SyrM1 of *Rhizobium* sp. NGR234 Activates Transcription of Symbiotic Loci and Controls the Level of Sulfated Nod Factors

Moez Hanin, Saïd Jabbouri, William J. Broughton, and Rémy Fellay

Laboratoire de Biologie Moléculaire des Plantes Supérieures (LBMPs), Université de Genève, 1 ch de l'Impératrice, 1292 Chambésy/Genève, Switzerland

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One or more transcriptional regulators of the LysR class control transcription of rhizobial nodulation genes. In *Rhizobium* sp. NGR234, two copies of *nodD* (*nodD1* and *nodD2*) are present on the symbiotic plasmid pNGR234a. The complete sequence of pNGR234a revealed two additional *nodD* homologues, *syrM1* and *syrM2*. Competitive RNA hybridization analyses involving a mutant of *syrM1* (NGRΔ*syrM1*) showed that a number of symbiotic genes (e.g., *nolXBTUVW*) are expressed in an *syrM1*-dependent manner. Assays in which regions upstream of *nolB* and *nolW* were fused to promoterless *lacZ* confirmed that *SyrM1* is required for their late induction. Mutation of *syrM1* also drastically reduced production of sulfated Nod factors as shown by reverse phase–thin layer chromatography (RP-TLC). *SyrM1* controls sulfation of Nod factors via one of the two chromosomal *nodPQ* loci. It thus seems likely that *syrM1* of NGR234 encodes a transcriptional activator that regulates the expression of genes involved in both the early and late stages of infection.

A number of genera of gram-negative soil bacteria, collectively known as rhizobia, form nitrogen-fixing nodules on the roots of legumes. Sequential exchange of signals between the symbionts controls specificity in these associations (for recent reviews see Dénarié et al. 1996; Spaïnk 1996; Schultze and Kondorosi 1996; Mergaert et al. 1997; Hanin et al., *in press*). Flavonoids released by legume roots, in conjunction with rhizobial NodD proteins, activate transcription of nodulation genes (*nod*, *nol*, and *noe*). Enzymes encoded by the *nod* genes are responsible for the production and secretion of a family of lipo-chitin-oligosaccharides (LCOs) called Nod factors. Nod factors are β-1,4 linked tri- to hexa-mers of *N*-acetyl-D-glucosamine, which are *N*-acylated at the nonreducing terminus (Lerouge et al. 1990). When transmitted to the host plant, these LCO messages trigger the initiation of nodule formation (Truchet et al. 1991; Relić et al. 1993b). Products of the *nodABC* genes synthesize the core oligosaccharide, while enzymes encoded by host-specific nodulation genes direct decoration of the core molecule with various adjuncts.

Transcriptional activation of nodulation genes by NodD appears to occur differentially in response to specific inducers (essentially flavonoids) derived from the roots of the various hosts. Correlations exist between the spectrum of flavonoids able to interact with NodD and promiscuity of nodulation. NodD proteins of narrow-host-range rhizobia such as *Rhizobium meliloti* and *R. leguminosarum* bv. *viciae* respond to a limited number of flavonoids while NodD1 of the broad-host-range *Rhizobium* sp. NGR234 reacts with a large spectrum of inducing compounds, including simple phenolic substances (e.g., vanillin, iso-vanillin) as well as compounds that have been shown to be inhibitory in other rhizobia (Györgypal et al. 1991; Le Strange et al. 1990).

The number of copies of *nodD* is strain dependent, and in some cases one constitutively expressed gene activates the expression of the others (Fellay et al. 1998). NodD regulators belong to the LysR family of prokaryotic transcriptional activators (Henikoff et al. 1988). All possess a helix-turn-helix DNA binding motif in their N termini that is responsible for recognition of *nod* gene promoters (*nod* boxes). Attachment of the NodD-flavonoid complex to a *nod* box results in bending of the DNA, which is thought to be necessary for transcription of downstream *nod* genes (Fisher and Long. 1993). Activation of *nod* gene expression via *nod*-box promoters is not the only regulatory system used in rhizobia, however. The *nolX* gene of *R. fredii* USDA257 lacks a *nod* box, yet it is regulated in the conventional flavonoid- and *nodD*-dependent manner (Kovács et al. 1995; Bellato et al. 1996).

It should also be noted that additional positive and negative regulators of *nod* gene expression have been identified. In *R. meliloti*, *syrM* (a *nodD* homologue) along with *nodD3* is involved in *nod* gene activation (Kondorosi et al. 1991a). *nolR* down-regulates transcription of *nodD1*, *nodD2*, and the *nodABC* genes by direct attachment to their overlapping promoters (Cren et al. 1995; Kondorosi et al. 1991b). By activating *nodD2* of *Bradyrhizobium japonicum*, *NolA* leads to repression of nodulation genes (Garcia et al. 1996). *B. japonicum* also possesses another, unique, regulatory system (NodV/NodW) (Sanjuan et al. 1994). NodV/NodW are members of the family of two-component regulatory systems. NodV is a sensor kinase that phosphorylates NodW, which is a response regulator (Loh et al. 1997).

Regulation of *nod* gene expression in NGR234 is poorly understood. Except for a few loci such as *nodPQ* (Perret et al.

Corresponding author: W. J. Broughton; LBMPs, 1 ch. de l'Impératrice, 1292 Chambésy/Genève, Switzerland; Telephone: 0041 22 906 17 40; Fax: 0041 22 906 17 41; E-mail: broughtw@sc2a.unige.ch

1991), most nodulation genes are grouped into several flavonoid-inducible loci on the large (536 kb) symbiotic plasmid pNGR234a (Fellay et al. 1995). Two copies of *nodD* (*nodD1* and *nodD2*) are present: Mutation of *nodD1* completely abolishes nodulation (Relić et al. 1993a). This is explained by the fact that, along with many others, *nodABC* is not transcribed in the *nodD1* mutant (Fellay et al. 1995). *nodD2*, which is involved in repression of the *nodABC* genes, is expressed in a *nodD1*-dependent manner, and is required for the formation of nitrogen-fixing nodules on roots of *Vigna unguiculata* and other NGR234 hosts (Fellay et al. 1998).

The complete sequence of pNGR234a (Freiberg et al. 1997) revealed 22 potential genes encoding transcriptional regulators. Among them, two copies of genes homologous to *nodD* were found: *syrM1* and *syrM2*. The molecular and biochemical analyses presented here show that *syrM1* is involved in activation of a number of symbiotic genes. These include the two *nodPQ* genes that help control the level of sulfated Nod factors.

RESULTS

Identification, sequence, and mutation of *syrM1*.

Initially, *syrM1* was mapped to pNGR234a by partial sequencing of a cosmid clone that originated from subtractive hybridizations performed between NGR234 and the closely related *R. fredii* strain USDA257 (Perret et al. 1994). The second copy of *syrM* (*syrM2*) was only found when the complete sequence pNGR234a became available (Freiberg et al. 1997). *syrM1* is more than 60% homologous to *syrM2*. Both are highly similar to the *syrM* genes of *R. meliloti* (Barnett and Long 1990) and *R. leguminosarum* bv. *phaseoli* (Michiels et al. 1993). The promoter region of *syrM1* contains a putative Shine-Delgarno sequence, followed by two possible initiation codons. GeneMark predictions (Borodovsky et al. 1994) with all known *syrM* genes suggest that the first of these, a GTG, is the probable start codon. On this basis, the *syrM1* open reading frame (ORF) is 1,014 bp in length, the deduced protein has 338 amino acids and a predicted molecular mass of approximately 37.5 kDa. A possible helix-turn-helix DNA-binding motif is present between residues 52 and 72.

Mutation of *syrM1* (giving NGRΔ*syrM1*) was performed by deleting a 1.1-kb *Bam*HI fragment (Fig. 1) and replacing it with the spectinomycin/streptomycin resistant Omega interposon (Ω Sp^r) (see Materials and Methods). This *Bam*HI fragment includes the first 107 amino acids of *syrM1* as well as the upstream region, which contains no apparent ORF (Freiberg et al. 1997).

SyrM1 is involved in activation of symbiotic genes.

To test whether *SyrM1* activates transcription of symbiotic genes, DNA from 23 cosmids selected to cover pNGR234a was used in a competitive RNA hybridization assay. Southern blots of these cosmids digested with *Xho*I were preincubated with nonlabeled RNA from apigenin-induced cultures of NGRΔ*syrM1*. Afterward, the blots were hybridized against ³²P-labeled RNA from apigenin-induced NGR234 cultures. Autoradiography revealed several cosmids containing labeled fragments (Fig. 2), which could be grouped into two sets: one includes pXB285, pXB182, and pXBS4 and the other contains pXB740, pXB110, pXB64, and pXB368. Most of the genes present in these fragments are listed in Table 1. Some of them (such as y4yR, y4xI, and y4wF) share significant homologies with genes in data bases while others (such as y4yP, y4yQ, and y4gE) do not show homology with any known gene. It is worth noting that many of these fragments contain *nod* boxes.

Included among the genes under the control of *syrM1* are *nolB* and *nolW*. Since they are divergently transcribed (Balatti et al. 1995; Freiberg et al. 1997), we attempted to verify this result by amplifying the intergenic region by polymerase chain reaction (PCR) and cloning it in both orientations in front of the promoterless *lacZ* gene of pMP220 (see Materials and Methods). Afterward, the resulting plasmids pM220B and pM220W were transferred to NGR234 and NGRΔ*syrM1* by tri-parental matings. Measurement of their β-galactosidase activities (Table 2) showed that both *nolB* and *nolW* are induced by apigenin, even though low but significant activities were detected under noninducing conditions (618 Miller Units = MUs with pM220B). After 24 h of induction with apigenin, the activity of NGR234(pM220B) increased approximately eightfold, while the activity of NGR234(pM220W) went up nearly 14 times. Interestingly, the activity of the *nolW* promoter was completely abolished in NGRΔ*syrM1*.

Fig. 1. Physical and genetic map of the *syrM1* locus of *Rhizobium* sp. NGR234. Restriction sites are as follows: *Bam*HI (B); *Bgl*II (Bg); *Cla*I (C); *Eco*RI (E); *Eco*RV (Ev); and *Hind*III (H). *syrM1* was mutated by replacing the internal *Bam*HI fragment by a spectinomycin-resistant Omega cassette and this is shown by Δ.

Mutation of *syrM1* decreases production of sulfated Nod factors.

NGR234 and NGR Δ *syrM1* were grown in RMM media supplemented with [1-¹⁴C]-D-glucosamine. Apigenin was used to induce *nod*-gene expression. LCOs extracted from both supernatants were analyzed by reverse phase–thin layer chromatography (RP-TLC). NGR234 produces two major spots on RP-TLC (Fig. 3), the upper of which, spotB ($R_f \cong 0.71$) corresponds to sulfated molecules, while the lower, spotA ($R_f \cong 0.54$) contains Nod factors that are either acetylated or nonsubstituted at the 2-*O*-methylfucose (Hanin et al.

1997). LCOs produced by NGR Δ *syrM1* also separated into two spots, although the ratio of spotB/spotA decreased 10-fold (determined by densitometric scanning of the corresponding autoradiographs). These data suggest that *SyrM1* is required for the optimal production of sulfated Nod factors. Moreover, complementation of NGR Δ *syrM1* with the wild-type copy on a broad-host-range plasmid (pBR-*syrM1*) restored the ability of the transconjugants to produce sulfated molecules at the wild-type level (Fig. 3). Furthermore, a deletion downstream of *syrM1* (deletion of the *Bgl*II fragment; Fig. 1) had no effect on levels of NodNGR[S] factors

Fig. 2. **A**, Competitive RNA hybridization to a Southern blot of cosmid DNA (digested with *Xho*I) covering pNGR234a. **B**, Genes controlled by *syrM1*. Restriction digests of the 23 overlapping cosmids covering pNGR234a were transferred to Nylon membranes, and competitive hybridizations performed as described in Materials and Methods. Loci that are under the control of *SyrM1* include *nolWBTUV* (open circle) and *nolX* (dark circle) genes. Lambda DNA digested with *BstEII* was used as the marker.

Table 1. Detection by competitive RNA hybridization of genes/open reading frames (ORFs) on pNGR234a that are under the control of *syrM1*^a

Cosmids	Size (kb)	Signal	ORFs	putative promoter
pXB740	10	+++	y4yP*, y4yQ*, y4yR, y4yS*, y4zA*	N.D
pXB740/pXB110	2.3	+	<i>hrcN</i> , <i>hrcQ</i> , y4yJ*	N.D
pXB110/pXB64	6.2	+	y4xI, y4xK*, y4xL*, <i>nodD2</i>	<i>nod</i> box
pXB110	4.17	++	<i>nolWBTUV</i>	Inducible promoter
pXB110	3.2	+	y4yA*, y4xO*	ND
pXB110	2.2	+++	<i>nolX</i>	Inducible promoter ?
pXB64	6.5	+++	y4wM	<i>nod</i> box
pXB64	4.5	+++	y4wF, y4wG*, y4wE	<i>nod</i> box
pXB368	4.7	+++	y4wD, y4wC	N.D
pXB182/pXB285	6.1	+++	y4hL*, y4hK*, y4hM	<i>nod</i> box
pXB285/pXBS4	4.2, 4.1, 3.8	+++	y4gE*, y4gF, y4gH, y4gG, y4gI, y4gJ*	<i>nod</i> box

^a DNA fragments were produced by digestion of appropriate cosmids with *Xho*I. Intensity of hybridization signals detected on autoradiographs are designated as follows: +++, strong; ++, medium; +, weak. Asterisks indicate genes coding for unknown functions. ND = not determined.

(data not shown), confirming that the observed phenotype is due to disruption of *syrM1*.

The most abundant of NodNGR[S] factors are N-acylated by either C_{18:1} or C_{16:1} fatty acids. Fast atom bombardment-mass spectrometric (FAB-MS) analysis of NodNGR[S] factors purified by reverse phase-high performance liquid chromatography (RP-HPLC) showed that the nonreducing *N*-methylglucosamine contains zero, 1, or 2 carbamates that gave, respectively, B1-ion fragments at *m/z* 526, 483, and 440 (C_{18:1} N-acylated LCOs) and B1-ion fragments at *m/z* 498, 455, and 412 (C_{16:1} N-acylated LCOs). This last fragmentation series was absent from sulfated LCOs produced by NGRΔ*syrM1*. On the other hand, the nonsulfated LCOs produced by both the wild-type and mutant strains were identical.

How does SyrM1 control sulfation of LCOs?

Genes required for sulfation of the reducing terminus of other LCOs have been described (Roche et al. 1991; Schwedock and Long 1992; Schwedock et al. 1994). *nodP* and *nodQ* code for enzymes responsible for the production of 3'-phosphoadenosine 5'-phosphosulfate (PAPS), the activated sulfate donor. In *R. meliloti*, the sulfate group is transferred from PAPS to the reducing terminus of LCOs by the *nodH* gene product (Ehrhardt et al. 1995; Schultze et al. 1995).

Two unlinked *nodPQ* loci are located elsewhere in the genome of NGR234 (Perret et al. 1991), while a Nod factor-specific sulfotransferase (NoeE) is present on pNGR234a (Hanin et al. 1997). NoeE also uses PAPS as the sulfate donor (D. Quesada-Vicens, *personal communication*). As *noeE* is under the control of a flavonoid-inducible *nod*-box promoter (Hanin et al. 1997) and mutation of *syrM1* drastically reduces the production of sulfated Nod factors, we asked whether SyrM1 is involved in the transcriptional activation of these sulfation genes. Plasmid pMPNB*noeE* in which the *nod* box of *noeE* is transcriptionally fused to a promoterless *lacZ* gene is carried on a broad-host-range plasmid, pMP220 (Spaink et al. 1987). When pMPNB*noeE* was transferred into both the mutant and wild-type strains, apigenin (24 h exposure) induced similar activities in NGRΔ*syrM1* (3,047 MUs) and NGR234 (3,085 MUs).

To test the effect of SyrM1 on expression of the *nodPQ* loci, we performed competitive RNA hybridization assays on Southern blots of three cosmids that contain the two *nodPQ* copies. Two overlapping cosmids, pXB559 and pXB1130, carry one copy of *nodPQ* (on a 3.1-kb *EcoRI* fragment) while the second is carried on a 4.3-kb *EcoRI* fragment of pXB1112. Competitive hybridization only labeled the 4.3-kb *EcoRI* fragment of pXB1112 (Fig. 4; the upper band corresponds to

the cosmid vector), suggesting that SyrM1 only activates one of the two copies of *nodPQ*.

SyrM1 and host specificity.

The influence of *syrM1* on nodulation of several NGR234 hosts possessing both determinate and indeterminate nodules was tested. NGRΔ*syrM1* produced fewer nodules than NGR234 on some hosts (Table 3), but the nodules formed on *Flemingia congesta* and *Robinia pseudoacacia* were less efficient in nitrogen fixation. Gas-chromatographic assays confirmed that nodules induced by NGRΔ*syrM1* on *Robinia pseudoacacia* reduced approximately 40% less acetylene to ethylene ($7.6 \pm 0.3 \mu\text{mol} \cdot \text{h}^{-1} \cdot \text{plant}^{-1}$) than when plants were inoculated with NGR234 ($11.9 \pm 0.3 \mu\text{mol} \cdot \text{h}^{-1} \cdot \text{plant}^{-1}$).

DISCUSSION

In *R. meliloti*, *syrM* induces *nod*-gene expression via *nodD3* and *exo*-gene expression via *syrA* (Mulligan and Long 1989; Maillet et al. 1990; Sharma and Signer 1990; Kondorosi et al. 1991a; Demont et al. 1994). As an example of this synergism, re-introduction of *syrM* together with *nodD3* on a multicopy plasmid into *R. meliloti* results in high-level expression of inducible *nod* genes even in the absence of flavonoids.

Fig. 3. Reverse phase-thin layer chromatography (RP-TLC) analysis of lipo-chitin-oligosaccharides (LCOs) purified from wild-type NGR234 and an *syrM1* mutant (NGRΔ*syrM1*). Appropriate cultures were labeled with [¹⁴C]-D-glucosamine, and the RP-TLC exposed to X-ray film. Spots A and B indicate the position of nonsulfated and sulfated LCOs, respectively.

Table 2. β-Galactosidase activity of NGR234 and NGRΔ*syrM1* harboring transcriptional fusions of *nodB* and *nodW* to a *lacZ* reporter gene

Strains	β-Galactosidase activity ^a (Miller units)	
	Noninduced	Apigenin-induced
NGR234(pMP220)	52 ± 8	76 ± 5
NGR234(pMP220B)	618 ± 14	4,811 ± 102
NGRΔ <i>syrM1</i> (pMP220B)	67 ± 16	2,087 ± 74
NGR234(pMP220W)	227 ± 72	3,146 ± 164
NGRΔ <i>syrM1</i> (pMP220W)	72 ± 3	109 ± 40

^a Values are the means ± standard errors of triplicate assays. Cultures were induced during 24 h with 10⁻⁶ M apigenin.

Two copies of *syrM*, *syrM1* and *syrM2*, are present on pNGR234a. RP-TLC analysis showed that an *syrM1* mutant produced 10 times fewer sulfated Nod factors than the wild-type bacterium. Apparently this is because SyrM1 is involved in activation of one of the two copies of *nodPQ*. In *R. meliloti*, NodPQ are required for the synthesis of PAPS, the activated sulfate donor. This suggests that the decrease in sulfated Nod factors may be due to a diminution in the pool of PAPS available for Nod factor synthesis. Sulfation of *R. tropici* Nod factors was also found to be limited by the intra-cellular concentrations of activated sulfate (Poupot et al. 1995). Three times more sulfated NodNGR factors are acylated with C_{18:1} than with C_{16:1}. As mutation of *syrM1* causes an overall reduction in Nod factor production, it is likely that the amounts of sulfated LCOs N-acylated by C_{16:1} are reduced to undetectable levels.

Competitive RNA hybridizations showed that SyrM1 is also a transcriptional regulator of several plasmid-borne loci. Genes such as *nolB* and *nolW* that are under the control of SyrM1 are only expressed late in infection (Fellay et al. 1995). In apparent contradiction to the results of Freiberg et al. (1997), who did not detect any activity in the absence of the inducer, *nolB* is still expressed, albeit at low levels. Perhaps this is because the corresponding transcript pool is so small that it cannot be detected by RNA hybridization.

Together, these data show that *syrM1* is involved both early in the symbiosis (in the production of sulfated Nod factors) and later during infection, suggesting that *syrM1* is continuously expressed during these stages. Several genes (but not *nolXWBTUV*) seem to be under the control of *nod*-box promoters and SyrM1 might interact directly or indirectly (via another

regulator) with them. As *syrM1* of *R. meliloti* needs *nodD3* to co-regulate *nod*-gene expression, *syrM1* of NGR234 may need other regulator(s). NodD1 is required for the induction of many of these genes (Fellay et al. 1995), and a regulatory cascade involving *nodD1* and *syrM1* may activate a number of symbiotic genes. Alternatively, *syrM1* could act cooperatively with *nodD1* to ensure optimal expression of these genes.

Regulation of *syrM1* itself has proven more difficult to study. Several transcriptional fusions between *lacZ* and the 5' region of *syrM1* were constructed but none permitted detection of β -galactosidase activity (data not shown). Similar, negative results were obtained with *syrM* of *R. meliloti* (Sharma and Signer 1990). Mutations in *syrM1* of NGR234 (this work) or *syrM* of *R. meliloti* (Barnett and Long 1990) have only minor effects on nodulation. One possibility is that *syrM1* might ensure a network of regulation whose biological role is to optimize factors controlling efficient nodulation and specific recognition between the symbiotic partners. A second copy of *syrM* also exists: *syrM2* may mask or complement the function of *syrM1*. In contrast, *syrM2* is under the control of a *nod*-box promoter and is induced by flavonoids (Freiberg et al. 1997). We are currently investigating the effects of single and multiple mutations in these regulators and their effects on host-specific nodulation.

MATERIALS AND METHODS

Media, growth conditions, and bacterial matings.

Bacterial strains and plasmids used in this study are listed in Table 4. *Rhizobium* strains were grown at 27°C on RMM medium (Broughton et al. 1986) containing succinate as the carbon source. Apigenin was added at 10⁻⁶ M. Antibiotics were added at the following concentrations: gentamycin (Gm), 15 μ g · ml⁻¹; rifampicin (Rif) and spectinomycin (Sp), 100 μ g · ml⁻¹; and tetracycline (Tc), 25 μ g · ml⁻¹. Broad-host-range plasmids were mobilized from *Escherichia coli* DH5 α into *Rhizobium* by tri-parental matings with the helper plasmid pRK2013 (Ditta et al. 1980).

Construction of an *syrM1* mutant.

A 5.3-kb *Hind*III-*Eco*RI fragment containing *syrM1* obtained from the cosmid pXB807 was cloned into pBluescript

Fig. 4. Competitive RNA hybridization to a Southern blot of cosmid DNA covering the two chromosomal copies of the *nodPQ* genes of *Rhizobium* sp. NGR234. Left, ethidium bromide-stained DNA of cosmids digested with *Eco*RI, and separated on a 1% (wt/vol) agarose gel. Right, autoradiograph obtained after hybridization with competitor RNA isolated from NGR Δ *syrM1* 24 h after induction with 10⁻⁶ M apigenin. The blot was probed with ³²P-labeled RNA isolated from NGR234 induced under the same conditions. Lambda DNA digested with *Bst*EII was used as the marker.

Table 3. Average numbers of nodules formed on plants following inoculation with NGR234 or NGR Δ *syrM1*^a

Plant ^{b,c}	NGR234	NGR Δ <i>syrM1</i>
<i>Cajanus cajan</i>	14 $\pm$ 1	13 $\pm$ 2
<i>Calopogonium caeruleum</i>	13 $\pm$ 2	9 $\pm$ 1
<i>Desmodium intortum</i>	19 $\pm$ 2	17 $\pm$ 1
<i>Flemingia congesta</i>	21 $\pm$ 2	18 $\pm$ 1
<i>Lablab purpureus</i>	35 $\pm$ 1	29 $\pm$ 1
<i>Leucaena leucocephala</i>	15 $\pm$ 2	15 $\pm$ 2
<i>Macroptilium atropurpureum</i>	31 $\pm$ 1	20 $\pm$ 4
<i>Pachyrhizus tuberosus</i>	5 $\pm$ 1.0	4 $\pm$ 1.5
<i>Psophocarpus palustris</i>	5 $\pm$ 0.9	5 $\pm$ 1.2
<i>Robinia pseudoacacia</i>	5 $\pm$ 0.5	4 $\pm$ 1.2
<i>Tephrosia vogeli</i>	22 $\pm$ 2	14 $\pm$ 2
<i>Vigna unguiculata</i>	32 $\pm$ 2	30 $\pm$ 2

^a Mean value for 16 to 24 plants. Standard deviations are indicated.

^b Plants were harvested 6 weeks after inoculation.

^c Botanical names and sources of seeds from S. G. Pueppke and W. J. Broughton (unpublished).

SK⁺, which was linearized with the same enzymes. Then, this fragment (p807HEX) was digested with *SpeI*-*ApaI* and cloned into the *SpeI*-*ApaI* sites of the suicide vector pJQ200 SK (Quandt and Hynes 1993) to give pJQ807HEX. Digestion of pJQ807HEX with *Bam*HI results in deletion of an internal 1.1-kb fragment of the *syrM1* gene (the *Bam*HI site in the polylinker was deleted after *SpeI*-*ApaI* digestion). A Sp^r Omega interposon (Prentki and Krisch 1984) was ligated into this deleted construct (pJQΔBH1.1). pJQ200SK carries the *sacB* gene of *Bacillus subtilis*, which is inducible by sucrose and lethal when expressed in gram-negative bacteria. The recombinant plasmid was then mobilized into NGR234 with tri-parental matings as described above. Transconjugants were selected and purified on RMM plates containing Rif (100 µg · ml⁻¹ and Sp (100 µg · ml⁻¹). Single colonies were grown in liquid RMM and spread on plates containing both antibiotics and 5% (wt/vol) sucrose. Marker exchange by double cross-over giving NGRΔ*syrM1* was confirmed by Southern transfer analysis.

Plant assays.

Nodulation tests were performed in Magenta jars as described previously (Lewin et al. 1990). All plants were grown at a day temperature of 28°C, a night temperature of 18°C, and a light phase of 16 h. Acetylene reduction assays on *Robinia pseudoacacia* were performed as described previously (Williams and Broughton 1979).

RNA extraction, labeling, and competitive hybridizations.

Rhizobium cultures were harvested at an optical density (*A*₆₀₀) of 0.4 to 0.6 and the RNA extracted as described previously (Fellay et al. 1995). Radioactively labeled RNA probes (10 µg) were prepared as follows: total NGR234 RNA was partially digested by treatment with 0.125 M NaOH for 25 min on ice. Terminal, 5' labeled fragments were prepared by incubating probe RNA with T4 polynucleotide kinase and [γ -³²P]-ATP for 90 min at 37°C. Finally, labeled RNA was separated from the unreacted components of the reaction mixture by passage through a Sephadex G50 column (Pharmacia Fine Chemicals, Uppsala, Sweden).

Competitive hybridizations were performed on GeneScreen Plus Nylon membranes (NEN Research Products, Boston,

MA) containing digested cosmid DNA (Fellay et al. 1995). Unlabeled RNA (100 µg) from NGRΔ*syrM1*-induced cultures was used for prehybridization (as competitor RNA) and 10 µg of labeled RNA extracted from NGR234-induced cultures was added as the probe.

Cloning and expression of the *nolB*-*nolW* intergenic region.

Two primers designed to amplify the *nolB*/*nolW* intergenic region—IBW1: 5'GGGGAGACTGTTAGAGATTG3'; IBW3: 5'GCAACGCGGCAGAGAGATCC3'—were synthesized. Together with the cosmid pXB110 (which contains the *nolXWBTUV* locus), these primers were used to amplify the intergenic region between *nolB* and *nolW*. Reactions were performed in a final volume of 50 µl containing 1 mM of each primer, 0.1 mM deoxynucleosides triphosphates, 1× thermophilic DNA buffer supplemented with 1.5 mM MgCl₂ and 1 unit *Taq* DNA polymerase. Cycles (35) at 94°C for 30 s, 52°C for 45 s, and 72°C for 30 s were followed by incubation at 72°C for 5 min. A 360-pb PCR product was cloned into both the *EcoRV*-*XbaI* sites of pBluescript KS⁺ and the *SmaI*-*XbaI* sites of pBluescript SK⁺. Both products were then cloned into pMP220, the first as a *KpnI*-*XbaI* fragment and the second as an *XbaI*-*PstI* fragment generating pM220W (carrying the *nolW* promoter) and pM220B (carrying the *nolB* promoter), respectively. *Rhizobium* strains containing pM220W or pM220B were grown in RMM media supplemented with 25 mg · ml⁻¹ tetracycline. When the optical density (*A*₆₀₀) reached 0.1, apigenin was added to a final concentration of 10⁻⁶ M. Levels of β-galactosidase were measured according to Miller (1972).

Purification of Nod factors.

Rhizobia were raised at 27°C in RMM medium with or without 10⁻⁶ M apigenin (Price et al. 1992). Cells were grown to an *A*₆₀₀ of 1. After centrifugation (7,000 × *g*, 30 min, 4°C), extracellular Nod factors were extracted from the supernatant and purified by RP-HPLC as described previously (Price et al. 1992; Jabbouri et al. 1995).

Analytical methods.

Rhizobium cultures were made radioactive by addition of [1-¹⁴C]-D-glucosamine (54 mCi/mmol) to the culture medium

Table 4. *Rhizobium* strains and plasmids used in this study

Strains/plasmids	Characteristics ^a	Source/reference
Strains		
NGR234	Rif ^r derivative of wild-type strain NGR234 from <i>Lablab purpureus</i>	Stanley et al. 1988
NGRΔ <i>syrM1</i>	<i>syrM1</i> deletion mutant, Rif ^r Sp ^r	This work
Plasmids		
pBluescript-KS ⁺	Amp ^r	Stratagene, La Jolla, CA
pBluescript-SK ⁺	Amp ^r	Stratagene
pJQ200 SK	pACYC184-derived (p15A) suicide vector, Gm ^r	Quandt and Hynes 1993
pMP220	<i>IncP</i> expression vector containing a promoterless <i>lacZ</i> gene, Tc ^r	Spaink et al. 1987
PMPNB <i>noeE</i>	960 bp <i>HindIII</i> - <i>SphI</i> fragment containing the <i>nod</i> box of <i>noeE</i> cloned in pMP220	Hanin et al. 1997
pM220B	Polymerase chain reaction (PCR) fragment containing the <i>nolB</i> promoter cloned in <i>XbaI</i> - <i>PstI</i> sites of pMP220	This work
pM220W	Polymerase chain reaction fragment containing the <i>nolW</i> promoter cloned in <i>KpnI</i> - <i>XbaI</i> sites of pMP220	This work
pBBR1-MCS5	Broad-host-range cloning vector, Gm ^r	Kovach et al. 1994
pBR- <i>syrM1</i>	A 5.6-kb <i>HindIII</i> - <i>EcoRI</i> fragment containing <i>syrM1</i> cloned in pBBR1-MCS5	This work
pRK2013	ColE1 replicon with RK2 tra genes, Nm ^r Km ^r	Figurski and Helinski 1979

^a Amp^r, ampicillin, Gm^r, gentamycin, Km^r, kanamycin, Rif^r, rifampicin, Sp^r spectinomycin, Tc^r, tetracycline, and Nm^r, neomycin, resistance.

and grown to the exponential phase. After centrifugation, the supernatant was applied to a C₁₈ reverse-phase Sep-Pak (Millipore, Milford, MA), and the columns rinsed with 5 ml of H₂O. Nod factors were eluted with 5 ml of methanol and, after vacuum drying were dissolved in methanol and applied to TLC plates (C₁₈ reverse phase—high performance thin layer chromatography, RP-HPTLC; Aldrich-Chemical, Gillingham-Dorset, UK). Development was performed with 9:1 (vol/vol) methanol/5.5 M ammonia as the running solvent. Mass spectra were recorded on an Autospec instrument (Fisons, VG-Analytical, Manchester, U.K.) fitted with a caesium-ion gun working at 20 kV. The acceleration voltage was 8 kV. A 1:1 mixture of *meta*-nitrobenzyl alcohol and glycerol was used as the matrix. Matrices were acidified with 0.1 M trifluoroacetic acid, or spiked with 10% sodium iodide for use in the positive-ionization mode.

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