of Sambrook et al. [42]. PCR reactions were done as described by Saiki [41]. Primers *nopA*f (5´-CCATGGCTGATGCCGACGAA-3´) and *nopA*r (5´-CTAGAGCCGTCGTACCAGTCA-3´) were used for amplification of a 518-bp fragment of *S. fredii* HH103 containing the complete *nopA* gene. Basic DNA sequence analysis and assembly was done with the UWGCG program [9]. Homology searches were carried out with the BLAST program [http://www.ncbi.nlm.nih.gov/BLAST/].

In order to generate an *rhcJ* mutant derivative of *S. fredii* HH103, a 3.6-kb *Hin*dIII–*Eco*RI fragment carrying the *rhcJ* gene was subcloned into pK18mob, a plasmid that is not replicable in rhizobial strains. A 2-kb *Sma*I fragment carrying the interposon Ω (Str^R, Spc^R) [39] was then subcloned into the unique *Eco*RV site of *rhcJ*. The resultant plasmid, pMUS750, was transferred by conjugation to strain SVQ269 (= HH103 Rif^R) and Rif^R Spc^R transconjugants were analyzed for sensitivity to kanamycin in order to identify double recombinants in which the wild-type *rhcJ* gene had been substituted by the mutated copy of the gene. Homogenotization of the *rhcJ*::Ω in several candidates was confirmed by hybridization, and strain SVQ518 (= SVQ269 *rhcJ*::Ω) was selected for further studies. Assays for β-galactosidase activity in liquid bacterial cultures on YM medium were done 16 h after induction, as described by Vinardell et al. [48]. At least three independent experiments performed in duplicate were carried out. Compounds tested as *nod* inducers were dissolved in ethanol and used at 1 μg/ml (approximately 3.7 μM for genistein and 3.9 μM for coumestrol). For time-course experiments, bacterial cultures at OD_{660} = 0.8–1.0 were diluted (10- to 100-fold) before the addition of flavonoids and cultivated, without shaking, to ensure that bacterial cultures had an OD_{660} in the range of 0.1–0.4 by the time that β-galactosidase activity was measured (0, 4, 8, 12, 16, 20, 24, 28, 32, 36, and 40 h after induction). Lipochitooligosaccharides (LCOs) from *S. fredii* strains grown on minimal B^- medium were analyzed by reverse-phase thin-layer chromatography (RP-TLC) as previously described [45]. Bacterial lipopolysaccharide crude extracts were obtained and analyzed by SDS-PAGE as described by Buendía-Clavería et al. [8].

Analysis of extracellular proteins. Extracellular proteins from *S. fredii* strains were recovered from 50 ml of YM bacterial cultures grown on an orbital shaker (180 rpm) for 40 h (approximately 10^9 bacteria/ml) as previously described [49]. Extracellular proteins were separated by SDS-PAGE using the discontinuous buffer system of Laemmli [30]. Electrophoresis was done in 1 mm-thick gels (15% acrylamide for the separation gel; 4% acrylamide for the stacking gel) in a Protean II xi Cell electrophoresis system (Bio-Rad, CA) at 25ºC and 150 V. Gels were silver-stained as previously described [46]. The 7-kDa extracellular protein secreted by *S. fredii* HH103 was transferred to

Table 1. Bacterial strains and plasmids

Strain or plasmid	Derivation and relevant properties	Reference
Sinorhizobium fredii		
HH103-1	HH103 Str^R	[7]
SVQ116	HH103-1 *nodA*::Tn*5*-*lacZ*	[8]
SVQ269	HH103 Rif^R	[32]
SVQ288	HH103-1 *rhcJ*::Tn*5*-*lacZ*	[49]
SVQ518	SVQ269 *rhcJ*:: Ω	This work
USDA257-1	USDA257 Str^R	This work
Escherichia coli		
DH5α	*SupE44*, Δ*lacU169*, *hsdR17*, *recA1*, *endA1*, *gyrA96* , *thi-1*, *relA1,* Nx^R	Stratagene
Plasmids		
pHP45Ω	Ap^R vector containing the Ω interposon (Spc^R Str^R)	[39]
pK18mob	Cloning vector, Km^R	[43]
pRK2013	Helper plasmid, Km^R	[11]
pMUS593	Cosmid pLAFR1 carrying genes of the *tts* cluster of *S. fredii* HH103	This work
pMUS750	pK18mob carrying a 5.6-kb *Hin*dIII–*Eco*RI fragment containing *rhcJ*::Ω	This work

PVDF membranes according to the method of Kennedy et al. [22]. The amino-terminal sequence of this protein was obtained from the Laboratorio Proteomics (Centro Nacional de Biotecnología, CSIC), which used a Precise Protein Sequencing System, model 494 (Applied Biosystems, CA).

Plant assays. Nodulation assays on *Glycine max* (L.) Merr. cvs. Williams and Peking, *Canavalia ensiformes* (L.) DC., *Crotolaria spectabilis* Roth., *Erythrina variegata* (L.), *Macroptilium atropurpureum* (Moc.& Sessé ex DC) Urb, *Macrotyloma axillare* (E. Mey) Verdc, and *Sesbania marginata* (L.) Merr were done as previously described [7]. Each Leonard jar contained two plants (one plant in the case of *E. variegata*). Each plant was inoculated with about 2×10^8 bacteria. Plants were grown for 42 days (90 days in the case of *E. variegata*) with a 16-h photoperiod at 25ºC in the light and 18ºC in the dark. Plant tops and nodules were dried at 80ºC for 48 h and weighed. Bacteria were isolated from surface-sterilized nodules as previously reported [7]. For competition studies on *Glycine max*, each plant was inoculated with approximately 10^8 bacteria of each co-inoculant, strains HH103 Str^R and SVQ288 (Str^R Km^R). Nodule occupancy by strain SVQ288 was determined in 100 nodules by assessing the presence of the Tn*5*-*lacZ* transposon (presence of the Km^R marker). For nodulation-blocking studies on *G. max* cv. Williams, each plant was inoculated with about 10^8 bacteria of the blocking strain (USDA257 Str^R) and 10^7 bacteria of strains HH103 Rif^R or SVQ518. Nodule occupancy was determined in at least 50 nodules by assessing the antibiotic resistance marker of each inoculant. The data presented represent the average of six jars containing two soybean plants each. Determinations were made 5 weeks after inoculation. The Mann-Whitney non-parametric test was employed for statistical analyses.

Sequence data. Nucleotide and/or amino acid sequence data are to be found at the EMBL database as accession numbers AY683479 and AY775562.

Results

Isolation of genes belonging to the *tts* cluster of *S. fredii* HH103. Strain SVQ288 belongs to a collection of Tn*5*-*lacZ* insertional mutants of *S. fredii* HH103 Str^R [29,32] that shows increased β-galactosidase activity in the presence of naringenin. The SVQ288 mutant carries a single insertion of the Tn*5*-*lacZ* transposon, which is located into the *rhcJ* gene (formerly *nolT*) [49]. This gene belongs to the *tts* cluster of *Rhizobium* sp. NGR234 as defined by Viprey et al. [51]. The pMUS593 cosmid was isolated from a *S. fredii* HH103 genomic library by using a DNA fragment containing part of the *rhcJ* gene as a hybridization probe.

A 10,264-bp segment of pMUS593 was sequenced (accession number AY683479). It comprise two partial and eleven complete ORFs showing homology to genes of the *tts* cluster of *S. fredii* USDA257 and *Rhizobium* sp. NGR234: *nopX* (from position 485 to 2275), *rhcC1* (formerly *nolW*, from 2557 to 3261), *nopB* (from 3417 to 3911), *rhcJ* (formerly *nolT*, from 3921 to 4790), *nolU* (from 4787 to 5425), *nolV* (from 5427 to 6053), *rhcN* (from 6050 to 7405), y4yJ (from 7381 to 7917), *rhcQ* (from 7871 to 9019), *rhcR* (from 9012 to 9680), *rhcS* (from 9677 to 9955), and the 5′ termini of y4yB (from 300 to 1) and *rhcT* (from 9964 to 10,264). Matching of the 1,0264-bp nucleotide sequence with the partial sequence obtained from mutant SVQ288 revealed that the Tn*5*-*lacZ* transposon was inserted 274 bp downstream of the putative translational start codon of *rhcJ* (Fig. 1).

The 10,264-bp sequenced fragment of *S. fredii* HH103 was 99 and 98% identical to the *tts* clusters of *S. fredii* USDA257 (accession number AF229441) and *Rhizobium* sp. NGR234 (accession numbers AE000106 and AE000107), respectively, and had the same genetic organization as the corresponding regions of these rhizobia. This high level of nucleotide sequence conservation was also present in the intergenic regions. The deduced protein sequences of strain HH103 were 94–100% identical to those of strains NGR234

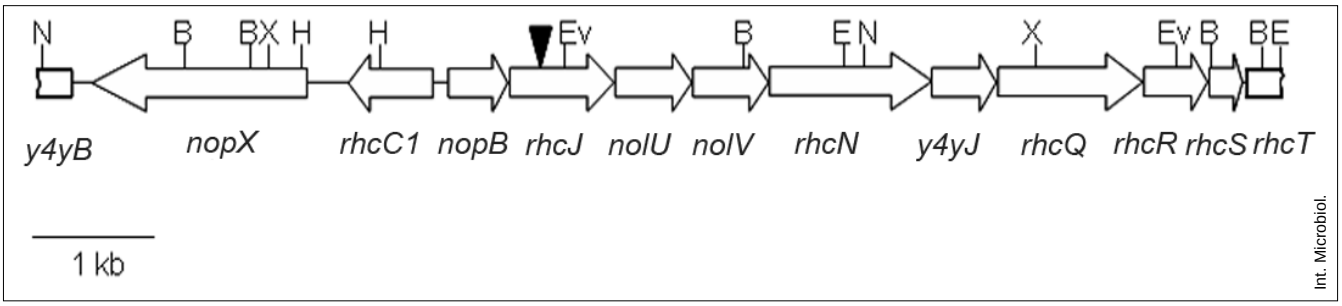

Fig. 1. Physical and genetic map of part of the *tts* cluster of *Sinorhizobium fredii* HH103. The black triangle indicates the site of the insertion of Tn*5*-*lacZ* in mutant SVQ288. N, *Nru*I; B, *Bam*HI; X, *Xho*I; H, *Hin*dIII; Ev, *Eco*RV; E, *Eco*RI.

and USDA257. The percentages of identity with the corresponding proteins of *M. loti* MAFF303099 and *B. japonicum* USDA110 were lower: 63–95% and 41–83%, respectively.

Characterization of mutant SVQ288. Mutant strain SVQ288 showed LCOs, lipopolysaccharide (LPS), and plasmid profiles indistinguishable from those of the parental strain HH103 Str^R, as demonstrated by RP-TLC, SDS-PAGE electrophoresis, and Eckhardt analysis, respectively (data not shown).

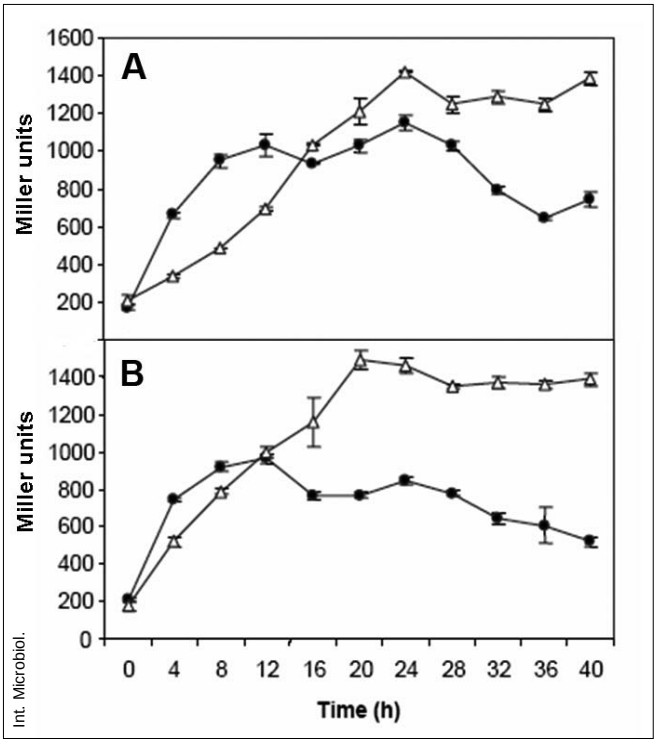

Fig. 2. Time-course β-galactosidase activity of strains SVQ116 (= HH103 *nodA*::*lacZ*), and SVQ288 (= HH103 *rhcJ*::*lacZ*) in the presence of (**A**) 3.7 μM genistein and (**B**) 3.9 μM coumestrol. SVQ288 (open triangles); SVQ116 (closed circles).

As indicated above, the level of *lacZ* expression of strain SVQ288 was increased in the presence of naringenin, indicating that transcription of the *S. fredii* HH103 *rhcJ* gene was activated by the presence of this flavonoid. The expression of this gene in the presence of genistein and coumestrol, two flavonoids that are good inducers of the *S. fredii nod* genes, was also studied. Strain SVQ116, a *nodA*::*lacZ* derivative of *S. fredii* HH103, was used as a control. The β-galactosidase activities of the *nodA*::*lacZ* and *rhcJ*::*lacZ* HH103 derivatives in the absence of flavonoids were 149.0 ± 5.6 and 187.7 ± 61.4, respectively. In the presence of genistein, the two genes were similarly induced (713.3 ± 30.6 and 855 ± 158.5). However, in the presence of coumestrol, expression of the *rhcJ* gene (1183.3 ± 185.5) was clearly stronger than that of the *nodA* gene (581.3 ± 82.0).

In previous studies [12,51], transcription analysis of the *tts* cluster of strain NGR234 showed that these genes are expressed later than *nod* genes involved in the synthesis and modification of Nod factors. Hence, the kinetics of *rhcJ* and *nodA* gene expression up to 40 h after induction with genistein or coumestrol were analyzed. As shown in Fig. 2, expression of both genes was observed along the entire time-course of the experiment. After induction with flavonoids, *nodA* reached its maximum level of expression before the *rhcJ* gene (at 12 h vs. 20–24 h). Upon induction with flavonoids, the maximum level of *nodA* expression was maintained between the 12- and 24-h time-points and then declined. The level of *rhcJ* gene expression increased faster with coumestrol than with genistein; sustained maximum values were reached at 20 and 24 h, respectively.

Nop profiles of strain SVQ288. In previous work, *S. fredii* HH103 was shown to secrete at least five proteins in response to flavonoids. The molecular masses of three of these proteins were 60, 40, and 20 kDa, and two of them were less than 6.5 kDa [49]. New SDS-PAGE experiments (Fig. 3A) revealed the presence of an additional protein of about 35 kDa

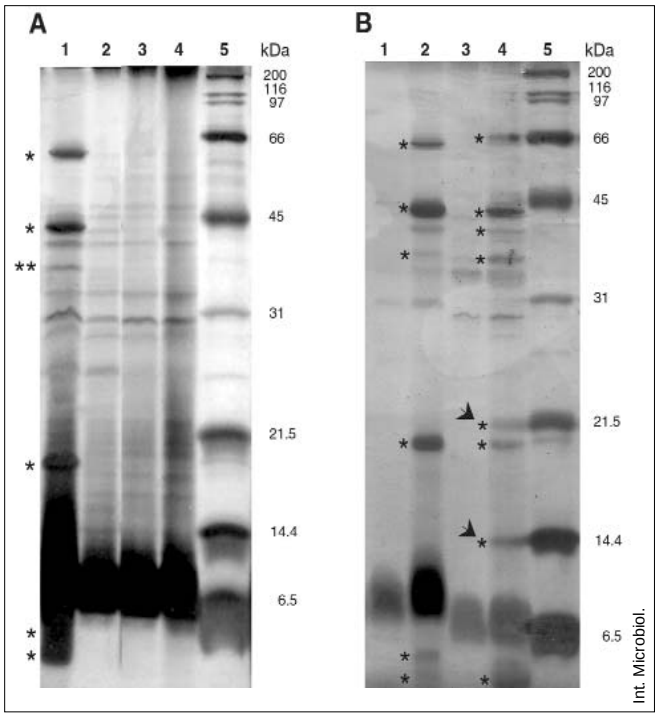

Fig. 3. Extracellular proteins profiles of *S. fredii* strains. (**A**) Lanes 1 and 2, HH103 Str^R; lanes 3 and 4, SVQ288; lane 5, molecular mass marker (SDS-PAGE standard broad-range from Bio-Rad, CA). Lanes 1 and 3: extracellular proteins from cultures grown in the presence of genistein 3.7 μM. Molecular masses of the marker are indicated on the left. Nops of *S. fredii* HH103 are indicated with an asterisk, except the 35-kDa protein, which is marked with a double asterisk. (**B**) Lanes 1 and 2, HH103 Str^R; lanes 3 and 4, USDA257. Lane 5: molecular mass marker (see **A**). Lanes 2 and 4: extracellular proteins from cultures grown in the presence of genistein 3.7 μM. Nops secreted by each *S. fredii* strain are indicated with an asterisk. Nops present in induced cultures of strain USDA257 but absent in those of strain HH103 are marked with an arrow.

in the extracellular medium of genistein-induced cultures of strain HH103 (marked with a double asterisk in the figure).

The involvement of the *S. fredii* HH103 *rhcJ* gene in Nop secretion was also investigated. SDS-PAGE experiments showed that the *rhcJ* mutant lacked the six proteins secreted by the wild-type strain upon flavonoid induction (Fig. 3A, lanes 3 and 4). In order to verify that the lack of Nop secretion observed for strain SVQ288 was caused by inactivation of the *rhcJ* gene, a HH103 *rhcJ*::Ω mutant, SVQ518, was constructed (see Materials and methods). The Nops profile of strain SVQ518 was similar to that of strain SVQ288 (data not shown).

The smallest of the proteins secreted by *S. fredii* HH103 was isolated and its amino terminus was sequenced. The amino acid sequence, KIGTVTSAVGAGAAAGQNVAAKG, was 95% identical to that of the putative NopA protein of *Rhizobium* sp. NGR234 [35]. The complete *nopA* gene of strain HH103 was PCR-amplified using primers designed from strain NGR234 and then sequenced (accession number AY775562). As in strain NGR234, the *nopA* gene of strain HH103 is located 322 nucleotides upstream of y4yQ and it codes for a protein of 71 amino acids. The deduced NopA protein of strain HH103 is 98% identical to that of strain NGR234 (YP 052973). NopA homologues are also present in *S. fredii* USDA257, *M. loti* MAF303099, and *B. japonicum* USDA110, with identities of 100, 64, and 44%, respectively, with respect to the *S. fredii* HH103 NopA protein.

Symbiotic phenotype of mutant SVQ288. Like the parental strain HH103 Str^R, mutant strain SVQ288 induced nitrogen-fixing nodules on *Erythrina variegata*, *Glycine max* cvs. Williams and Peking, *Macrotyloma axillare*, and *Macroptilium atropurpureum*, but was not able to nodulate *Crotolaria spectabilis*, *Canavalia ensiformis*, and *Sesbania marginata*. However, the symbiotic capacity of mutant strain SVQ288 with *G. max* and *E. variegata* was clearly different from that of its parental strain HH103 Str^R.

In both Williams and Peking soybeans (Table 2), the number of nodules formed following inoculation with strain SVQ288 was significantly lower (α = 5%) than in plants inoculated with the parental strain HH103 Str^R. In Williams soybean, the plant-top dry weight of plants inoculated with strain SVQ288 was also significantly lower than that of plants inoculated with strain HH103 Str^R. In competition experiments carried out in Williams soybean, mutant SVQ288 was out-competed by parental strain HH103 Str^R, as the mutant strain occupied only the 4% of the nodules formed. Similar results were observed in Peking soybean, in which strain SVQ288 was out-competed by strain HH103 Str^R (strain SVQ288 occupied 16% of the nodules) when the mutant strain was coinoculated with the parental strain at a 1:1 ratio.

Plant tests with *E. variegata* showed different results (Table 2). In this plant, the nodulation capacity and nitrogen-fixation efficiency of strain HH103 were low. In fact, no differences in plant-top dry weight were detected between non-inoculated plants and plants inoculated with strain HH103 Str^R or strain HH103 Rif^R. The number and fresh-weight of the nodules as well as the plant-top dry-weight were significantly higher (α = 5%) in plants inoculated with strain SVQ288 than in those inoculated with strain HH103 Str^R. Mutant SVQ518 (HH103 Rif^R *rhcJ*::Ω) also showed improved symbiotic capacities with *E. variegata* compared to its parental strain HH103 Rif^R.

***S. fredii* HH103 and USDA257 may secrete a different set of Nops**. *S. fredii* USDA257 does not nodulate American soybeans, but these soybean varieties are

Table 2. Plant responses to inoculation of *Glycine max* cvs. Williams and Peking, and *Erythrina variegata* with *Sinorhizobium fredii* strains HH103, SVQ288, and SVQ518[a]

Legume tested, inoculant[b]	Plant-top dry-weight (g)	Number of nodules	Nodule weight (g)[d]
Glycine max cv. Williams[c]			
None	0.46 ± 0.07	0	0
HH103 Str^R	9.83 ± 1.72	297.0 ± 75.9	0.87 ± 0.12
SVQ288	7.41 ± 1.51*	178.3 ± 45.4*	0.67 ± 0.16
Glycine max cv. Peking[c]			
None	0.78 ± 0.25	0	0
HH103 Str^R	2.79 ± 0.75	134.4 ± 27.4	0.23 ± 0.70
SVQ288	1.89 ± 0.41	77.4 ± 13.0*	0.17 ± 0.38
Erythrina variegata[c]			
None	1.20 ± 0.33	0	0
HH103 Str^R	1.37 ± 0.44	47.0 ± 11.8	1.72 ± 0.77
SVQ288	6.14 ± 1.17*	174.3 ± 99.4*	6.57 ± 0.71*
HH103 Rif^R	1.31 ± 0.64	12.5 ± 14.1	0.74 ± 0.84
SVQ518	5.12 ± 1.25*	127.2 ± 51.1*	5.77 ± 0.99*

[a]Data represent averages of 6 jars for soybean Williams and 5 jars for soybean Peking. Each jar contained two soybean plants. Determinations were made 6 and 5 weeks after inoculation for soybean Williams and soybean Peking, respectively. For *Erythrina variegata* plants, data represent averages of 4 plants. Determinations were made 90 days after inoculation.

[b]Bacteria isolated from 20 nodules formed by each inoculant showed the expected resistance markers.

[c]Each mutant was individually compared to its parental strain (HH103 Str^R or HH103 Rif^R) by using the Mann-Whitney non-parametrical test. Numbers on the same column followed by an asterisk are significantly different at the level α =5%.

[d]For soybean and *Erythrina variegata* plants, numbers represent nodule dry-weight and nodule fresh-weight, respectively.

nodulated by USDA257 mutants affected in different genes that belong to the *tts* cluster. However, the capacity of these mutants to nodulate American soybeans is strongly reduced when they are coinoculated with the parental strain. These results are consistent with the fact that Nop secretion is responsible for the inability of strain USDA257 to nodulate American soybeans [2].

In this context, it is paradoxical that strain HH103, a *S. fredii* strain that secretes Nops, nodulates American soybeans. Abolition of Nop secretion by this strain impaired nodulation capacity with Williams soybean. One possible explanation for this finding is that the set of Nops secreted by strains USDA257 and HH103 differed, and that only the Nops secreted by strain USDA257 were detrimental for nodulation with American soybeans. Nops secreted by each of the *S. fredii* strains were compared in SDS-PAGE experiments (Fig. 3B). Although the Nops profiles of strains USDA257 and HH103 showed several common proteins, some of the Nops secreted by strain USDA257, such as a 21.5- and a 14.4-kDa protein (marked with arrows in Fig. 3B), were apparently absent in induced cultures of strain HH103. If strains USDA257 and HH103 indeed secreted different sets of Nops, then the presence of strain USDA257 would decrease the nodulation ability of strain HH103 with American soybeans. In order to test this hypothesis, experiments were carried out in which strain USDA257 was coinoculated with either strain HH103 or its *rhcJ* derivative strain SVQ518 on Williams soybean.

The presence of strain USDA257 Str^R reduced significantly (α = 5%) the number of nodules induced by strains HH103 Rif^R (from 147.3 to 25.3) and SVQ518 (from 73.4 to 16.0) on Williams soybean compared to the number of nodules formed when strain HH103 or strain SVQ518 was present as a single inoculant. Strain USDA257 Str^R was not present in any nodule. Significant reductions of the nodule fresh-weight (from 2.09 to 0.37 g for strain HH103 Rif^R; from 1.35 to 0.39 g for strain SVQ518) and of the plant-top dry-weight (from 3.55 to 1.06 g for strain HH103 Rif^R; from 2.39 to 1.09 g for strain SVQ518) were also observed in these coinoculation experiments.

Discussion

The symbiotic role of Nops has been mainly investigated in *Rhizobium* sp. NGR234, a broad-host-range rhizobial strain that does not nodulate soybean, and *S. fredii* USDA257, a strain that nodulates Asiatic but not American varieties of soybean. Nop secretion has been postulated to determine symbiotic incompatibility between *S. fredii* USDA257 and many American soybean cultivars, as mutants in the *nolXWBTUV* locus fail to secrete the respective proteins and concomitantly gain the ability to nodulate these soybean varieties [28,36].

This work analyzed the symbiotic role of Nops in *Sinorhizobium fredii* HH103, a strain that, paradoxically, produces Nops [49] and is able to induce the formation of nitrogen-fixing nodules in both American and Asiatic soybeans. The results showed that Nop secretion by strain HH103 is not detrimental, but rather beneficial for nodulation with the agronomically improved American soybean cultivars.

Isolation and sequencing of part of the *tts* cluster of *S. fredii* HH103, including the complete *nolXWBTUV* locus, showed that the sequenced genes are highly homologous to those of *S. fredii* USDA257 and *Rhizobium* sp. NGR234. Indeed, the putative proteins encoded by these genes had identities in the range of 94–100%.

As is the case for the transcription of *nod* genes, responsible of the biosynthesis and secretion of Nod factors, Nop secretion is also a process dependent on NodD and flavonoids [28,51]. As expected, both genistein and coumestrol, two flavonoids that are present in soybean exudates [40], activated expression of the *S. fredii rhcJ* gene. A previous report showed that, in *S. fredii* USDA257, the responsiveness of *nopX* to coumestrol was higher than that of *nodC* [40]. Similarly, in the present work, the inducing effect of coumestrol was stronger for the *S. fredii* HH103 *rhcJ* gene than for the *nodA* gene. Taken together, these results suggest that there are slight differences in the fine-tuning of the regulation of Nop and Nod factor secretion in *S. fredii*.

Time-course experiments (Fig. 2) showed that, although the *S. fredii nodA* gene is transcriptionally activated in the presence of flavonoids prior to the *rhcJ* gene, the level of induction of *rhcJ* became higher than that of *nodA* 16 h after induction. Moreover, *nodA* expression declined 24 h after induction whereas expression of *rhcJ* was maintained at its maximum level during the entire period examined. These results are in agreement with previous observations showing that the expression of *nod* genes in *Rhizobium* sp. NGR234 is turned on earlier than the expression of genes involved in Nop secretion [12,24].

Inactivation of *S. fredii* HH103 *rhcJ* had a strong impact on both flavonoid-dependent protein secretion and bacterial symbiotic capacity with soybeans and *Erythrina variegata*. As described for *S. fredii* USDA257 [28], mutation of *rhcJ* resulted in the absence of Nops in the extracellular medium. This result is in agreement with a putative role for RhcJ as a component of the TTSS apparatus [51]. As previously reported for mutants of strain USDA257 with impaired Nop secretion, and for a *nopX* derivative of strain HH103 [5,27], the *rhcJ* derivative of strain HH103 showed improved symbiotic behaviour on *E. variegata*. However, in contrast to strain USDA257, *rhcJ* inactivation in strain HH103 had a clear detrimental effect on bacterial symbiotic capacity with American soybeans, since the final number of nodules produced by the HH103 *rhcJ* mutant and its competitive capacity to nodulate were clearly reduced. Recent studies of *B. japonicum* USDA110 also showed a negative effect following abolition of Nop secretion, as it resulted in delayed nodulation of this bacterium with the American soybean Williams [25]. In this case, however, the total number of nodules formed by USDA110 mutants impaired in Nop secretion was not significantly different from that induced by the wild-type strain.

SDS-PAGE experiments (Fig. 3B) suggested that *S. fredii* strains USDA257 and HH103 secrete different sets of Nops. This is consistent with the fact that the presence of strain USDA257 led to a dramatic reduction in the number of nodules induced by strains HH103 or its *rhcJ* derivative SVQ518 on soybean Williams. Several reports have shown that the families of Nod factors produced by these two strains are essentially equivalent [4,15]. We cannot exclude the possibility that differences in surface polysaccharides (such as in KPS, a strain-specific capsular polysaccharide, or LPS) are also involved in the blocking capacity of strain USDA257 against strain HH103 with Williams soybean. In fact, mutations in the *nolWXBTUV* genes of strain USDA257 not only enabled this strain to nodulate American soybean cultivars but also significantly altered bacterial LPS and K-antigens [21].

Clear differences between the sequence of the *tts* genes of strains HH103 and that of USDA257 were not detected, either in their coding sequences or in their intergenic regions. Comparisons among the sequences of three genes (*nopX*, *nopB*, and *nopA*) of strain HH103 that code for proteins secreted by the TTSS and their respective orthologues in strain USDA257 showed that they were 99, 100, and 100% identical. Nevertheless, an exhaustive characterization of the Nops secreted by the two strains is required to definitively elucidate whether their different symbiotic behaviors on American soybeans are exclusively due to differences in the respective sets of Nops.

Most *S. fredii* strains (including strains HH103 and USDA257) were isolated from nodules of soybean plants growing in soils (or in the presence of soil samples) of the People's Republic of China, which is to be expected given that soybean is native to this country. With the exception of strain USDA191, the initially characterized *S. fredii* strains failed to establish effective symbiosis with American soybeans [23]. This finding led to the notion that most *S. fredii* strains are effective within only a narrow range of soybean genotypes from Asia. Recently, we had the opportunity of isolating hundreds of new *S. fredii* strains from different Chinese soybean-cropping areas. Two hundred of these strains have now been characterized [47] and an extensive description of

them is available at [www.soybeanrhizobia.net]. Most of these new *S. fredii* strains showed the capacity to nodulate with both American and Asiatic soybeans, indicating that strain HH103 is genuinely representative of the symbiotic properties of large *S. fredii* natural populations. Thus, strains HH103 and USDA257 constitute two possible models of symbiotic interactions, compatible and incompatible, between *S. fredii* strains and American soybean cultivars.

Acknowledgements. This work was supported by CICYT grants BOS2002-04164-C03-02 and AGL2002-04188-C06-04.

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La inactivación del gen *rhcJ* de *Sinorhizobium fredii* HH103 elimina la secreción de las proteínas externas de nodulación (Nops) y disminuye la capacidad de simbiosis con la soja

Resumen. Se ha propuesto que las proteínas externas de nodulación (Nops) impiden la nodulación efectiva de *Sinorhizobium fredii* USDA257 con las sojas americanas. *S. fredii* HH103 nodula de forma natural tanto con las sojas asiáticas (no comercializadas) como con las americanas (comercializadas). La inactivación del gen *rhcJ* de HH103, que pertenece a la agrupación génica *tts* (secreción de tipo III), anuló la secreción de Nops y redujo la capacidad simbiótica de esta bacteria con las dos variedades de soja. Las cepas HH103 y USDA257 de *S. fredii*, que sólo nodula sojas asiáticas, mostraron perfiles SDS-PAGE diferentes de Nop, lo cual sugiere que estas cepas podrían secretar distintos conjuntos de Nops. Cuando las cepas USDA257 y HH103 fueron inoculadas conjuntamente, la capacidad de nodulación de esta última cepa con el cultivar americano Williams de soja se redujo significativamente. Estos resultados indican que las Nops secretadas por *S. fredii* pueden actuar como factores simbióticos tanto positivos como negativos dependiendo de la cepa-cultivar rizobiana. Se detectaron también diferencias entre la expresión mediada por flavonoides del gen *rhcJ* y del *nodA*. Además, una de las Nops secretadas por la cepa HH103 fue identificada como NopA. [**Int Microbiol** 2006; 9(2):125-133]

Palabras clave: *Sinorhizobium fredii* HH103 · gen *rhcJ* · sistema de secreción de tipo III · proteínas externas de nodulación (Nops) · soja

A inactivação do gene *rhcJ* de *Sinorhizobium fredii* HH103 elimina a secreção das proteínas externas de nodulação (Nops) e diminui a capacidade de simbiose com a soja

Resumo. Postulou-se que as proteínas externas de nodulação (Nops) impedem a nodulação de *Sinorhizobium fredii* USDA257 com as sojas americanas. *S. fredii* HH103 nodula de forma natural tanto com as sojas asiáticas (não comercializadas) como com as americanas (comercializadas). A inactivação do gene *rhcJ* de HH103, que pertence ao agrupamento genético *tts* (secreção tipo III), anulou a secreção de Nops e reduziu a capacidade simbiótica desta bactéria com as duas variedades de soja. As cepas HH103 e USDA257 de *S. fredii*, que somente nodulam com sojas asiáticas, mostraram perfis SDS-PAGE diferentes de Nop, o que sugere que estas cepas poderiam secretar diferentes conjuntos de Nops. Quando as cepas USDA257 e HH103 foram inoculadas conjuntamente, a capacidade de nodulação desta última cepa com a variedade americana Williams reduziu-se significativamente. Estes resultados indicam que as Nops secretadas por *S. fredii* podem actuar como factores simbióticos tanto positivos como negativos, dependendo da cepa-cultivar rizobiana. Encontraram-se diferenças entre a expressão induzida por flavonoides dos genes *rhcJ* e *nodA*. Além disso, uma das Nops secretadas pela cepa HH103 foi identificada como NopA. [**Int Microbiol** 2006; 9(2):125-133]

Palavras chave: *Sinorhizobium fredii* HH103 · gene *rhcJ* · sistema de secreção de tipo III · proteínas externas de nodulação (Nops) · soja