Identification and Characterization of a Gene on *Rhizobium meliloti* pSyma, *syrB*, That Negatively Affects *syrM* Expression

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The *Rhizobium meliloti* SyrM protein activates transcription of *nodD3* and *syrA*. Regulation of *syrM* is complex and may involve as yet undiscovered genes. Here we report the isolation of insertion mutants showing increased expression of a *syrM-gusA* gene fusion. Characterization of one mutant strain, designated SYR-B, revealed a mutation consisting of a transposon insertion linked to a large deletion. The corresponding wild-type DNA was cloned as a 5.3-kb *Bam*HI fragment. Genetic and physical analysis of this DNA demonstrated that an open reading frame (ORF) near one end of the fragment, encoding the 16.5-kDa SyrB protein, is responsible for the repression of *syrM* activity. Results of complementation experiments with the 5.3-kb *Bam*HI DNA led us to hypothesize that other genes within this DNA fragment interfere with the expression or activity of SyrB. Our analysis showed that the region upstream of *syrB* contains three ORFs. One ORF is similar to the Ros repressor of *Agrobacterium tumefaciens* and the MucR repressor of *R. meliloti*.

Rhizobium meliloti (also termed *Sinorhizobium meliloti*; De Lajudie et al. 1994) induces nitrogen-fixing nodules on certain species of leguminous plants. Early events in nodule initiation are triggered by an exchange of signals between the bacterium and host plant. The signal synthesized by *R. meliloti* is a β -1,4 linked tetramer or pentamer of *N*-acetyl-glucosamine that is mono-*N*-acylated on the terminal nonreducing subunit and 6-*O*-sulfated on the reducing sugar (Lerouge et al. 1990). The *nod* and *nol* genes encode proteins responsible for the synthesis of these extracellular lipochitooligosaccharide compounds (for reviews see Fisher and Long 1992; Dénarié and Cullimore 1993).

In all species of *Rhizobium* studied thus far, expression of the *nod* genes is controlled by the transcriptional activator, NodD (reviewed in Schlaman et al. 1992). NodD proteins bind to a specific sequence in *nod* gene promoters called the *nod* box (Rostas et al. 1986; Fisher et al. 1988; Fisher and Long 1989). *R. meliloti* has three *nodD* genes (Honma and Ausubel 1987; Györgypal et al. 1988). NodD1 activates *nod* gene transcription in the presence of the plant flavonoids lute-

olin and methoxychalcone (Peters et al. 1986; Maxwell et al. 1989). Betaines, such as trigonelline and stachydrine, as well as methoxychalcone, stimulate *nod* gene expression via NodD2 (Hartwig et al. 1990; Phillips et al. 1992). NodD3 activates expression independently of any plant-produced compound (Mulligan and Long 1989; Maillet et al. 1990; Kondorosi et al. 1991; Swanson et al. 1993). Instead, NodD3 depends on the *syrM* gene product for its expression (Maillet et al. 1990; Swanson et al. 1993). SyrM has amino acid sequence similarity to NodD proteins (Barnett and Long 1990) and like NodD is a member of the LysR family of positive transcriptional activators (Henikoff et al. 1988). NodD3 itself can activate transcription of *syrM*, probably by interacting with a *nod* box located upstream of the *syrM* transcription start site (Swanson et al. 1993; Barnett et al. 1996). SyrM can also activate its own expression, but to much lower levels than with NodD3 (Swanson et al. 1993).

SyrM also activates transcription of *syrA* (Mulligan and Long 1989); overexpression of *syrA* results in extremely mucoid colonies, probably via effects on the exopolysaccharide (EPS) synthesis pathway (Mulligan and Long 1989). Therefore, SyrM may be viewed as a global regulatory protein that influences both Nod factor synthesis and EPS I production.

The *syrM-nodD3-syrA* regulatory circuit appears unique to *R. meliloti* and little is known about the precise role of *syrM* in symbiosis. Perhaps *syrM* plays a role in host specificity; overexpression of *syrM*, *nodD3*, and *syrA* extends the host range of *R. meliloti* to siratro (*Macroptilium atropurpureum*), although the mechanism by which this occurs is not known (Kondorosi et al. 1991). Strains with mutations in *nodD3* and *syrM* form effective nodules on alfalfa (*Medicago sativa*), albeit with a slight delay (Mulligan and Long 1989; Kondorosi et al. 1991); however, these strains do not form effective nodules on another host plant, *Medicago truncatula* (L. Smith, unpublished data).

SyrM likely activates expression of other genes. Overexpression of *syrM* and *nodD3* leads to production of C18-C26 (ω -1)-hydroxylated fatty acid-containing Nod factors (Demont et al. 1994). Synthesis of these factors also occurred when *syrM* was overexpressed in the absence of *nodD3* (Demont et al. 1994).

Other data suggest additional roles for SyrM. Although *syrM* is expressed at low levels when grown in aerobic or microaerobic cultures, it is also expressed in the nitrogen fixa-

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tion zone of mature nodules (Sharma and Signer 1990; Swanson et al. 1993). Because *nodD3* is not expressed in nitrogen-fixing cells (Sharma and Signer 1990; Swanson et al. 1993), the symbiotic role of *syrM* in planta is probably not the activation of *nodD3*. Similarly, *syrA* is expressed in mature nodules, but *syrM* is not required for its expression (M. Barnett and J. Swanson, unpublished data). These observations suggest that *syrM* may perform different functions in planta than in free-living bacteria. We therefore hypothesized that *syrM* expression is maintained at low levels in the free-living state via repression. Here we report the isolation of a mutant that is derepressed for *syrM* expression, and the genetic and physical characterization of the corresponding locus, *syrB*.

RESULTS

Isolation of mutants with increased expression of *syrM*.

Previous experiments have shown that *syrM* is expressed at low levels in free-living *R. meliloti* (Swanson et al. 1993). If the low level of *syrM* expression observed in bacterial cultures is maintained via repression, then transposon insertion in the repressor should permit increased *syrM* expression in free-living cells. We used Tn5 to mutagenize *R. meliloti* strain JM142, which contains a transcriptional *gusA* fusion in the *syrM* open reading frame (ORF) (Swanson et al. 1993), and screened for increased levels of β -glucuronidase (GUS) activity with a fluorescence assay as described in Materials and Methods.

Of 3,200 Tn5 mutants, three showed GUS activity threefold to fivefold higher than that of the JM142 parental strain (Table 1). MB100 showed the largest increase in GUS activity, about fivefold greater than that of JM142 (Table 1). N3 transduction failed to link the Tn5 insertion in MB100 to *syrM*. The phenotype transduced 100% with neomycin resistance of Tn5. This mutant, designated SYR-B (SYR = SYmbiotic Regulator), was further characterized.

A second mutant, MB110, also had higher GUS activity than the JM142 parent strain, but this phenotype was unlinked to neomycin resistance of the Tn5 and therefore not a result of the Tn5 insertion. In contrast to MB100, the phenotype of MB110 displayed linkage to *syrM*; transduction showed that the mutation was about 40 kb from *syrM*. Since *nodD2* is located about 40 kb from *syrM*, we predicted that the mutation in MB110 was in *nodD2*. pRmM137, which contains *nodD2* on a 6.8-kb *EcoRI* fragment (Fisher et al. 1988), suppressed the mutant phenotype of MB110 (Table 1), while a control plasmid containing *syrB*, pMB154, failed to suppress completely the mutation in MB110. However, a derivative of JM142 containing a *nodD2::Tn5* insertion, MB114, failed to show increased expression of the *syrM::gusA* fusion (Table 1).

A third mutant, MB120, showed the least elevation of GUS activity of the three mutants. Here the phenotype always transduced with Tn5, localizing it to within 1.5 kb of *syrM*.

None of the three mutants were impaired in nodulation ability or conferred an altered pattern of *syrM-gusA* expression in the symbiotic state (data not shown).

Cloning *syrB*.

As a first step to further characterization of the SYR-B mutation, we transduced the Tn5 from mutant strain MB100 into wild-type *R. meliloti* Rm1021 to create MB101. From this

strain, we cloned DNA adjacent to the Tn5 and used this DNA to screen a genomic library (see Materials and Methods). All positive clones contained a 5.3-kb *BamHI* fragment that hybridized to the SYR-B::Tn5 probe (Fig. 1A). We confirmed the location of the Tn5 by sequencing across the Tn5 junction and comparing this sequence to that of the 730-bp *BamHI-SstI* fragment on the left end of the 5.3-kb *BamHI* fragment shown in Figure 1A.

Complementation of the *syrB* mutant phenotype.

In order to test the ability of the putative *syrB* clone to complement the Tn5 mutation, we constructed a variety of subclones (Fig. 1B) in the broad host range expression vector pTE3 (Egelhoff and Long 1985). pTE3 contains a polylinker that lies between the *Salmonella typhimurium trp* promoter, from which expression is strongly constitutive in *R. meliloti*, and an *Escherichia coli rpoC* terminator. All inserts, with the exception of pMB121, were cloned in both orientations downstream of the *trp* promoter. These plasmids were conjugated into MB100 and the SYR-B phenotype was evaluated by performing GUS assays as described in Materials and Methods. A clone complementing the mutant phenotype should suppress GUS activity to levels observed in the JM142 *syrM::gusA* fusion strain. The results of these assays are shown in Table 2.

The 5.3-kb *BamHI* fragment in either orientation behind the *trp* promoter failed to complement the SYR-B mutant phenotype (Table 2, plasmids pMB109 and pMB110). A 3-kb *BamHI-EcoRI* fragment also failed to complement the mutation (Table 2, plasmids pMB111 and pMB112). However, a 2-kb *EcoRI* fragment and a 1.6-kb *PstI-BamHI* fragment each suppressed the SYR-B mutant phenotype (Table 2, plasmids pMB154 to 157). Complementation was somewhat dependent on the *trp* promoter: the lowest GUS levels were observed when the insert was cloned such that the *trp* promoter directed expression from left to right as shown in Figure 1 (Table 2, plasmids pMB155 and pMB157). pMB155 conferred levels of GUS activity about 15-fold lower than those of the SYR-B mutant without a plasmid. In JM142, plasmids pMB154, pMB155, pMB156, and pMB157 suppressed *syrM* expression to levels lower than those observed for this strain without a plasmid (Table 2).

That DNA in the left half of the 5.3-kb *BamHI* insert (Fig. 1) interferes with ability to complement was confirmed by deleting a 2-kb *SstI* fragment from pMB109. This deletion allowed complementation by the remainder of the 5.3-kb *BamHI* DNA (Table 2, plasmid pMB121).

Table 1. β -glucuronidase assays of mutant strains^a

Strain	Units β -glucuronidase ^b
JM142 (<i>syrM-gusA</i>)	9.2 $\pm$ 1.4
MB100 (JM142, Tn5 #31-16)	45.8 $\pm$ 9.9
MB110 (JM142, Tn5 #75-2)	37.4 $\pm$ 1.3
MB110 pMB154	17.1 $\pm$ 2.2
MB110 pRmM137	12.3 $\pm$ 0.3
MB114 (JM142, <i>nodD2::Tn5</i>)	7.4 $\pm$ 1.2
MB120 (JM142, Tn5 #51-8)	33.2 $\pm$ 1.2
MB120 pMB154	13.6 $\pm$ 1.8

^a Spectrophotometric assay (see Materials and Methods).

^b Each data point represents the average of at least three independent assays. Standard deviation from the mean is given as a $\pm$ value. Units = [OD₄₁₅/(time $\times$ cell volume $\times$ OD₆₀₀)]100,000.

The SYR-B mutant strain contains a deletion.

In designing the complementation experiments, we expected DNA from the leftmost region of the 5.3-kb *Bam*HI clone to complement the SYR-B mutation because the Tn5 insertion was located here. Results from Southern hybridization analyses with the 1.6-kb *Pst*I-*Bam*HI fragment as a probe suggested that the Tn5 insertion is linked to a deletion in

MB100. We mapped the extent of this deletion by Southern blots, hybridizing the 730-bp *Bam*HI-*Sst*I probe to Rm1021 and MB101 genomic DNA that had been digested with restriction enzymes that would allow us to detect a shift in band size in the *syrB* mutant, compared with wild-type Rm1021 DNA. The results of one such Southern hybridization experiment are shown in Figure 2A. When Rm1021 DNA is digested with *Eco*RV a 9-kb band is detected. The corresponding band in MB101 is greater than 20 kb (lanes 1 and 2). A 12-kb hybridizing band is seen when Rm1021 DNA is digested with *Sfi*I. This same enzyme detects an 8-kb band in DNA from the mutant. Because previous Southern hybridizations with a Tn5 probe had shown the entire Tn5 likely to be intact and a 2-kb *Sst*I probe showed no hybridization to mutant DNA (data not shown), we conclude that the SYR-B mutation consists of a Tn5 insertion that is linked to a deletion of about 9 kb. *Eco*RI, which cuts both mutant and wild-type DNA at a position about 6 kb from the leftmost *Bam*HI site, was used in double digests with *Eco*RV and *Sfi*I to map restriction sites in the deleted DNA (Fig. 2B).

These mapping results and the complementation assays support the idea that the *syrB* locus is contained within the 1.2-kb *Pst*I-*Eco*RI fragment of the larger *Bam*HI clone (Fig. 1A). The nucleotide sequence of this region revealed a single ORF encoding a protein larger than 15 kDa. The DNA sequence is shown in Figure 3 along with the ORF that encodes a protein of 16.5 kDa. This ORF specifies a protein that contains no cysteines and is highly charged, consisting of 15 mol% glutamate and aspartate, and 20 mol% histidine, lysine, and arginine. We found one plausible match in the GenBank data base: ORF3 of *Agrobacterium rhizogenes* has no known function and is located downstream of the *rep* genes of plasmid pRiA4b (accession no. X04833; Nishiguchi et al. 1987).

Table 2. Complementation of SYR-B mutant^a

Strain ^b	Plasmid ^c	Units β -glucuronidase ^d
JM142	None	9.2 $\pm$ 1.4
MB100	None	45.8 $\pm$ 9.9
MB100	pMB109	20.0 $\pm$ 1.4
MB100	pMB110	32.3 $\pm$ 2.5
MB100	pMB111	35.0 $\pm$ 2.7
MB100	pMB112	35.1 $\pm$ 3.7
MB100	pMB121	8.4 $\pm$ 1.5
MB100	pMB154	11.6 $\pm$ 2.4
MB100	pMB155	3.1 $\pm$ 1.1
MB100	pMB156	14.2 $\pm$ 1.2
MB100	pMB157	7.3 $\pm$ 1.0
MB100	pMB360	39.9 $\pm$ 5.0
MB100	pMB376	45.4 $\pm$ 5.2
MB100	pMB377	36.3 $\pm$ 9.4
JM142	pMB154	5.9 $\pm$ 0.4
JM142	pMB155	2.8 $\pm$ 0.1
JM142	pMB156	5.2 $\pm$ 0.3
JM142	pMB157	4.3 $\pm$ 0.5

^a Spectrophotometric assay (see Materials and Methods).

^b JM142 is the parental *syrM-gusA* fusion strain. MB100 is *syrM-gusA* with Tn5 #31-16, SYR-B.

^c pMB109-pMB157 are diagrammed in Figure 1. pMB360 is pMB155 with *lacZ*YA fusion in *syrB*. pMB376 and pMB377 contain *syrB* with frameshift mutation.

^d Each data point represents the mean of at least two independent assays. Standard deviation from the mean is given as a $\pm$ value. Units = [OD₄₁₅/(time $\times$ cell volume $\times$ OD₆₀₀)]100,000.

Fig. 1. Map of *syrB* region and clones. **A**, Restriction map of the 5.3-kb *Bam*HI clone containing *syrB*. Location of the Tn5 insertion in MB100 is marked with an arrow. **B**, Clones used in complementation assays of the *syrB* mutant. Each line represents a subclone of the 5.3-kb *Bam*HI clone of **A** that was tested in pTE3 for ability to complement the *syrB* mutant. Arrowheads show orientation of each insert with respect to the *trp* promoter of pTE3. A neomycin resistance cassette that replaces the 2-kb *Sst*I fragment is indicated by a hatched line. Abbreviations: B, *Bam*HI; P, *Pst*I; S, *Sst*I; RI, *Eco*RI.

Mutations in *syrB* abolish complementation.

To demonstrate that this ORF encodes *syrB*, we created two different mutant derivatives of the complementing plasmid, pMB155, and assayed them for their ability to complement the *syrB* mutation. The first mutant plasmid was discovered by screening a set of pMB155-Tn3::lacZYA derivatives for loss of ability to complement. One derivative, pMB360, contained a Tn3 insertion in the fifth codon of the putative *syrB* gene. This insertion also creates an active fusion to *lacZYA*. The second mutant plasmid, pMB376, was created by site-directed mutagenesis as described in Materials and Methods. This mutant has a G residue inserted at position 581, as shown in Figure 3; translation of this mutated ORF would result in a truncated protein. When mated into MB100 and screened for GUS activity, both of these mutants failed to complement the SYR-B phenotype, supporting the hypothesis that the 16.5-kDa ORF encodes *SyrB*. That *syrB* is expressed is supported by the fact that we obtained Tn3-*gusA* gene fusions in the *syrB* ORF with high levels of GUS activity (Fig. 4). The insertion points of these gene fusions, nos. 38, 95, and 99, are shown in Figure 3.

syrB is located on pSymb.

R. meliloti possesses three replicons. Many of the genes necessary for symbiotic functions are located on the two large plasmids, pSymb and pSymA. It is possible to mobilize each of the *sym* plasmids separately into *Agrobacterium tumefaciens*, thereby isolating the *sym* plasmids from each other and from the *R. meliloti* chromosome (Schwedock and Long 1992). To determine the location of *syrB* we used a 730-bp

*Bam*HI-*Sst*I fragment to probe a Southern blot that contained DNA from several such *A. tumefaciens* strains (Fig. 5). This *syrB*-region probe hybridizes to a 5.3-kb *Bam*HI and a 9-kb-*Eco*RI DNA (Fig. 5, arrowheads). These bands are seen only in DNA from *A. tumefaciens* strain A136 that carries pSymb, not in wild-type A136 or A136/pSymb. We concluded that the *syrB* locus is probably located on pSymb and attempted genetically to link *syrB* to another locus on pSymb. The loci characterized thus far are contained within a 260-kb region of the 1,340-kb megaplasmid (Honeycutt et al. 1993). *syrB* did not transduce with known loci at opposite ends of this region (data not shown).

The 5.3-kb *Bam*HI DNA contains at least three additional genes.

We determined the nucleotide sequence of the entire 5.3-kb *Bam*HI DNA. In addition to the *syrB* ORF, this sequence analysis revealed three upstream ORFs (Fig. 4). We used Tn3-pHoHoGUS, as described in Materials and Methods, to introduce β -glucuronidase gene fusions onto the 5.3-kb *Bam*HI DNA (Fig. 4). As mentioned above, three very active fusions, nos. 38, 95, and 99, are located in *syrB*.

Several less active fusions map to ORF2 (nos. 1, 15, 17, 42,

Fig. 2. Southern hybridization analysis of SYR-B mutant. **A**, Southern blot of Rm1021 and MB101 DNA that was hybridized to a 730-bp *Bam*HI-*Sst*I probe from pMB99 as described in Materials and Methods. All lanes labeled "A" contain DNA from Rm1021; lanes labeled "B" contain DNA from MB101. **B**, Map showing extent of deletion. Location of the Tn5 is marked with an arrow; DNA deleted from the mutant is represented by a dashed line. A restriction map of the same region in wild-type Rm1021 is shown below. Extent of the 5.3-kb *Bam*HI insert of pMB99 is designated with a hatched line. Abbreviations: B, *Bam*HI; P, *Pst*I; S, *Sst*I; RI, *Eco*RI; RV, *Eco*RV; Sf, *Sfi*I.

Fig. 3. Nucleotide sequence of the *syrB* locus. Nucleotide in the first position corresponds to nucleotide no. 3721 of sequence available in GenBank (accession no. U90220). Predicted amino acid sequence of the *syrB* open reading frame is shown below the DNA sequence. Insertions of Tn3-*lacZYA* (hatched triangle) and Tn3-*gusA* (plain triangles) are marked, as is location of the introduced frameshift mutation, G581 (G). A potential ribosome binding site is overlined.

and 156), which overlaps and is transcribed divergently from ORF3. ORF2 encodes a polypeptide of 15.5 kDa and may begin with either a GTG or TTG start codon. The amino acid sequence of ORF2 is 53% similar to that of the *ros* repressor of *Agrobacterium tumefaciens* (Fig. 6). *Ros* is a specific repressor of the *A. tumefaciens virC* and *virD* operons (Cooley et al. 1991) and has been shown to bind a specific sequence called the "Ros box" (D'Souza-Ault et al. 1993). ORF2 is also 50% similar to MucR (Keller et al. 1995). Mutations in *mucR* confer a mucoid colony morphology to *R. meliloti* (Zhan et al. 1989) because MucR negatively regulates transcription of genes required for galactoglucan (EPS II) synthesis (Keller et al. 1995). Neither overexpression of ORF2 nor mutations in ORF2 affected *R. meliloti* colonial morphology.

The *Ros* and *MucR* repressors possess a zinc fingerlike motif of the C₂H₂ type (Berg 1990), but this motif is not conserved in ORF2 (marked with asterisks in Figure 6). We do not know whether ORF2 functions as a repressor in *R. meliloti*.

ORF3 has several in-frame start codons and may encode a polypeptide of up to 50.8 kDa. ORF3 shows good *R. meliloti* codon usage, but has a weak ribosome binding site consensus sequence upstream of the first potential methionine start codon. The third in-frame methionine is preceded by a somewhat better consensus and begins an ORF of 46 kDa. A TFASTA search of GenBank revealed no significant sequence similarity to any other ORF in the data base. No Tn3 insertions in ORF3 that expressed β -glucuronidase were obtained in either screen, so in order to determine whether ORF3 is expressed in *R. meliloti*, we made a transcriptional β -glucuronidase fusion to ORF3. This fusion expressed β -glucuronidase both when plasmid-borne and when recombined into the *R. meliloti* genome (Fig. 5).

Most Tn3 insertions expressing high levels of β -glucuronidase mapped to a region downstream of ORF3. These insertions include nos. 76, 86, 87, 105, 110, 114, and 121. Sequence analysis confirmed the location of an ORF in this re-

Fig. 5. Hybridization of DNA from *Agrobacterium tumefaciens* pSym strains to *syrrB* region probe. DNA was digested separately with *Bam*HI and *Eco*RI, transferred, and hybridized as described in Materials and Methods; a 730-bp *Bam*HI-*Sst*I probe from pMB99 was used for hybridization. This probe hybridizes to a 5.3-kb *Bam*HI DNA and to a 9-kb *Eco*RI DNA (arrowheads). *A. tumefaciens* pSym strains were constructed as described previously (Schwedock and Long 1992).

Fig. 4. Tn3-*gusA* insertions in the *syrrB* clone. Positions of various Tn3-HoHoGUS insertions are indicated by open circles. Direction of *gusA* transcription is shown by two bold arrows above and below map: insertions on top half of map are oriented so that the *gusA* gene reads from left to right; those below are oriented so that *gusA* is expressed from right to left. Gray bars denote average levels of β -glucuronidase activity for plasmid-borne *gusA* gene fusion. Fusions with fewer than 20 units of β -glucuronidase are not shaded. Open triangle marks location of a *gusA* fusion in open reading frame (ORF) 3. Locations of Tn3 insertions were determined by restriction mapping, except for insertion nos. 38, 42, 86, 95, 99, 105, 150, and 156, whose locations were determined by DNA sequencing. Bold arrows denote locations of ORFs determined by DNA sequencing. GenBank accession number for nucleotide sequence of the 5.3-kb *Bam*HI DNA fragment is U90220.

gion with good *R. meliloti* codon usage. This ORF4 encodes a protein of 21.6 kDa that is composed of about 30 mol% charged residues. Both *syrB* and ORF4 encode small, highly charged proteins. Like *SyrB*, ORF4 contains no cysteine residues. ORF4 showed no sequence similarity to any other protein in the GenBank data base; however, a 39 amino acid segment in the carboxy terminus of ORF4 showed 53% amino acid sequence similarity to *SyrB*.

One insertion, no. 150, is located downstream of the *syrB* ORF and has not been assigned to an ORF because we lack additional downstream sequence.

Strains containing insertions in each of the four ORFs, and insertion no. 150, were able to form effective nodules on *M. sativa* (data not shown).

Phenotypes of *syrB* deletion strains.

We attempted to re-create the *SYR-B* mutant phenotype by constructing several deletion strains with the 5.3-kb *Bam*HI DNA as starting material. These derivatives along with the corresponding β -glucuronidase assay results are shown in Figure 7.

Two deletion derivatives that remove *syrB*, MB243 and MB240, showed a slight increase in *syrM* levels over background. The larger deletion of MB240 confers a larger increase in *syrM* expression. This deletion removes *syrB* as well as the region of DNA that interferes with complementation. It is plausible that whatever is contained within this interfering region has an additional effect on *syrM* expression.

MB202 contains an intact *syrB* gene and a 2-kb deletion of the *Sst*I fragment upstream of *syrB*; it produces slightly lower levels of *syrM-gusA* expression than the JM142 control. This lower activity might indicate that whatever interferes with *syrB* complementation when carried on a plasmid also curtails the activity of *syrB* when present in single copy in the Rm1021 genome.

None of the deletion strains derepress *syrM::gusA* expres-

sion to the same extent as the original *SYR-B* mutant strain. It seems likely from these results that DNA flanking the 5.3-kb *Bam*HI fragment and missing in MB100 contains a function additive or redundant to that of *SyrB*. Southern blots hybrid-

Strain	Units β -glucuronidase
MB202	6.1 $\pm$ 1.5
MB243	12.7 $\pm$ 0.4
MB240	17.7 $\pm$ 1.5
JM142	9.2 $\pm$ 1.4
MB100	45.8 $\pm$ 9.9

Fig. 7. Deletion mutants of *syrB* region. Hatched segment of each map shows the extent of DNA deleted from the 5.3-kb *Bam*HI clone. Deleted DNA was replaced with a 1.5-kb neomycin resistance cassette as described in Materials and Methods. Each deletion was marker exchanged into Rm1021 and then transduced into JM142 to assay the effect of each deletion on *syrM* expression. Assay results for each strain are shown compared with a strain containing only *syrM::gusA* (JM142) and the original MB100 mutant strain. Each number is a mean of four or more independent assays $\pm$ standard deviation from the mean.

ORF2	VPSFRPKYLH	SASLDFIGNR	LRRRSLTET.	..RSNGRRLE	LTSRIVSAYL
Ros			MTETA	YGNAQDLLVE	LTADIVAAYV
MucR			MTETS	LGTSNELLVE	LTAEIVAAYV
ORF2	SRNVIAPDEL	PYLIQQTYGS	LNETSGPGET	PPAVEEQRPA	VPIKKSVTDD
Ros	SNHVVPVTEL	PGLISDVHTA	LSGTSAPASV	AVNVEKQKPA	VSVRKSVDQD
MucR	SNHVVPVAEL	PTLIADVHSA	LNNITAPAPV	VVPVEKPKPA	VSVRKSVDQD
ORF2	FIVCLEDGKK	FKSLKRHLTT	KYGMTPDQYR	EKWKLPSSEYP	MTARNYALQR
Ros	HIVCLECGGS	FKSLKRHLTT	HHSMTPEEYR	EKWDLFVDYP	MVAPAYAEAR
MucR	QITCLECGGT	FKSLKRHLMT	HHNLSPEEYR	DKWDLFADYP	MVAPAYAEAR
ORF2	SKLARAMGLG	KSRALK			
Ros	SRLAKEMGLG	QRRKANR			
MucR	SRLAKEMGLG	QRRKRRGK			

Fig. 6. Amino acid sequence alignment of open reading frame (ORF) 2 with Ros and MucR. Alignment of ORF2 with *Agrobacterium tumefaciens* Ros (Cooley et al. 1991) and *Rhizobium meliloti* MucR (Keller et al. 1995) was performed with the PILEUP program (GCG software package; Devereux et al. 1984). Residues identical and conserved with respect to ORF2 are shaded. A putative C₂H₂ zinc fingerlike motif conserved between Ros and MucR is marked with asterisks. Conserved groupings are Ile, Leu, Val; Asp, Glu; Arg, His, Lys.

ized at high stringencies to a 1.6-kb *Pst*I-*Bam*HI DNA probe containing *syrB* do show additional hybridizing bands (data not shown). However, these bands were seen when DNA from the original *syrB* mutant strain was used for Southern analysis. If a nearby locus contributes to *syrM* repression, it is not structurally similar to *syrB* at a level detectable by high-stringency Southern hybridization analysis.

DISCUSSION

The positive transcriptional activator *syrM* is expressed at low levels in free-living *R. meliloti* strains. We have isolated three Tn5 mutants displaying increased *syrM* expression. These mutants still did not express *syrM* at levels as high as those seen when *nodD3* is overexpressed (Swanson et al. 1993). This suggests that the regulatory circuit controlling *syrM* expression involves multiple genes.

Two of the mutations were linked by transduction to *syrM*. In one of these mutant strains, MB120, the insertion is located within 1.5 kb of *syrM*. It is known that Tn5 insertions can have polar effects on nearby genes (Berg et al. 1980). Possibly this Tn5 insertion is near the *syrM* promoter and causes increased transcription of *syrM* from a Tn5 promoter. This idea is supported by the observation that the MB120 mutation was partially suppressed by a clone containing *syrB*, pMB154 (data not shown). We would expect this result if *SyrB* represses *syrM*.

The other strain contained a mutation, unlinked to the Tn5 insertion, that mapped about 40 kb from *syrM*. Since this mutation is not marked, we were not able to characterize it directly. Instead we found that a clone containing *nodD2* appeared to suppress its phenotype. However, a Tn5 insertion in *nodD2* did not have a stimulatory effect on *syrM-gusA* expression. Possibly the mutant phenotype in MB110 results from a *nodD2* mutation such as a missense mutation that is not mimicked in phenotype by a Tn5 null mutation. Alternatively, the mutation in MB110 may not be in *nodD2*, but in a nearby locus contained on the suppressing pRmM137. Finally, pRmM137 may restore *syrM* repression by an indirect mechanism rather than by true complementation.

Kondorosi et al. (1991) observed that, in *R. meliloti* strain Rm41, a *nodD2* mutation resulted in decreased activity of a plasmid-borne *syrM-lacZ* gene fusion. In Rm1021, we failed to find a stimulatory effect on activity of a *syrM-gusA* fusion when *nodD2* was overexpressed in the presence of appropriate inducer compounds (M. J. Barnett and J. A. Swanson, unpublished). These observations may reflect either strain-specific differences in *syrM* regulation or an indirect effect of *nodD2* on *syrM* expression.

In only one of these mutant strains, MB100, was the Tn5 insertion both linked to the mutation and unlinked to *syrM* (the SYR-B mutation). The mutation in MB100 is not a simple Tn5 insertion. We determined that this strain contains a deletion of about 9 kb adjacent to the Tn5 insertion site. DNA that contains the *syrB* ORF was sufficient to restore the phenotype of this complex mutant as well as to repress *syrM* expression in the JM142 parental strain.

syrB encodes a protein of 16.5 kDa and, through mutational analysis, was determined to be responsible for complementing the *syrB* mutant phenotype. The *syrB* coding sequence showed similarity to only one ORF in the GenBank data base, ORF3

from *A. rhizogenes* (accession no. X04833; Nishiguchi et al. 1987). ORF3 is located downstream from the *rep* genes of plasmid pRiA4b, but it is unclear whether ORF3 is actually required for replication functions (Nishiguchi et al. 1987).

When present on the same plasmid, a region upstream of *syrB* interfered with the ability of *syrB* to complement the SYR-B mutation. A 2-kb deletion in this upstream region restores complementation by *syrB*. Genetic and physical analysis revealed three ORFs within this deleted region. ORF2 has homology to known repressors *Ros* and *MucR*. ORF3 and ORF4 showed no significant similarity to sequences in the GenBank data base.

We tried to demonstrate inhibitory effects of this region on the *syrB-gusA* gene fusions. ORF2 had no significant effect of *syrB* expression when present alone, when present with ORF3, or when present with ORF3, ORF4, and *syrB* on a multicopy plasmid (data not shown). However, *syrB* alone on a plasmid, pMB155, did decrease expression of *gusA* fusions in ORF3, ORF4, and *syrB*. Basal levels of genomic ORF2-*gusA* fusions were too low to detect repression by *syrB*.

That regulation of *syrM* is complex is further demonstrated by the fact that we were unable to construct mutants similar in phenotype to the original MB100 mutant strain. Deleting *syrB* caused only a slight elevation of *syrM* expression, which increased a bit more if additional sequence upstream of *syrB* was deleted. None of the deletion strains had the same phenotype as the original mutant, which indicates there may be an additional locus contained within the region of DNA deleted in the original MB100 mutant strain that has a negative effect on *syrM* expression. If such a protein does exist it may, like *syrB*, decrease expression of upstream ORFs. This might explain why we were unable to demonstrate repression of *syrB-gusA* gene fusions by upstream DNA: in the SYR-B mutant this protein is missing; therefore, the upstream gene products may interfere with *syrB* expression or activity. In the *syrB-gusA* gene fusions this protein would be present and would prevent these same effects.

SyrB contains a high proportion of charged residues, a feature common to DNA-binding proteins; however, we could not identify any structural motifs similar to those in known DNA-binding proteins (Pabo 1992). *SyrB* has no amino acid sequence similarity to *NolR*, a repressor of *nodD1* and *nodD2*, previously identified in *R. meliloti* strain Rm41 (Kondorosi et al. 1989; Cren et al. 1995). The gene for this repressor is present in Rm1021, but encodes an inactive protein due to an insertional point mutation (Cren et al. 1994). The *syrM* transcription start site has been mapped (Barnett et al. 1996) and is upstream of a DNA fragment that demonstrates gel mobility shifting in both Rm1021 and Rm41 (Kondorosi et al. 1991). The protein responsible for this shifting has not been identified, but its putative binding to DNA downstream from the transcription initiation site is suggestive of a repressor. Future experiments will determine whether *SyrB* represses *syrM* expression by binding to *syrM* control sequences, or has an indirect effect on *syrM* expression.

MATERIALS AND METHODS

Bacterial genetic techniques.

Strains and plasmids used in this study are listed in Table 3. *E. coli* strains were grown in either Luria-Bertani (LB) or 2×

Table 3. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant characteristics ^a	Source
<i>Escherichia coli</i>		
MT616	MM294 pRK600, Cm ^r	Finan et al. 1986
pHoHoGUS, Tn3- <i>gusA</i> delivery system		B. Staskawicz
pSShe		
pHoHoLAC, Tn3- <i>lacZYA</i> delivery system		B. Staskawicz
pSShe		
<i>Rhizobium meliloti</i>		
Rm1021	<i>R. meliloti</i> RCR2011 Str ^r	Meade et al. 1982
JM142	Rm1021, <i>syrM::gusA</i> , Sp ^r	Swanson et al. 1993
MB100	JM142, Tn5 #31-16, SYR-B	This work
MB101	Rm1021, Tn5 #31-16, SYR-B	This work
MB110	1021, Tn5 #75-2	This work
MB114	JM142, <i>nodD2::Tn5</i>	This work
MB120	Rm1021, Tn5 #51-8	This work
MB202	JM142, 2-kb <i>SstI</i> Δ in <i>syrB</i> region	This work
MB240	JM142 <i>SstI-EcoRI</i> Δ <i>syrB</i> region	This work
MB243	JM142, 2-kb <i>EcoRI</i> Δ in <i>syrB</i> region	This work
<i>Agrobacterium tumefaciens</i>		
A136	<i>Agrobacterium tumefaciens</i>	Garfinkel et al. 1981; Watson et al. 1988
A136	A136 with pSyma from Rm5321	Schwedock and Long 1992
A136	A136 with pSymb from Rm5300	Schwedock and Long 1992
Plasmids		
pALTER	Cloning vector, Tc ^r	Promega (Madison, WI)
pBRNeo	pBR322 containing Nm ^r	D. Bramhill
pRmM137	pLAFR3, <i>nodD2</i> , Tc ^r	Fisher et al. 1988
pMB76	pUC119, 4-kb <i>BamHI</i> Tn5 31-16 Km ^r	This work
pMB98	<i>syrB</i> , 5.3-kb <i>BamHI</i> in pUC119	This work
pMB99	Same as pMB98, insert in opposite orientation	This work
pMB104	2-kb <i>SstI</i> from pMB98 in pUC119	This work
pMB109	<i>syrB</i> , 5.3-kb <i>BamHI</i> in pTE3	This work
pMB110	Same as pMB109, insert in opposite orientation	This work
pMB111	<i>syrB</i> , 3-kb <i>BamHI-EcoRI</i> in pTE3	This work
pMB112	Same as pMB111, insert in opposite orientation	This work
pMB121	<i>syrB</i> , 5.3-kb <i>BamHI</i> , <i>SstI</i> Δ in pTE3	This work
pMB154	<i>syrB</i> , 1.6-kb <i>PstI</i> in pTE3	This work
pMB155	Same as pMB154, insert in opposite orientation	This work
pMB156	2-kb <i>EcoRI</i> in pTE3	This work
pMB157	Same as pMB156, insert in opposite orientation	This work
pMB180	<i>syrB</i> , 5.3-kb <i>BamHI</i> in pSW213	This work
pMB360	pMB155, Tn3 <i>lacZYA</i> #177	This work
pMB376	pTE3, 1.6-kb <i>PstI</i> , 581 G point mutation	This work
pMB377	Same as pMB377, insert in opposite orientation	This work
pMB500	pR751, Tn3::gusA, Tp ^r , Km ^r	This work
pPHIJ1	IncP, Sp ^r Gm ^r	Hirsch et al. 1984
pR751	IncP, Tp ^r	Jobanputra and Datta 1974
pRK602	Tn5 delivery plasmid	Leigh et al. 1985
pSW213	IncP, Tc ^r	Chen and Winans 1991
pTE3	pLAFR1, <i>trp</i> promoter	Egelhoff and Long 1985
pUC119	Cloning vector, ColE1, Ap ^r	Vieira and Messing 1987

^a Ap^r, Cm^r, Km^r, Sp^r, Str^r, Tc^r, and Tp^r denote ampicillin, chloramphenicol, kanamycin, spectinomycin, streptomycin, tetracycline, and trimethoprim resistance, respectively.

YT media at 37°C (Sambrook et al. 1989). *R. meliloti* strains were grown on LB agar plates or in liquid TY medium at 30°C (Beringer 1974). Antibiotic concentrations were as follows. For *R. meliloti*: neomycin, 150 µg/ml, or spectinomycin, 200 µg/ml; gentamycin, 25 µg/ml; tetracycline, 10 µg/ml; streptomycin, 500 µg/ml; trimethoprim, 600 µg/ml. For *E. coli*: kanamycin, 25 µg/ml; tetracycline, 10 µg/ml; ampicillin, 50 or 100 µg/ml; chloramphenicol, 50 µg/ml; spectinomycin, 40 µg/ml; nalidixic acid, 50 µg/ml. Triparental conjugations were performed with MT616 as the helper plasmid strain. *R. meliloti* DNA containing an antibiotic resistance gene was marker exchanged into the *R. meliloti* genome with pPHIJ1, pR751, or pMB500 as previously described (Ruvkun and Ausubel 1981). Transductions were performed with N3 phage (Martin and Long 1984).

Tn5 mutagenesis and screening.

JM142 (*syrM::gusA*, spectinomycin resistant) was Tn5 mutagenized with pRK602 (Leigh et al. 1985) as the Tn5 delivery plasmid. Approximately 3,200 spectinomycin- and neomycin-resistant colonies were patched and then screened for increased GUS activity as follows: patches were transferred into wells of microtiter plates that contained 50 mM sodium phosphate pH 7, 10 mM β-mercaptoethanol, 0.1% Triton X-100, and 1 mM 4-methylumbelliferyl β-D-glucuronide, a fluorescent substrate of β-glucuronidase. The plates were illuminated with 366 nm UV light and those with increased fluorescence, when compared with the JM142 parent strain, were colony purified and rescreened.

β-glucuronidase assays.

R. meliloti strains were grown to mid to late log phase in TY medium (Beringer 1974). β-glucuronidase activity was determined by spectrophotometric assay as previously described (Swanson et al. 1993). Cells (500 µl was typically used) were pelleted before resuspending in lysis buffer.

Cloning of Tn5-tagged DNA.

Genomic DNA was prepared from *R. meliloti* strain MB101 as previously described by a cetyltrimethyl ammonium bromide extraction method (Ausubel et al. 1987), modified to include a lysozyme treatment step. DNA was digested with *BamHI* and resolved on a 1% agarose gel. DNA of approximately 4-kb was isolated from a gel slice by the freeze squeeze method (Tautz and Renz 1983) and ligated into pUC119. Ampicillin- and kanamycin-resistant colonies were screened by miniprep for plasmid content and were shown to contain inserts consisting of *R. meliloti* genomic DNA linked to the half of Tn5 that carries kanamycin resistance (pMB76).

Isolation of λ clones.

A 700-bp *BamHI-HpaI* probe from pMB76 was used to screen a *R. meliloti* λ bank made from *BamHI*-digested DNA (D. Bramhill, *unpublished*). Positive clones were plaque purified and further screened by Southern hybridization of miniprep DNA to the same *BamHI-HpaI* probe. The probe hybridized to a 5.3-kb *BamHI* band present in all positive clones. This 5.3-kb *BamHI* fragment was cloned into pUC119 in both orientations to yield pMB98 and pMB99.

DNA sequencing.

The Tn5-*Rhizobium* DNA junction was sequenced with clone pMB76 and a primer specific for the Tn5 end. The entire 5.3-kb *Bam*HI insert was sequenced on both strands as described. Subclones for sequencing were generated either by restriction digestion with specific enzymes or by Exonuclease III digestion as previously described (Barnett and Long 1990). All DNA sequencing was done with Sequenase (U.S. Biochemical, CITY, STATE) as previously described (Barnett and Long 1990) with the exception of a 2.8-kb *Hind*III subclone that was sequenced at the Protein and Nucleic Acid Facility of Stanford University with an Applied Biosystems 373A DNA sequencer. The University of Wisconsin Genetics Computer Group software (Devereux et al. 1984) was used for DNA sequence analysis.

Southern hybridization analysis.

Hybridizations with *R. meliloti* DNA were conducted at high stringency as previously described (Barnett and Long 1990). Washes were done at 65°C, except for the Southern hybridization shown in Figure 4, which was washed at 60°C.

Tn3 mutagenesis.

We used the pHoHoGUS/pSShe Tn3 delivery system (D. Dahlbeck and B. Staskawicz, *unpublished*; Stachel et al. 1985) to introduce β -glucuronidase (*gusA*) gene fusions onto a 5.3-kb *Bam*HI DNA fragment that was cloned in pSW213 (pMB180; Chen and Winans 1991). Initial characterization of insertions was done either by physical analysis or phenotypic screening. For physical screening, insertions were characterized by means of restriction digestion to determine if the insert DNA carried Tn3. These plasmids were then conjugated into Rm1021 for further characterization. Phenotypic screening was carried out by conjugating a pool of mutagenized plasmids into Rm1021 and screening for blue colonies on M9 sucrose plates with antibiotic selection and 50 μ g/ml of X-Gluc substrate. This identified plasmids with fusions expressing *gusA* from insertions. The lower limit of detection with this plate screen was about 100 units of β -glucuronidase activity as determined by spectrophotometric assay. Tn3-*lacZYA* mutagenesis of pMB155 was performed in a similar manner except that X-Gal substrate was used for screening. The exact insertion points of Tn3 insertions were determined by subcloning Tn3-Rm1021 DNA and sequencing across the junction. Genomic fusions were constructed via marker exchange and subsequent transduction into a clean background.

Site-directed mutagenesis.

The Altered Sites mutagenesis kit (Promega, Madison, WI) was used according to manufacturer's instructions to introduce a frameshift in *syrB*. An oligonucleotide that had a G residue inserted after position 581 in the *syrB* ORF was synthesized (5'CGGCTGCCGAACCGAGCTCAACCAAAGGC 3'). This insertion created an *Sst*I site, which was used to screen for plasmids containing the introduced mutation. *Pst*I inserts (1.6-kb) containing the mutation were cloned from the pALTER vector into pTE3 in both orientations (pMB376 and pMB377).

Construction of ORF3 fusion.

pMB246 contains *gusA* from pRAJ260 (Jefferson et al. 1986) and the *aadA* gene from Tn21 encoding spectinomycin

resistance (Sundström et al. 1988). This cassette was digested from pMB246 as a *Hind*III DNA, blunted with Klenow, and cloned into pMB104 that had been digested with *Xho*I and blunted with Klenow. To obtain a genomic fusion in ORF3, the GUS cassette was first marker exchanged onto pMB110 to form pMB338. The fusion was then marker exchanged into the Rm1021 genome and transduced into a clean background.

Construction of *syrB* deletion strains.

MB202. The ends of the 1.5-kb *Hind*III-*Sa*I fragment from pBRNeo were filled in with Klenow enzyme, *Sst*I linkers were added, and the resulting DNA was cloned into pMB109 that had been digested with *Sst*I, creating pMB121. pPH1J1 was used to marker exchange the deletion into Rm1021. Neomycin resistance was transduced from this strain into JM142 to create MB202.

MB240. The 5.3-kb *Bam*HI fragment from pMB98 was cloned into the *Bam*HI site of pUC119 that had the *Eco*RI and *Sst*I sites deleted. The resulting plasmid was digested with *Eco*RI and *Sst*I and the ends filled in with Klenow enzyme. A neomycin cassette was inserted and recombined into the genome as described for MB202, above.

MB243. Construction was the same as for MB240 except that the starting plasmid was digested with *Eco*RI only.

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