***Sinorhizobium fredii* HH103 Has a Truncated *nolO* Gene Due to a –1 Frameshift Mutation That Is Conserved Among Other Geographically Distant *S. fredii* Strains**

Nuria Madinabeitia,¹ Ramón A. Bellogín,¹ Ana M. Buendía-Clavería,¹ María Camacho,² Teresa Cubo,¹ M. Rosario Espuny,¹ Antonio M. Gil-Serrano,³ María C. C. P. Lyra,¹ Ahmed Moussaid,¹ F. Javier Ollero,¹ M. Eugenia Soria-Díaz,³ José M. Vinardell,¹ Jing Zeng,⁴ and José E. Ruiz-Sainz¹

¹Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, Apdo. 1095, 41080-Sevilla, Spain;

²Las Torres-Tomejil, Apdo. 41200, Alcalá del Río, Sevilla, Spain; ³Departamento de Química Orgánica, Facultad de Química, Universidad de Sevilla, Apdo. 553, 41071-Sevilla, Spain; ⁴Department of Microbiology, College of Biology, China Agricultural University, 2 Yuan Ming Yuan West Road, 100094 Beijing, China

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Strain SVQ121 is a mutant derivative of *Sinorhizobium fredii* HH103 carrying a transposon Tn5-*lacZ* insertion into the *nolO*-coding region. Sequence analysis of the wild-type gene revealed that it is homologous to that of *Rhizobium* sp. NGR234, which is involved in the 3 (or 4)-*O*-carbamoylation of the nonreducing terminus of Nod factors. Downstream of *nolO*, as in *Rhizobium* sp. NGR234, the *noeI* gene responsible for methylation of the fucose moiety of Nod factors was found. SVQ121 Nod factors showed lower levels of methylation into the fucosyl residue than those of HH103, suggesting a polar effect of the transposon insertion into *nolO* over the *noeI* gene. A *noeI* HH103 mutant was constructed. This mutant, SVQ503, produced Nod factors devoid of methyl groups, confirming that the *S. fredii* *noeI* gene is functional. Neither the *nolO* nor the *noeI* mutation affected the ability of HH103 to nodulate several host plants, but both mutations reduced competitiveness to nodulate soybean. The Nod factors produced by strain HH103, like those of other *S. fredii* isolates, lack carbamoyl residues. By using specific polymerase chain reaction primers, we sequenced the *nolO* gene of *S. fredii* strains USDA192, USDA193, USDA257, and 042B(s). All the analyzed strains showed the same –1 frameshift mutation that is present in the HH103 *nolO*-coding region. From these results, it is concluded that, regardless of their geographical origin, *S. fredii* strains carry the *nolO*-coding region but that it is truncated by the same base-pair deletion.

Additional keywords: lipochitooligosaccharides, *nolN*.

Soil bacteria from genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Rhizobium* induce the formation of nodules on the roots or stems of their leguminous host plants. In the nodules, these bacteria are able to fix atmospheric nitrogen. The production of an effective, nitrogen-fixing nodule is a complex biological event that requires genetic de-

terminants from both the plant and the bacterium (Freiberg et al. 1997; Pueppke 1996; Spaink 1996, 2000). The bacterial genes (*nod*, *noe*, and *nol*) involved in the formation of the nodules are called, generically, *nod* genes. Transcription of *nod* genes requires both flavonoids, compounds that are secreted by the roots of the legumes, and the bacterial protein NodD, a member of the LysR family of transcriptional regulators (Henikoff et al. 1988). As a result of *nod* gene activation, the bacteria synthesize signal molecules, known as Nod-factors or lipochitooligosaccharides (LCOs). LCOs are N-acylated oligomers of *N*-acetyl-D-glucosamine that play a key role in the symbiotic recognition, acting as morphogens that initiate the nodulation program of the host plant and allow bacterial entry (Dénarié et al. 1996; Lerouge et al. 1990; Spaink 1996, 2000). Principal differences among LCOs of the various rhizobia concern the length of the *N*-acetyl-D-glucosamine backbone, as well as substitutions to both the reducing and nonreducing residues. Host plants respond only to Nod factors with a defined composition. Most rhizobial strains do not produce one single LCO, and it might be that diverse Nod factors act cooperatively to induce a full plant nodulation response (Minami et al. 1996).

The *nod* genes participate in different ways in Nod factor biosynthesis. Whereas the *nodABC* genes are required for the biosynthesis of the oligosaccharide backbone (Atkinson et al. 1994; Röhring et al. 1994; Spaink et al. 1994) and the *nodIJ* genes are involved in secretion of LCOs (Spaink et al. 1995), the so-called host-specific *nod* (*hsn*) genes are necessary for the specific substitutions carried on the LCOs of each rhizobial strain and, consequently, for the interaction with certain legumes (Hanin et al. 1999; Spaink 2000; van Rhijn and Vanderleyden 1995). The *hsn* genes are not functionally or structurally conserved among rhizobia. A number of these genes have been characterized. For example, the *nodS* gene is involved in the production of N-methylated Nod factors in different *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* species (Göttfert et al. 1990; Jabbouri et al. 1995; Lewin et al. 1990). NodU is the protein responsible for the 6-*O*-carbamoylation of the nonreducing terminus of Nod factors of different rhizobial species (D'Haeze et al. 1999; Jabbouri et al. 1995; Lewin et al. 1990). On the other hand, *NolO* is involved in 3 (or 4)-*O*-carbamoylation of the nonreducing terminus in the *Rhizobium* sp. NGR234 LCOs (Jabbouri et al. 1998). The *noeL*, *nolK*, and

Corresponding author: José E. Ruiz-Sainz; Telephone: +34-954557121; Fax: +34-954557830; E-mail: rsainz@us.es.

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nodZ genes are involved in the C-6 fucosylation of the Nod factors of *B. japonicum*, *Rhizobium* sp. NGR234, *A. caulinodans*, and *S. fredii* (Lamrabet et al. 1999; López-Lara et al. 1996; Mergaert et al. 1996; Quesada-Vincens et al. 1997; Stacey et al. 1994). The fucose group of Nod-factors of *Rhizobium* sp. NGR234, *B. japonicum* USDA110, and *S. fredii* USDA257 is mostly 2-O-methylated by the product of the *noel* gene (Perret et al. 2000).

S. fredii includes fast-growing strains that nodulate soybean (*Glycine max* L. Merr.) in a cultivar-specific manner and can enter into symbiosis with many other legume species (Buendía-Clavería et al. 1989; Keyser et al. 1982; Pueppke and Broughton, 1999). One of the most interesting features of *S. fredii* is its exceptionally broad host range, probably due in part to the fact that this bacterium produces at least 16 different Nod factors. None of these LCOs have sulfate or carbamoyl residues (Bec-Ferté et al. 1994; Gil-Serrano et al. 1997).

We have isolated and identified the *nolO* region and the *noel* gene of *S. fredii* HH103. Mutation of the *noel* gene totally abolished the 2-O-methylation of the fucosyl residue of *S. fredii* LCOs, whereas a Tn5-*lacZ* insertion into the *nolO* region reduced the production of Nod factors bearing a methyl substitution on the fucosyl residue. The *nolO* region is present in *S. fredii* strains, although these bacteria produce LCOs that are devoid of carbamoyl residues. We show in this work that a -1 frameshift mutation (caused by 1-bp deletion) in the *nolO* coding region splits the *nolO* gene into two computer-predicted open reading frames (ORFs) that are probably nonfunctional. This frameshift mutation is conserved in five different strains isolated from distant areas of China.

RESULTS

Isolation of Tn5-*lacZ* mutants with flavonoid-inducible β -galactosidase activity.

Random Tn5-*lacZ* mutagenesis of *S. fredii* HH103-1, a streptomycin-resistant derivative of strain HH103, was carried out in our laboratory (Lamrabet et al. 1999). About 2,800 Km' transconjugants were patched onto Bergensen's medium and assayed individually for elevated β -galactosidase activity in

the presence of the flavonoid naringenin, an effective *nod* gene inducer of HH103 (Vinardell et al. 1993). Different presumptive HH103-1 mutants expressed higher β -galactosidase activity under these conditions, suggesting that they harbored Tn5-*lacZ* insertions into nodulation genes. One of these clones, named SVQ121, was selected for further analysis. Expression of β -galactosidase activity by mutant SVQ121 was elevated fourfold in the presence of 3.7 μ M naringenin. This mutant strain harbors only one copy of the Tn5-*lacZ* transposon, as indicated by hybridization experiments using an internal fragment of Tn5-*lacZ* as a probe (data not shown).

Genetic and sequence analyses of the mutated region of strain SVQ121.

Plasmid pMUS508 was obtained using the strategy described below. This plasmid contains the rhizobial DNA that is adjacent to the IS50R of Tn5-*lacZ* in strain SVQ121. Plasmid pMUS508 was partially sequenced from the IS50R terminus of Tn5-*lacZ*, and the exact position of the transposon insertion in SVQ121 was determined. The rhizobial DNA sequenced (approximately 300 bp) showed a high degree of homology to the *nolO* gene of *Rhizobium* sp. NGR234. An *Xho*I 750-bp rhizobial DNA fragment from pMUS508 (containing the 300 bp sequenced) was used as a probe to identify and isolate cosmid pMUS629 from a *S. fredii* HH103 genomic library. A 4.1-kb *Sac*I-*Sma*I fragment (Fig. 1) from pMUS629 showing positive hybridization to this probe was identified and 3,183 bp were sequenced (accession number AF228683).

One partial and three complete adjacent ORFs were localized in the sequenced fragment (Fig. 1). All putative ORFs showed the same polarity and a high probability of encoding proteins, as indicated by the Testcode algorithm. The ORF partially sequenced was homologous to the *nodJ* 3' termini of *Rhizobium* sp. NGR234 (95.5% identity) and *B. japonicum* USDA110 (63% identity). The following two ORFs were homologous (98.1% identity) to the 5' terminus or to the central part and 3' terminus of the *nolO* gene of NGR234, and they were designated *nolO'* and ORF2, respectively. *nolO'* and ORF2 were also homologous to the *nolN* (73.4% identity) and *nolO* (71.1% identity) genes of USDA110, respectively (Fig. 1).

Fig. 1. Organization of the *nolO* region in different rhizobia strains and restriction map of this region in *Sinorhizobium fredii* HH103. The position of the Tn5-*lacZ* insertion into SVQ121 is shown by a black triangle; the arrow indicates the sense of transcription of the *lacZ* gene. The sequenced fragment, the fragment used as a probe, and the fragment used for complementation studies are indicated. The *nolO* gene and homologous open reading frames are shown as dashed arrows. Other genes are represented as solid arrows, except the 3' end of *nodJ*, which is shown as a broken dotted arrow. The positions of primers *pnolO* (r) and *pnolO* (f) are indicated by arrows. Restriction sites shown are Ap, *Apa*I; C, *Cla*I; E, *Eco*RI; P, *Pst*I; S, *Sac*I; Sm, *Sma*I; and X, *Xho*I.

The last ORF of the HH103 sequenced fragment showed high similarity (99.4% identity) to the NGR234 *noI* gene.

The *noI*O' gene of *S. fredii* HH103 begins at position 232 of the sequenced fragment and extends to the right for 392 bp. It encodes a putative polypeptide of 130 amino acids, whereas the complete *Rhizobium* sp. NGR234 NoI protein is 680 amino acids long. Matching of the nucleotide sequence with that obtained from plasmid pMUS508 revealed that Tn5-*lacZ* is inserted 4 bp after the putative translational start codon of *noI*O' in mutant strain SVQ121. The *noI*O' coding region of HH103 presented 1-bp deletion (A) between nucleotides 462 and 463 of the sequenced fragment (corresponding to 231 and 232 in the *noI*O' coding region) when compared with the homologous region of *Rhizobium* sp. NGR234 (Fig. 2). This deletion originates a premature stop codon at position 622 (corresponding to position 391 of the *noI*O'-coding region) in the DNA of *S. fredii* HH103. The deduced HH103 NoI'O' protein is 97.4% identical in its first 77 amino acids (encoded by the nu-

cleotide sequence preceding the deletion) to the NGR234 NoI protein (Fig. 3). As mentioned above, 35 bp after the stop codon of HH103 *noI*O', it appears in the HH103 sequence of a second ORF (ORF2) showing homology to the central part and 3' terminus of the *Rhizobium* sp. NGR234 *noI*O' gene. ORF2 extends between positions 660 and 2,291 of the sequenced fragment and encodes for a 543-amino acids-long deduced polypeptide that is 98.7% identical to the NGR234 NoI protein segment between residues 144 and 680 (Fig. 3).

Our results indicate that the *S. fredii* HH103 *noI*O' gene is truncated. A similar situation is found in *B. japonicum* USDA110. In this strain, the *noI*N gene is homologous to the 5' region of the NGR234 *noI*O' gene, but a 1-bp deletion (G), located 433 nucleotides after the start codon, generates a premature stop codon (Fig. 2). The HH103 *noI*O' and USDA110 *noI*N show the start and stop codons at the same position, and their encoded polypeptides are 75% homologous in the first 76 amino acids (Fig. 3).

Fig. 2. Alignment of DNA sequence containing the *noI*O' region of *Sinorhizobium fredii* [strains HH103, 042B(s), USDA192, USDA193, and USDA257], the *noI*O 5' terminus of *Rhizobium* sp. NGR234, and the *noI*N gene of *Bradyrhizobium japonicum* USDA110. Primers used to amplify the *S. fredii* *noI*O' region are shown in bold italic. The putative translational start and stop codons are marked in bold, except the GTG start codon of *B. japonicum* USDA110 *noI*O, which is underlined. The 1-bp deletions in the *S. fredii* and *B. japonicum* USDA110 sequences are indicated by black triangles. Nucleotide mismatches and deletions are marked with white squares.

No10 NGR234	1	MLCGLSGGLSIIHENSLLPNTFHDGAAVLVRDGOVIAAVEEERLNRKHSNKLPRRS
No10' HH103	1	MLCGLSGGLSKIYENLLPNTFHDGAAVLVRDGRVIAAVEEERLNRKHSNKLPRRS
No1N USDA110	1	MLSGLSGGLDIYESSPLPNTFHDGAAVLVDGRVIAAVEEERLNRKHSNKFEENS
No10 NGR234	61	IQYCLEYAGVQLSDIDCIAYYATEACNAMLERLLVSQSHMSIPLDAKLLLRGLLAQEFG
No10' HH103	61	IQYCLEYAGVQLSDIDCSRIMQPRHAMLCSSACSFLSRTCQYRCMPSCCCVGCSSRNLV
No1N USDA110	61	IRYCLSTAGVELGDIDRIAFYATEACKAMLERLSVSQPVPLDPKLLLRQLLARELGAEI
No10 NGR234	121	TEVDPSRISFVSHHLSHAWSAFMSGFEQSLLTIDGGGDEFASGLAAGSGTEKPLAIF
ORF2 HH103	1	MSGFEQSLLTIDGGGDEFASGLAAGSGTEKPLAIF
No10 USDA110	1	MNHHEVHAVSAFAMSGFEQSLFAIDGGGDEFASGLAAGSGTVLAQLVSE
No10' HH103	121	LRSIRRESHS
No1N USDA110	121	DPSGFPS
No10 NGR234	181	PESDSLGLLYLETFYLGYGMFDEYKVMGLAPYGDPAHRLLFEQFYELLDNGGYRYLD
ORF2 HH103	38	PESDSLGLLYLETFYLGYGMFDEYKVMGLAPYGDPAHRLLFEQFYELLDNGGYRYLD
No10 USDA110	51	PEHNSLGLFYLETFYLGCGMFDEYKVMGLAPYGDPDYRLLFAQFYELLDSSGGYRHLD
No10 NGR234	241	RIGPTLLRSTIEVRRKGMPFTQQHDSASLQEALERIVFHVLRHHSEITGKRLSLAGGV
ORF2 HH103	98	RIGPTLLRSTIEVRRKGMPFTQQHDSASLQEALERIVFHVLRHHSEITGKRLSLAGGV
No10 USDA110	111	RIGPALLRNIOVRRKGMPFTQQHDSASLQEALERIVFHTLRHHKATGTRLCLAGGV
No10 NGR234	301	AHNCTLNGKLLRSGIFQDIFVQPAAH DAGCALGAALMSNELGQSAPRERLQFVYWGPD
ORF2 HH103	158	AHNCTLSGKLLRSGIFQDIFVQPAAH DAGCALGAALMSNELGQSAPRERLQFVYWGPD
No10 USDA110	171	AHNCTLNGKLLYSGIEDDIFVQPAAH DAGCALGAALMSSELGRPAPRERLPQVYWGPD
No10 NGR234	361	GSRRAVEQELIAWGGHIEIERCDDASRAAWADGAVIGWQGRSEFGPRALGNRSILA
ORF2 HH103	218	GSRRAVEQELIAWGGHIEIERCDDASRAAWADGAVIGWQGRSEFGPRALGNRSILA
No10 USDA110	231	GNEQATELELNAWSGHIEIORSDDASSAAWANGAVIGWQGRSEFGPRALGNRSILA
No10 NGR234	421	DPRPATNKDRINAVKKREGYRPFAPSVLEEDANEFFELPD SRQEFPFMNFVVPVRESKR
ORF2 HH103	278	DPRPATNKDRINAVKKREGYRPFAPSVLEEDANEFFELPD SRQEFPFMNFVVPVRESKR
No10 USDA110	291	DPRPAENKDRINAVKKRESYRPFAPSALEEDANEFFELPDGTRQLPFMNFVVRVREAKG
No10 NGR234	481	NELGATHVDGTARLQTVSRNINQAYWVINAFKRTGPILLNTSFNNNVEPIVDSVAD
ORF2 HH103	338	NELGATHVDGTARLQTVSRNINQAYWVITAFKRTGPILLNTSFNNNVEPIVDSVAD
No10 USDA110	351	NELGATHVDGTARLQTVSRKTNPAYWVINAFKRTSPILLNTSFNNNAEPIVQSVSD
No10 NGR234	541	ATTFLTTDL DGLVVGSLKRTASPEDWSRLALSLPPYSSLHQVRAFTALDRQETVCE
ORF2 HH103	398	ATTFLTTDL DGLVVGSLKRTASPEDWSRLALSLPPYSSLHQVRAFTALDRQETVCE
No10 USDA110	411	ATTFLTTDL DGLVVGPLKRPASLQDWSALASLPPYASLHRVRSHTAPDRQETVCE
No10 NGR234	601	IRTCGPSSREAVRISSELFELMRIUGEAPLGLDLIAPNQNQREALNELRGWEQRSV
ORF2 HH103	458	IRTCGPSSREAVRISSELFELMRIUGEAPLGLDLIAPNQNQREALNELRGWEQRSV
No10 USDA110	471	IRMCHSAISSRISPELFEELMRIUGEASLGSFDTALLDQAKREDLKELRLEWELRGV
No10 NGR234	661	RLHPTADSAAPSSPINL
ORF2 HH103	518	RLHPTAGSAAPSSINLSSGARL
No10 USDA110	531	RLHPTAACRHENQSGT

Fig. 3. Comparison of the deduced proteins encoded by *Sinorhizobium fredii* HH103 *no10'* and ORF2 with the *Rhizobium* sp. NGR234 *No10* and the *Bradyrhizobium japonicum* USDA110 *No1N* and *No10* proteins. Identical residues are marked with black squares. Not identical and not identical but at least similar residues are marked with white and gray squares, respectively.

The *nolO* gene of USDA110, whose start codon (GTG) overlaps with the stop codon (TGA) of the *nolN* gene, encodes a putative polypeptide of 548 amino acids that would be 73% identical to the ORF2 encoded polypeptide of HH103.

The *nolM* gene lies between the *nodJ* and *nolN* genes of *B. japonicum* USDA110. No homology with this gene has been found in *S. fredii* HH103 or in any other rhizobia.

The DNA sequence preceding the *S. fredii* HH103 *noeI* gene also shows high similarity (96.4%) to the NGR234 *nolO-noeI* intergenic region. However, 12 base pairs are missing in *S. fredii* HH103.

The HH103 *noeI* gene begins at position 2,458 and extends downstream for 725 bp. The deduced NoeI protein comprises 241 amino acids, which is two residues shorter than the NoeI protein of NGR234, due to a 6-bp deletion located at position 2,811 of the HH103-sequenced fragment. The amino acid identity between both NoeI proteins is 98.3% (Fig. 4), whereas the genes are 99.4% identical.

Characterization of LCOs produced by mutant SVQ121.

LCOs produced by mutant strain SVQ121 were purified by high-performance liquid chromatography into six fractions (designated F-1 to F-6) that were identified by fast atom bombardment mass spectrometry (FABMS). Carbohydrate composition and methylation analyses were done for some of these fractions of gas chromatography-mass spectrometry (GC-MS). The LCOs identified in each chromatographic fraction and their approximate molar ratios (on the basis of the intensity of the pseudo molecular ion in the FABMS analysis) are shown in Table 1.

LCOs secreted by mutant SVQ121 show lower levels of methylation at the fucosyl residue than those observed in the LCOs produced by the parental strain HH103. Moreover, some molecular species carrying nonmethylated fucosyl residues (such as III [C_{18:0}], III [C_{18:1}], or IV [C_{18:0}]) were detected only in mutant SVQ121 (Table 1).

Construction of a *noeI* mutant of *S. fredii* HH103.

Mutant strain SVQ121 contains transposon Tn5-B20 inserted into a truncated version of *nolO* upstream of the *noeI* gene. The fact that mutant SVQ121 is still able to produce LCOs that carry methylated fucosyl residues suggests that its *noeI* gene can still be expressed. Alternatively, strain HH103

might contain another gene whose encoded product can methylate the fucosyl residue of Nod factors. To investigate this possibility, a *noeI* mutant derivative (SVQ503) of *S. fredii* HH103 was generated by insertion of the Ω interposon as described below.

Nod factors of mutant SVQ503 were purified and characterized as described for strain SVQ121. All the LCOs produced by SVQ503 were devoid of 2-*O*-methylation at the fucosyl residue (Table 1), demonstrating that the production of methyl-fucosylated LCOs by SVQ121 is only due to the expression of the *noeI* gene. This result indicates that the -1 frameshift mutation present in *nolO* is only partially polar over *noeI*.

A 2-kb *PstI*-*ApaI* HH103 fragment containing the entire *noeI* gene of HH103 (Fig. 1) was subcloned into the broad-host range vector pMP92, generating plasmid pMUS588. Transfer of plasmid pMUS508 to mutant strains SVQ121 and SVQ503 restored their ability to produce LCOs with similar levels of methylation at the fucosyl residue to those of *S. fredii* HH103 LCOs (Table 1).

Symbiotic phenotype of SVQ121 and SVQ503 mutants.

The symbiotic properties of mutant strains SVQ121 and SVQ503 with seven leguminous plants were studied. Like the parental strain HH103, both mutants induced nitrogen-fixing nodules on *Glycine max* cv. Williams, *Cajanus cajan*, *Desmodium canadense*, *Glycine soja*, *Macroptilium atropurpureum*, *Macrotyloma axillare*, and *Vigna radiata*.

The symbiotic interaction of these mutant strains with *G. max* cv. Williams was further analyzed. No differences ($\alpha = 5\%$) were found between strain HH103 and its mutant derivatives SVQ121 and SVQ503 in either nitrogenase activity of nodules or in plant-top dry weights, fresh weight of nodules, number of nodules at 5 weeks after inoculation, or nodulation kinetic.

Competition experiments between mutant strains (SVQ121 and SVQ503) and their respective parental strains (HH103-1 and SVQ269) were carried out with soybean cultivar Williams. When competitors were mixed at a 1:1 inocula ratio (8×10^8 cells per competitor), nodule occupancy by SVQ121 and SVQ503 was only 28% and 36%, respectively. In both cases, differences in nodule occupancy between the parental strains and their respective mutant derivatives were statistically significant ($\alpha = 5\%$) by the chi-square test. Transfer of plasmid

NoeI HH103	1	MEVGRYLKEGLLISLQQLREFAEIG	CKPTSSLKVHRQIVSLLEKPDVPVILDIGCNDGSD
NoeI NGR234	1	MEVGRYLKEGLLISLQQLREFAEIG	CKPTSSLKVHRQIVSLLEKPDVPVILDIGCNDGSD
NoeI HH103	61	ARRFLQLRPKAQLFCFEPDPRAAARCRENMGPLDRMLFEVAISDRNGRIDFHPSN	..GD
NoeI NGR234	61	ARRFLQLRPKAQLFCFEPDPRAAARCRENMGPLDRMLFEVAISDRNGRIDFHPSN	GDGD
NoeI HH103	119	AKewDLsgsIRQPKNHLSEYQWVRFDGPISVETRRLDDWCSEAGLESIDLIMDVQGAES	
NoeI NGR234	121	AKewDLsgsIRQPKNHLSEYQWVRFDGPISVETRRLDDWCSEAGLESIDLIMDVQGAES	
NoeI HH103	179	DVIAGGKETLNN	TRFIYTEYSDQELYEGQLPLRAILDLLPSFELVAQFPRGVEGDVLLRN
NoeI NGR234	181	DVIAGGKETLTK	TRFIYTEYSDQELYEGQLPLRAILDLLPSFELVAQFPRGVEGDVLLRN
NoeI HH103	239	TFL	
NoeI NGR234	241	TFL	

Fig. 4. Comparison of the predicted amino acid sequence of the NoeI proteins from *Sinorhizobium fredii* HH103 and *Rhizobium* sp. NGR234. Identical residues are marked with black squares. Not identical and not identical but at least similar residues are marked with white and gray squares, respectively.

pMUS588 (carrying the *noeI* gene) to mutants SVQ121 and SVQ503 did not restore the bacterial competition ability to nodulate soybean cultivar Williams. However, most of the isolates from nodules formed by these mutants carrying pMUS588 were Tc^S, indicating that plasmid pMUS588 had been lost.

Analysis of the *noeI* sequence of other *S. fredii* strains.

By using the primers pnoIof and pnoIor (discussed below), a fragment covering the whole *noeI* region was amplified by polymerase chain reaction (PCR) from *S. fredii* strains 042B(s), B15, B50, HH17, S30, SMH15, USDA192, USDA193, USDA201, USDA205, USDA257, as well as from *S. xinjiangensis* CCBau110 and *Rhizobium* sp. NGR234 (Fig. 5). All the amplified PCR fragments showed a similar size to that of *S. fredii* HH103 (457 bp). PCR fragments obtained from *S. fredii* strains USDA192, USDA193, USDA257, and 042B(s) were isolated and sequenced. The sequences of these PCR products (accession numbers AF358780, AF358781, AF358782, and AF358783, respectively) showed 100% identity to that of HH103. Hence, the -1 frameshift mutation in comparison with the NGR234 *noeI* gene that is present in the HH103 *noeI* coding region is conserved in the homologous sequences of all *S. fredii* strains studied in this work (between nucleotides 280 and 281 of the PCR fragments; Fig. 2). Therefore, at least these five *S. fredii* strains have a putative interrupted version of the *noeI* gene.

DISCUSSION

We have carried out transposon Tn5-*lacZ* mutagenesis to identify genes showing elevated expression in the presence of the flavonoid naringenin, an effective *nod* gene inducer of *S. fredii* HH103 (Vinardell et al. 1993). Following this strategy, we have screened 2,800 HH103 mutants, each carrying a random Tn5-*lacZ* insertion. In this work, we have analyzed one of these mutants, SVQ121, which contains a Tn5-*lacZ* insertion into the *S. fredii* HH103 symbiotic plasmid.

In order to determine the exact position of the transposon insertion, we constructed plasmid pMUS442. This plasmid specifically allows the isolation of the rhizobial DNA that is adjacent to the IS50R of transposon Tn5-*lacZ*, regardless of the genome region in which the transposon is inserted.

DNA sequencing of the SVQ121 DNA that is adjacent to the Tn5-*lacZ* insertion showed homology to the 5' terminus of the *Rhizobium* sp. NGR234 *noeI* gene. Isolation of cosmid pMUS629 allowed the sequencing of the homologous wild-

type region from strain HH103. Computer analysis of this HH103 region predicts two putative ORFs, which were designated *noeI*' and ORF2. Alignment of the homologous regions from HH103 and NGR234 shows a 1-bp deletion in the HH103 *noeI* coding sequence. Hence, we conclude that the *noeI* region of strain HH103 is split into two computer-predicted ORFs by the creation of an internal stop codon that renders a truncated protein. The NGR234 *noeI* protein contains 680 amino acids while the *S. fredii* HH103 *noeI*' ORF would encode for a truncated *noeI*' protein of only 130 amino acids. The fact that LCOs produced by *S. fredii* HH103 are devoid of carbamoyl residues (Gil-Serrano et al. 1997) supports the idea that even if the putative polypeptides encoded by either *noeI*' or ORF2, or both, were produced, they should not be able to carbamoylate LCOs. Moreover, introduction of the NGR234 *noeI* gene in *S. fredii* USDA257, whose LCOs also lack carbamoyl residues (Bec-Ferté et al. 1994), results in production of carbamoylated LCOs (Jabbouri et al. 1998), indicating that *S. fredii* LCOs can be carbamoylated in the presence of a functional *noeI* gene.

A similar situation is found in *B. japonicum*, another soybean nitrogen-fixing symbiont. The putative *B. japonicum* *noeI* and *noeI* proteins are highly homologous to the deduced polypeptides encoded by HH103 *noeI*' and ORF2, respectively. In fact, nodulation factors produced by *B. japonicum* are also devoid of carbamoyl residues (Carlson et al. 1993). Interestingly, although the HH103 *noeI*' and USDA110 *noeI* ORFs have their stop codons at the same position, the -1 frameshift mutations responsible for the appearance of these premature stop codons are different (Fig. 2).

The *S. fredii* HH103 *noeI* gene is 99.4% identical to that of NGR234, and it is also located downstream of the *noeI* region, probably belonging to the same operon, *nodABCInoeI*. Although the whole operon is most probably transcriptionally activated from the *nodA* promoter, the transposon insertion into *noeI*' (mutant SVQ121) does not completely abolish transcription of *noeI*. This idea is supported by the fact that mutant SVQ121 produces methyl-fucosylated LCOs, although the MeFuc/Fuc ratio is lower than that of LCOs produced by the wild-type strain HH103-1. In *S. fredii* HH103, methylation of the fucosyl residue is only due to the action of *NoeI*, since the *noeI* mutant SVQ503 produces fucosylated LCOs that are devoid of methyl residues and introduction of the wild-type *noeI* gene in this mutant restores the production of methylfucosylated LCOs. Trisaccharide LCOs appear in minor quantities that are not always detected in mass spectrometry experiments. For instance, III(C_{16:0}, MeFuc) has been found to be produced by

Table 1. Mass spectrometric structural assignments for lipochitooligosaccharides (LCOs) from *Sinorhizobium fredii* HH103 and its derivatives SVQ121, SVQ503, SVQ121(pMUS588), and SVQ503(pMUS588)^a

Pairs of LCOs containing Fuc or MeFuc	HH103 ^b	SVQ121	SVQ503	SVQ121 (pMUS588)	SVQ503 (pMUS588)
III(C _{16:0} ,Fuc) / III(C _{16:0} ,MeFuc)	A/A	F-4: 1/2, F-5: 1/2	F-4: P/A	A/A	A/A
III(C _{16:1} ,Fuc) / III(C _{16:1} ,MeFuc)	F-2: A/P	F-2: 3/2	F-2: P/A	A/A	A/A
III(C _{18:0} ,Fuc) / III(C _{18:0} ,MeFuc)	F-6: A/P	F-6: 2/3	A/A	A/A	A/A
III(C _{18:1} ,Fuc) / III(C _{18:1} ,MeFuc)	F-5: A/P	F-6: 2/3	A/A	A/A	A/A
IV(C _{16:0} ,Fuc) / IV(C _{16:0} ,MeFuc)	F-3: A/P	F-3: 2/1	F-3: P/A	F-3: A/P	F-3: A/P
IV(C _{16:1} ,Fuc) / IV(C _{16:1} ,MeFuc)	F-1: 2/3	F-1: 4/1	F-1: P/A, F-2: P/A	F-1: 1/2	F-1: 1/3
IV(C _{18:0} ,Fuc) / IV(C _{18:0} ,MeFuc)	F-6: A/P	F-6: 3/2	F-6: P/A	F-6: A/P	F-6: A/P
IV(C _{18:1} ,Fuc) / IV(C _{18:1} ,MeFuc)	F-4: 1/4	F-4: 3/1, F-5: 3/1	F-4: P/A, F-5: P/A	F-4: 1/2, F-5: 1/5	F-4: 1/3, F-5: A/P
V(C _{16:0} ,Fuc) / V(C _{16:0} ,MeFuc)	F-2: 1/5	F-2: 1/3	F-2: P/A	F-2: 1/5	F-2: 1/3
V(C _{16:1} ,Fuc) / V(C _{16:1} ,MeFuc)	F-1: 1/2	F-1 ^c : A/P	A/A	F-1: 1/4	F-1: A/P
V(C _{18:0} ,Fuc) / V(C _{18:0} ,MeFuc)	F-6: A/P	F-6: A/P	F-6: P/A	F-6: A/P	F-6: A/P
V(C _{18:1} ,Fuc) / V(C _{18:1} ,MeFuc)	F-3: 1/3, F-4: A/P	F-3: 1/3	F-3: P/A, F-4: P/A	F-3: A/P	F-3: 2/7

^a The approximate molar ratios between pairs of fucosylated or methyl-fucosylated LCOs sharing the same fatty acid and backbone skeleton are indicated in bold. A = Absent or not detected. P = Present in the F fractions obtained by HPLC.

^b As described by Gil-Serrano and associates (1997).

^c Very low level detected.

wild-type strain HH103 in other experiments that also showed that methyl-fucosylated LCOs produced by HH103 are more abundant than LCOs carrying nonmethylated fucosyl residues (data not shown). Similarly, the absence of trisaccharide LCOs among LCOs produced by SVQ121(pMUS588) and SVQ503(pMUS588) could be simply due to their low amounts in the bacterial culture.

The lower level of LCOs methylation in mutant SVQ121 (in comparison with that of HH103 or SVQ121 carrying pMUS588) is probably due to a polar effect of the Tn5-*lacZ* insertion over the *noeI* expression. A similar situation has been described in *B. japonicum* USDA110 in which Tn3-*lacZ* insertions into *nolN* or *nolO* reduce the expression of the downstream gene *nodZ* (Luka et al. 1993).

Mutant strains SVQ121 and SVQ503 retained their nodulation and nitrogen-fixation abilities with all the legumes tested. Similar results have been reported for an NGR234 *noeI* mutant (Jabbouri et al. 1998; Perret et al. 2000). However, both mutants SVQ121 and SVQ503 showed decreased competition ability to nodulate soybean cultivar Williams. These results indicate that complete suppression, or even reduction, of the level of 2-*O*-methylfucosylation of HH103 Nod factors affects bacterial competition ability. Since we have isolated HH103 derivatives carrying a Tn5-*lacZ* insertion that do not show any significant reduction of their competition ability (Madinabeitia, unpublished data), we can conclude that the reduced competition ability of SVQ121 is due not to the transposon Tn5-*lacZ* by itself but to the mutation caused.

Transfer of plasmid pMUS588, a Tc^R plasmid containing the HH103 *noeI* gene, to mutants SVQ121 and SVQ503 complemented the production of wild-type levels of 2-*O*-methylfucosylated LCOs. This plasmid, however, did not restore the competition ability of these strains to nodulate soybean cultivar Williams. Most of the nodule isolates (identified by the markers Km^r or Spc^r, respectively) were Tc^s, indicating that although plasmid pMUS588 was initially present in the bacterial inocula, it had been lost during the nodulation process.

By using primers designed from the flanking DNA sequences of *S. fredii* HH103 *nolO'*, we could amplify a PCR fragment from different *S. fredii* strains, as well as from the closely related bacteria *S. xinjiangensis* CCBAU110 and *Rhizobium* sp. NGR234. In all the cases, the fragment size was identical to that of the fragment amplified from HH103 (Fig. 5), indicating that the *nolO'* region is well conserved in *S. fredii*. Moreover, the sequences of these fragments were identical in the five *S. fredii* strains analyzed, which were originally isolated from various provinces of east-central China (HH103,

USDA192, USDA193, and USDA257) and from far western China [042B(s)]. Thus, at least in these five strains, the *nolO* gene is disrupted by a conserved -1 frameshift mutation that renders a putative, nonfunctional, truncated Nod protein.

The *nolO'* and ORF2 genes of HH103 are not involved in carbamoylation of *S. fredii* Nod factors, but we cannot discard the possibility that these genes encode polypeptides with an unknown function in the cell.

The high homology between the *S. fredii* HH103 sequenced fragment and the *Rhizobium* sp. NGR234 *nolO/nolI* locus is similar to that found between other symbiotic loci of these bacteria (Lamrabet et al. 1999). This fact suggests that these bacteria are closely related. Thus, the 1-bp deletion detected in this region of *S. fredii* must be very recent from an evolutionary point of view and could explain why apparently nonfunctional sequences are conserved in several *S. fredii* strains.

The *Rhizobium* sp. NGR234 *nodU* gene is not linked to the *nolO* gene, and it is also involved in carbamoylation of Nod factors (Jabbouri et al. 1995). This gene is also present in *S. fredii* USDA257, and again, it is nonfunctional because its promoter region contains a defective *nod*-box (Krishnan et al. 1992). Assuming that other *S. fredii* strains could contain a nonfunctional *nodU* gene, since *S. fredii* LCOs are noncarbamoylated (Bec-Ferté et al. 1994; Gil-Serrano et al. 1997), it might be interesting to determine whether a defective *nod*-box, identical to that of strain USDA257, is also present.

MATERIALS AND METHODS

Strains, plasmids and media.

The bacterial strains and plasmids used in this work are listed in Table 2. *Sinorhizobium* strains were grown on tryptone-yeast extract (TY) medium (Beringer 1974), yeast extract-mannitol medium (Vincent 1970), B⁻ medium (Spaink et al. 1992) or Bergersen's minimal medium (Bergersen 1961) at 28°C. *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). Flavonoids were dissolved in ethanol and used at 1 μg ml⁻¹ (approximately 3.7 μM). Bacterial strains were maintained as stocks in 15%:0.5% glycerol/peptone at -80°C.

Genetic manipulations.

Transposon mutagenesis of strain HH103-1 was carried out using the suicide plasmid pSUP102-Gm that harbors transposon Tn5-B20 (Tn5 fused to a *lacZ* gene) as described by Lamrabet and associates (1999). Transconjugants were selected on TY medium containing kanamycin and streptomycin. Single colonies were screened for β-galactosidase activity on Bergersen's medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (32 μg ml⁻¹) and the inducer naringenin at a concentration of 3.7 μM. The positive clones were assayed for β-galactosidase activity in liquid bacterial cultures as described by Miller (1972).

Plasmids were transferred by conjugation as described by Simon (1984). Recombinant DNA techniques were performed according to the general protocols of Sambrook and associates (1989). DNA sequencing was done using the dideoxy-chain termination method (Sanger et al. 1977).

To identify the gene affected by the Tn5-B20 insertion in mutant SVQ121, plasmid pMUS442 was transferred to this strain. The plasmid pMUS442 (Cm^R, Tc^R) is a pSUP202 derivative carrying a 0.9-kb *Pst*I fragment that contains part of the kanamycin-resistance gene from Tn5. This plasmid cannot replicate in *Sinorhizobium* strains. Thus, it can only be maintained in SVQ121 by specific integration into the Tn5-*lacZ*

Fig. 5. Amplification of a 457- to 458-bp DNA fragment covering the HH103 *nolO'* open reading frame in different *sinorhizobia* strains. Lane 1, HH103-1; lane 2, USDA205; lane 3, USDA257; lane 4, NGR234; lane 5, USDA201; lane 6, USDA192; lane 7, USDA193; lane 8, CCBAU110; lane 9, 042B(s); lane 10, B15; lane 11, molecular weight marker (500-bp ladder, Roche); lane 12, B50; lane 13, HH17; lane 14, S30; lane 15, SMH15; and lane 16, control without DNA.

transposon through homologous recombination between the kanamycin-resistance gene of Tn5-B20 and that of plasmid pMUS442. Total genomic DNA of a Tc^R SVQ121 transconjugant was digested with *Eco*RI, self-ligated, and used to transform *E. coli*. Following this strategy, a new plasmid (pMUS508) containing part of pMUS442 and the SVQ121 rhizobial DNA that is adjacent to the IS50R of transposon Tn5-B20 was isolated. Primer padad2 (5'-AGATTTAGCCCAGTCG), which was designed from the nucleotide sequence of Tn5-B20 IS50R, was used to sequence the cloned rhizobial DNA adjacent to the right arm of the transposon in plasmid pMUS508.

In order to generate a *noeI* mutant derivative of *S. fredii* HH103, the Ω interposon (Str^R, Spc^R; Prentki and Krisch 1984) was subcloned into the unique *Cl*AI site of this gene. For this purpose, a 4-kb *Pst*I-*Kpn*I fragment carrying the *noeI*:: Ω was subcloned into pK18mob, a plasmid that is not replicable in rhizobia strains. The resultant plasmid, pMUS594, was transferred by conjugation to strain SVQ269. Rif^R Spc^R HH103 transconjugants were analyzed for sensitivity to kanamycin in order to identify double recombinants where the wild-type *noeI* gene had been substituted by the mutated copy of the gene.

Mutant strain SVQ503 (= SVQ269 Rif^R *noeI*:: Ω) was obtained, and the substitution of the wild-type *noeI* gene by the *noeI*:: Ω was confirmed by hybridization.

The *nolO*' region from various *S. fredii* strains, *S. xinjiangensis* CCBau110, and *Rhizobium* sp. NGR234 were PCR amplified from genomic DNA as described by Saiki (1990) using primers p_{nol}of (5'-CGGAGCACAGGGCCT) and p_{nol}or (5'-GCGACAGGTGATGGC). PCR fragments (457 bp) were subcloned into plasmid pGEM-T (Promega, Madison, WI, U.S.A.), and sequenced. DNA and deduced protein sequences were analyzed with the GCG program (Genetics Computer Group, Madison, WI, U.S.A.) (Devereux et al. 1984).

Plant assays.

The nodulation ability of strains SVQ121 and SVQ503 was tested on *G. max* cultivar Williams, *Cajanus cajan* (L.) Millsp., *Desmodium canadense* (L.) DC., *G. soja*, *Macroptilium atropurpureum* (Moc. & Sessé ex DC) Urb, *Macrotyloma axillare* (E. Mey.) Verdc, and *Vigna radiata* (L.) Wilczek as described by Buendía-Clavería and associates (1989). Fåhræus (Vincent 1970) or Rigaud-Puppo (Rigaud and Puppo 1975) plant nutritive solutions were used. Each plant was inoculated with about

Table 2. Bacterial strains and plasmids

Strain	Derivation and relevant properties ^a	Source or reference
<i>Sinorhizobium fredii</i>		
042B(s)	Wild-type strain	S. S. Yang
B15	Wild-type strain	S. S. Yang
B50	Wild-type strain	S. S. Yang
HH103-1	HH103 Str ^R	Buendía-Clavería et al. 1989
HH17	Wild-type strain	S. S. Yang
S30	Wild-type strain	S. S. Yang
SMH15	Wild-type strain	Rodríguez-Navarro et al. 1996
SVQ121	HH103-1 <i>nolO</i> ::Tn5-B20	This work
SVQ269	HH103 Rif ^R	This work
SVQ503	SVQ269 <i>noeI</i> :: Ω	This work
USDA192	Wild-type strain	Keyser et al. 1982
USDA193	Wild-type strain	Keyser et al. 1982
USDA201	Wild-type strain	Keyser et al. 1982
USDA205	Wild-type strain	Keyser et al. 1982
USDA257	Wild-type strain	Keyser et al. 1982
<i>S. xinjiangensis</i>		
CCBAU110	Wild-type strain	Chen et al. 1988
<i>Rhizobium</i> sp.		
NGR234	Wild-type strain	Trinick 1980
NGR234-2	NGR234 Rif ^R	This work
<i>Escherichia coli</i>		
DH5 α	<i>supE44</i> , Δ <i>lacU169</i> , <i>hsdR17</i> , <i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>relA1</i> , Nx ^R	Sambrook et al. 1989
HB101	Restriction-minus, <i>recA</i> background, Str ^R	Boyer and Roulland-Dossoix 1969
Plasmids		
pBluescript	Cloning vector, Ap ^R	Stratagene
pGEM-T	Cloning vector, Ap ^R	Promega
pK18mob	Cloning vector, Km ^R	Schafer et al. 1994
pMP92	Broad-host-range cloning vector, Tc ^R	Spaick et al. 1987
pMUS442	Plasmid derived from pSUP202 carrying a 0.9-kb <i>Pst</i> I fragment that contains part of the kanamycin resistance gene from Tn5.	This work
pMUS508	pMUS442 derivative containing a 1.2-kb fragment of the <i>S. fredii</i> HH103 <i>nolO</i> region and part of Tn5-B20.	This work
pMUS531	pBluescript containing a 2.8-kb <i>Eco</i> RI- <i>Sma</i> I fragment from pMUS629 that carries <i>noeI</i> and part of the <i>nolO</i> region	This work
pMUS567	pBluescript carrying a 2-kb <i>Pst</i> I- <i>Apa</i> I fragment from pMUS531 containing HH103 <i>noeI</i>	This work
pMUS588	pMP92 carrying a 2-kb <i>Pst</i> I- <i>Kpn</i> I fragment from pMUS567 containing HH103 <i>noeI</i>	This work
pMUS594	pK18mob carrying a 4-kb <i>Pst</i> I- <i>Kpn</i> I fragment containing <i>noeI</i> :: Ω	This work
pMUS596	pBluescript carrying a 2.7-kb <i>Sac</i> I- <i>Cl</i> A1 fragment of pMUS629 containing <i>nolO</i> ', ORF2, part of <i>nodJ</i> and part of <i>noeI</i>	This work
pMUS629	Cosmid pLAFR1 carrying nodulation genes of <i>S. fredii</i> HH103	This work
pSUP102-Gm::Tn5-B20	Suicide plasmid carrying the Tn5- <i>lacZ</i> transposon Tn5-B20	Simon et al. 1989
pSUP202	pBR325 containing the Mob region subcloned in <i>Sau</i> 3A, Ap ^R , Cm ^R , Tc ^R	Simon et al. 1983

^a R indicates resistance.

5×10^8 bacteria. Nitrogen fixation was assessed by acetylene reduction assays (Buendía-Clavería et al. 1986). Plant tops were dried at 80°C for 48 h and weighed. Bacteria were isolated from surface-sterilized nodules as previously reported (Buendía-Clavería et al. 1986). Competitive studies were carried out on *G. max* cultivar Williams as described by Romero and associates (1993).

LCO purification.

Bacteria were grown in 10 l of B⁻ medium (Spaink et al. 1992) in the presence of genistein at 3.7 µM. LCOs were extracted and purified from bacterial cultures as described by Lamrabet and associates (1999).

Carbohydrate composition and methylation analysis of LCOs.

Glycosyl composition analyses were carried out after methanolysis using anhydrous methanolic 0.625 M HCl (16 h, 80°C). The samples were reacylated using 1:1 Ac₂O-pyridine, and then trimethylsilylated with 1:1 pyridine-BSTFA and analyzed by GC-MS (Chaplin 1982).

The LCOs were permethylated (Ciucanu and Kerek 1984), and samples were hydrolyzed with 2 M CF₃COOH (1 h, 120°C), reduced, and acetylated using the method described by Blakeney and associates (1983). The permethylated alditol acetates were analyzed by GC-MS. The GC-MS instrument, column, and conditions for the different derivatizations have been described by Gil-Serrano and associates (1995).

FABMS analysis.

Positive FABMS was performed on a Kratos MS80-RFA instrument (Kratos Analytical, Manchester, U.K.) with a xenon atom beam at an accelerating voltage of 4 kV. Thioglycerol was used as the matrix and NaI as the cationizing agent. Samples were dissolved in Me₂SO.

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