Multiple Genetic Controls on *Rhizobium meliloti syrA*, a Regulator of Exopolysaccharide Abundance

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ABSTRACT

Exopolysaccharides (EPS) are produced by a wide assortment of bacteria including plant pathogens and rhizobial symbionts. *Rhizobium meliloti* mutants defective in EPS production fail to invade alfalfa nodules. Production of EPS in *R. meliloti* is likely controlled at several levels. We have characterized a new gene of this regulatory circuit. *syrA* was identified by its ability to confer mucoid colony morphology and by its ability to suppress the colonial phenotype of an *exoD* mutant. Here we show that *syrA* encodes a 9-kD hydrophobic protein that has sequence similarity to two other EPS regulatory proteins: ExoX of *Rhizobium* NGR234 and *R. meliloti*, and Psi of *R. leguminosarum* bv. *phaseoli*. The *syrA* transcription start site lies 522 nucleotides upstream of a non-canonical TTG start codon. The *syrA* promoter region is similar to the promoter region of the nodulation regulatory protein, *nodD3*. We found that in free-living bacteria, *syrA* expression is activated by the regulatory locus, *syrM*, but not by *nodD3*. *In planta*, *syrM* is not required for expression of *syrA*. Instead, expression of the nitrogen fixation (*nifHDKE*) genes upstream of *syrA* plays a role. Specific and distinct sets of genetic controls may operate at different times during nodule invasion.

THE development of a nitrogen-fixing alfalfa nodule by the symbiotic soil bacterium, *Rhizobium meliloti* (also termed *Sinorhizobium meliloti*; De Lajudie *et al.* 1994), is a complex process involving sequential changes both in the bacterium and the host plant. Compounds produced by the plant induce expression of rhizobial *nod* genes (Mulligan and Long 1985; Peters *et al.* 1986). The protein products encoded by these genes synthesize lipo-oligosaccharide signal molecules that cause the earliest events in nodule inititiation: root hair membrane depolarization, root hair deformation, and root cortical cell divisions (Dénarié *et al.* 1996; Long 1996). Later in nodule development, the *nod* genes do not appear to be expressed (Schlaman *et al.* 1991; Sharma and Signer 1990).

The interaction of a bacterial cell with a root hair results in the formation of an inwardly growing tunnel of plant cell wall. The bacteria proliferate in this infection thread, penetrating through the root hair into the root cortex (reviewed by Brewin 1991; Hirsch 1992; Kijne 1992). After release from the infection thread, bacteria differentiate morphologically into bacteroids. These developmental changes involve the eventual cessation of cell division and DNA replication and the induction of genes necessary for nitrogen fixation. The *nif* genes encode the subunits of nitrogenase and other genes necessary for nitrogenase function. The *fix* genes encode functions unique to symbiotic nitrogen fixation.

In other free-living, nitrogen-fixing bacterial species, regulation of the *nif* and *fix* genes occurs via the *ntrA* and *ntrC* gene products in response to nitrogen levels (reviewed in de Bruijn *et al.* 1990; Merrick 1992). However, induction of the *nif* and *fix* genes occurs differently in *R. meliloti* during symbiosis (de Bruijn *et al.* 1990; Merrick 1992). NtrC is not required; instead, *nif*/*fix* gene expression is regulated in response to oxygen levels via the two-component regulators, FixL and FixJ (David *et al.* 1988). FixL senses oxygen tension and phosphorylates FixJ, which activates *nifA* and *fixK* (David *et al.* 1988; Batut *et al.* 1989). NifA and NtrA activate expression from *nif* and *fix* promoters (Gussin *et al.* 1986; Szeto *et al.* 1984).

nod gene expression requires a positive transcriptional activator, NodD. NodD proteins are members of the LysR activator family and bind to *nod* boxes, conserved regions upstream of the *nod* operons. Most Rhizobium species possess multiple *nodD* genes. The *R. meliloti* genome encodes three different NodD proteins (Gyorgypal *et al.* 1988; Honma and Ausubel 1987). NodD1 and NodD2 activate *nod* promoters in concert with inducers synthesized by the host plant (Maxwell *et al.* 1989; Mulligan and Long 1985; Peters *et al.* 1986; Phillips *et al.* 1992). NodD3 acts independent of plant inducer compounds and requires another LysR family member, SyrM, for *nod* gene expression (Mulligan and Long 1989; Swanson *et al.* 1993). The *nod* genes appear to be expressed early in nodule initia-

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tion, whereas *syrM* appears to be produced at intermediate stages of development (Sharma and Signer 1990; Swanson *et al*. 1993).

The regulatory circuit controlling *nod* gene expression is probably most important during the early stages of symbiosis, whereas the *nif*/*fix* circuit is required later in nodule development. During intermediate stages of development, other genes are required for successful invasion of a developing nodule. For example, *exo* genes, which encode biosynthetic enzymes necessary for the production of acidic exopolysaccharide (also called EPS I or succinoglycan) are necessary for *R. meliloti* to invade alfalfa nodules (reviewed by Leigh and Walker 1994). EPS I consists of repeating octasaccharide subunits assembled on a lipid carrier, then polymerized and secreted (Aman *et al*. 1981; Reuber and Walker 1993).

R. meliloti strain SU47 can produce a second exopolysaccharide, EPS II or galactoglucan, which can substitute for EPS I in nodule invasion of alfalfa, but not in invasion of other plant hosts (Becker *et al*. 1997; Glazebrook and Walker 1989; Keller *et al*. 1995; Zhan *et al*. 1989). EPS II is only made in Exo$^-$ mutant strains when phosphate is limiting, or in strains overexpressing the EPS II biosynthetic (*exp*) genes (Glazebrook and Walker 1989; Zhan *et al*. 1989; Zhan *et al*. 1991).

Many aspects of *exo* and *exp* gene regulation remain mysterious. Nitrogen or phosphate limitation stimulates EPS I production, but otherwise the physiological signals that regulate expression of these genes are not known. *Exo* structural genes are expressed during nodule invasion and their expression decreases during bacteroid maturation (Reuber *et al*. 1991). The regulatory genes that control expression of the *exo* structural genes include *exoR* and *exoS* which negatively affect the amount of EPS I, probably by repressing genes in the *exo* synthesis pathway (Doherty *et al*. 1988; Reed *et al*. 1991b; Reuber *et al*. 1991). ExoX, a small, probably membrane-associated protein, exerts negative effects on EPS I production, perhaps by a posttranslational mechanism involving ExoY (Reed *et al*. 1991a; Zhan and Leigh 1990). *expR* represses genes in the EPS II biosynthetic pathway (Glazebrook and Walker 1989). The *exp* genes are affected positively by *mucS* and negatively by *mucR* and *expR* (Astete and Leigh 1996; Glazebrook and Walker 1989; Zhan *et al*. 1989). *mucR* may also control EPS I synthesis (Zhan *et al*. 1989).

Another locus that affects the amount of EPS I is *syrA*. *syrA* carried on a low-copy plasmid, pRmJT5, that contained about 20 kb of *R. meliloti* DNA, conferred a mucoid colony phenotype to Rm1021 (Mulligan and Long 1989). An insertion in either of two loci, *syrM* or *syrA*, abolished this mucoid phenotype (Mulligan and Long 1989). Because SyrM activates transcription of *nodD3*, it was hypothesized to activate transcription of *syrA* as well (Mulligan and Long 1989; Swanson *et al*. 1993).

Leigh *et al*. (1985) independently isolated a plasmid carrying *syrA* based on its ability to suppress the calcofluor-dim phenotype of an *exoD* mutant. This plasmid failed to compensate for the invasion defects of the *exoD* mutant strain; instead, *exoD* mutants could invade nodules if the plant growth media was buffered to a slightly acidic pH (Reed and Walker 1991b). These and other observations led to the hypothesis that *exoD* encodes a membrane protein necessary for the bacteria to survive the alkaline conditions encountered during nodule invasion and that the effects of *exoD* mutations on EPS production are probably indirect (Reed and Walker 1991b).

We report here that *syrM* is necessary and sufficient to activate expression of *syrA*, which in turn confers the mucoid-colony phenotype: *nodD3* participates only indirectly as an activator of *syrM* expression. We determined that the requirement for *syrM* in activating *syrA* could be obviated by cloning DNA containing the *syrA* locus downstream of a strong exogenous promoter. We used mutational and DNA sequence analysis to locate the *syrA* open reading frame. *syrA* is a small hydrophobic protein possessing sequence similarity to other proteins involved in EPS biosynthesis.

We also found that although *syrM* activates expression of *syrA-gusA* gene fusions in free-living cells, *syrM* is not required for the expression of *syrA* in bacterioids. Instead, *syrA* expression is dependent on expression of an upstream *nifHDK* operon.

MATERIALS AND METHODS

Plasmids and strains: All plasmids and strains used in this study are listed in Table 1. *R. meliloti* strains were grown in TY (Beringer 1974) or on lb agar plates. *Escherichia coli* strains were grown in lb.

Bacterial genetic techniques: Broad-host range plasmids were transferred into *R. meliloti* strains via triparental conjugation using pRK2013 (Ditta *et al*. 1980) or pRK600 (Finan *et al*. 1986) as helper plasmids. Replacement of the Nm^r of Tn*5* with Sp^r/Gm^r of Tn*5*-233 was performed as previously described (De Vos *et al*. 1986). N3 phage transduction was used to construct strains with multiple insertions (Martin and Long 1984).

Plasmid constructions: The 1.8-kb *Bam*HI-*Pvu*II from pM144 was inserted into *Bam*HI-*Sma*I digested pUC119 to create pMB54. This insert was removed as an *Eco*RI-*Hin*dIII fragment and cloned into pLAFR3 to make pMB57. A 3.1-kb *Bam*HI-*Bgl* II partial digestion product was cloned into the *Bam*HI site of pTE3 to create pMB51 and pMB52 and into pLAFR3 to create pMB56. The insert in pMB51 is oriented such that *nodD3* is expressed from the *lac* promoter, whereas in pMB52 it is oriented such that *syrA* is expressed from the *lac* promoter. On pMB56, *nodD3* is expressed from the *trp* promoter. pMB55 contains the 2.6-kb *Sph*I insert from pMB2 (Barnett and Long 1990) in the *Sph*I site of pUC1318 (Kay and McPherson 1987). The 2.6-kb *Bam*HI fragment from pMB55 was cloned into the *Bam*HI site of pMB57 to create pMB64 and into the *Bam*HI site of pMB56 to create pMB61. A 1.1–kb *Sty*I deletion derivative of pMB54 was cloned as an *Eco*RI-*Hin*dIII fragment, into pLAFR3 that had been digested with *Eco*RI and *Hin*dIII to make pMB131. A *Hin*dIII-linkered derivative of the

TABLE 1

Strains and plasmids

Strain or plasmid	Description or relevant genotype	Source or reference
Rhizobium meliloti		
Rm 1021	Str^r derivative of RCR2011	Meade *et al.* (1982)
JAS105	*syrM*::Tn*5*–233	Swanson *et al.* (1993)
JAS142	1021, Tn*3-gusA* 1–24, *syrM*::Tn*5*–233 No. 701	This study
JAS143	1021, Tn*3-gusA* 4–11, *syrM*::Tn*5*–233 No. 701	This study
JAS144	1021, Tn*3-gusA* 1–24, *ntrA*::Tn*5*–233	This study
JAS145	1021, Tn*3-gusA* 4–11, *ntrA*::Tn*5*–233	This study
JAS303	1021, Tn*3-gusA* 1–24	This study
JAS304	1021, Tn*3-gusA* 4–11	This study
MB303	1021, Tn*3-gusA* 4–11, *nifD*::Tn*5*–233	This study
MB304	1021, Tn*3-gusA* 1–24, *nifD*::Tn*5*–233	This study
MB309	1021, Tn3-*gusA* 4–11, *fixH*::Tn*5*–233	This study
MB310	1021, Tn*3-gusA* 1–24, *fixH*::Tn*5*–233	This study
Rm1312	1021, *nifD*::Tn*5*	Ruvkun *et al.* (1982)
Rm1312Sp	1021, *nifD*::Tn*5*–233	This study
Escherichia coli		
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 Δ(lacZYA-argF) U169 deoR*	Hanahan (1985)
JM109	*F′ traD36 lacI^q Δ(lacZ)M15 proA+proB+ e14–Δ(lac-proAB) thi gyrA96 endA1 hsdR17 relA1 supE44 recA1*	Yanisch-Perron *et al.* (1985)
MT616	MM294A, pRK600. Cm^r	Finan *et al.* (1986)
Plasmids		
pALTER	cloning vector, Tc^r Ap^5	Promega
pBrNeo	pBR322, Nm^r	D. Bramhill
pD3–25	2-kb *Bgl* II, *nodD3*	L. Zumstein
pE65	pTE3, *nodD3*	Fisher *et al.* (1988)
pGMI515	RP–4 prime, *nifHDKE*	Truchet *et al.* (1985)
pHOHOGUS/pSShe	Tn*3-gusA* delivery system	B. Staskawicz
pJWR127	*exoD*	Reed and Walker (1991a)
pLAFR3	IncP, *lac* promoter vector	B. Staskawicz
pM113	*syrM nodD3*	Mulligan and Long (1989)
pM136	*nodD3 syrA*	Mulligan and Long (1989)
pM111	pJT5, *syrA*::Tn*5* 29b	Mulligan and Long (1989)
pM149	pUC119, 1.1-kb *Bgl* II-*Bam*HI from pM144	J. Mulligan
pM150	same as pM149, opposite orientation	J. Mulligan
pM144	*Xba*I-*Bam*HI from pRmJT5	J. Mulligan
pMB2	*syrM*, 2.6-kb *Sph*I in pUC119	Barnett and Long (1990)
pMB51	pTE, *nodD3*, *syrA*	This study
pMB52	same as pMB51, opposite orientation	This study
pMB54	*Bam*HI-*Pvu*II from pM144 in pUC119	This study
pMB55	pUC1318, *syrM*	This study
pMB56	pLAFR3, *nodD3*, *syrA*	This study
pMB57	pLAFR3, *syrA*	This study
pMB61	pLAFR3, *syrM*, *nodD3*, *syrA*	This study
pMB64	pLAFR3, *syrM*, *syrA*	This study
pMB70	*Apa*I-*Pvu*II from pM144 in pUC119	This study
pMB81	pTE3, 0.9-kb *Pst*I from pM149	This study
pMB82	same as pMB81, opposite orientation	This study
pMB85	pTE3, 1.8-kb *Bam*HI-*Pvu*II from pM144	This study
pMB86	same as pMB85, opposite orientation	This study
pMB87	pTE3, 1.55-kb *Bst*EII-*Pvu*II from pM144	This study
pMB88	same as pMB88, opposite orientation	This study
pMB89	pTE3, 1.3-kb *Cla*I-kb-*Pvu*II from pM144	This study
pMB90	same as pMB89, opposite orientation	This study
pMB116	pTE3, 1.1-kb *Bam*HI-*Bgl*II	This study
pMB117	same as pMB116, opposite orientation	This study

TABLE 1

Continued

Strain or plasmid	Description or relevant genotype	Source or reference
Plasmids *(continued)*		
pMB131	pLAFR3, 1.1–kb *Sty*I-*Pvu*II, syrA	This study
pMB132	pTE3, 1–kb *Sty*I-*Pvu*II from pM144	This study
pMB133	same as pMB132, opposite orientation	This study
pMB134	pTE3, 0.9–kb *Pst*I from pM144	This study
pMB135	pLAFR3, 1.1–kb *Sty*I-*Pvu*II, *syrA*, *syrM*	This study
pMB137	pTE3, 1.2–kb *Hpa*I-*Pvu*II from pM144	This study
pMB138	same as pMB137, opposite orientation	This study
pMB308	pALTER 0.55–kb *Cla*I-*Bgl*II from pMB70	This study
pMB313	pTE3, *Bam*HI from pMB308	This study
pMB314	same as pMB313, opposite orientation	This study
pMB315	pMB313, 940 C→A point mutation No. 5	This study
pMB316	same as pMB315, opposite orientation	This study
pMB317	pMB313, 838 T→A point mutation No. 1	This study
pMB318	same as pMB317, opposite orientation	This study
pMB319	pMB313, 882 C→T point mutation No. 4	This study
pMB320	same as pMB319, opposite orientation	This study
pMB333	pMB313, 948 CT→TC point mutation No. 6	This study
pMB334	same as pMB333, opposite orientation	This study
pMB372	pMB313, 838 G→A point mutation No. 2	This study
pMB373	same as pMB372, opposite orientation	This study
pMB374	pMB313, 835 T→A point mutation No. 3	This study
pMB375	same as pMB374, opposite orientation	This study
pMH0	Tn*5* subclone from pRmM111	This study
pPH1JI	IncP, Sp^r, Gm^r	Hirsch and Beringer (1984)
pRK2013	ColEI, provides RK2 transfer functions	Figurski and Helinski (1979)
pRmJT5	20–kb cosmid clone	Swanson *et al.* (1987)
pS73	pTE3; *syrM*	Swanson *et al.* (1993)
pS701	pJT5, *syrM*::Tn*5*	Swanson *et al.* (1987)
pS801	pJT5, *nodD3*::Tn*5*	Swanson *et al.* (1987)
pTE3	pLAFR1, Trp promoter	Egelhoff and Long (1985)
pUC119	cloning vector, ColE1	Vieira and Messing (1987)
pUC1318	ColE1, cloning vector	Kay and McPherson (1987)

2.6–kb *Bam*HI insert was cloned into the *Hin*dIII site of pMB131 to make pMB135.

Construction of pTE3 expression clones: The 1.8–kb insert of pMB54 was removed with digestion by *Eco*RI and *Hin*dIII and blunted with Klenow enzyme. *Bam*HI linkers were added and the fragment was ligated into the *Bam*HI site of pTE3 in both orientations to form pMB85 and pMB86. Inserts from deletion derivatives of pMB54 were cloned into pTE3 in a similar fashion. The 0.55–kb *Cla*I-*Bgl* II fragment from pMB70 was blunted and *Bam*HI linkers were added before ligation into the *Bam*HI site of pALTER to form pMB308. Mutant derivatives of pMB308 were cloned as *Bam*HI fragments into the *Bam*HI site of pTE3.

Assays for mucoid phenotype: Strains were streaked to single colonies on M9 sucrose plates (Meade and Signer 1977) with appropriate antibiotic selection. At 5 days' growth, colonies were scored for mucoid morphology.

Site-directed mutagenesis: Site-directed mutagenesis was performed using an Altered Sites mutagenesis kit (Promega, Madison, WI) Double-stranded miniprep DNA was made from ampicillin-resistant colonies and screened by DNA sequencing for presence of the introduced mutation. The following oligonucleotides were used: Nucleotide 948 C→A, 5′-GCTGGCTTGATTGCTGCTC; 838 T→A, 5′-CGTCAT TGACCTTCAGA; 882 C→T, 5′-CTGTGCGCTAGTTCTC TCG; 948 CT→TC, 5′-CGACGCTGGCTTGTGTGCTGCTCT TCC; 837 G→A, 5′-GGAGAACGTCATTATCCTTCAG; 835 T→A, 5′-GGAGAACGTCAATGTCCTTCAG

DNA sequence analysis: The 1.1–kb *Bam*HI-*Bgl*II fragment from pM144 was sequenced on both strands using Sequenase (United States Biochemical, Cleveland). This sequence has been assigned Genbank accession U90221. That the *Bgl* II site of this fragment represents the same *Bgl* II site of the 2-kb *Bgl* II fragment containing *nodD3* (Rushing *et al.* 1991) was confirmed by synthesizing a specific primer and sequencing across the *Bgl* II site of pM144. DNA sequence was analyzed using the University of Wisconsin GCG software (Devereux *et al.* 1984). A codon usage table of 55 *R. meliloti* SU47 genes and the program Codonpreference of the GCG software were used to generate codon usage profiles. *R. meliloti* DNA containing Tn*3* insertions was subcloned as *Bgl* II fragments into pUC119 and the insertion points were determined using a primer specific to the Tn*3* end to sequence across the junction. The insertion point of Tn*5* 29b was determined in a similar fashion using a primer specific to the Tn*5* end. The locations of Tn*5* No. 1005 and No. 213 were reported by Rushing *et al.* 1991.

Primer extension analysis of *syrA* transcript: RNA was isolated from a strain that has *syrM*, *nodD3* and *syrA* present on a low copy number plasmid, 1021 pRmJT5 (Swanson *et al.*

1987). RNA purification and primer extension mapping was done according to Barnett *et al.* 1996. Two different oligonucleotides were used to map the *syrA* start site. One was complementary to a region 108 nucleotides downstream of the *syrA* transcription start site: 5′-CCACGATCCGCAGAAAT CTTGAGCTCGGGTAAGCGGCG. The other was located 547 nucleotides downstream of the *syrA* transcription start site in the *syrA* open reading frame: 5′-GTAGATTGCGAGAGA ACTGGCGCACAGGAGCAGCCATAG.

Construction of *syrA::gusA* fusion strains: Mutagenesis of plasmid pM136 was performed as previously described by Stachel *et al.* (1985) and Swanson *et al.* (1993). Mutagenized plasmids were screened for insertions in *syrA* by conjugating into Rm1021 and looking for colonies that were less mucoid. *syrA* gene fusions were marker exchanged into the *R. meliloti* genome using pPH1JI (Hirsch and Beringer 1984).

β-glucuronidase assays: TY grown cultures were assayed for β-glucuronidase activity at mid-log phase as previously described (Swanson *et al.* 1993). For *in planta* assays, nodules were sectioned and stained as described by Swanson *et al.* (1993). Nodules were counted and observed every few days from 10 days post inoculation (dpi) to 45 dpi.

RESULTS

***syrM*, *nodD3* and *syrA*: effects on mucoid phenotype:** Our previous data suggested that both *syrM* and *syrA* are required for the mucoid phenotype, with SyrM most likely acting as a positive activator of *syrA*. Overproduction of acidic exopolysaccharide (EPS I) is pre-

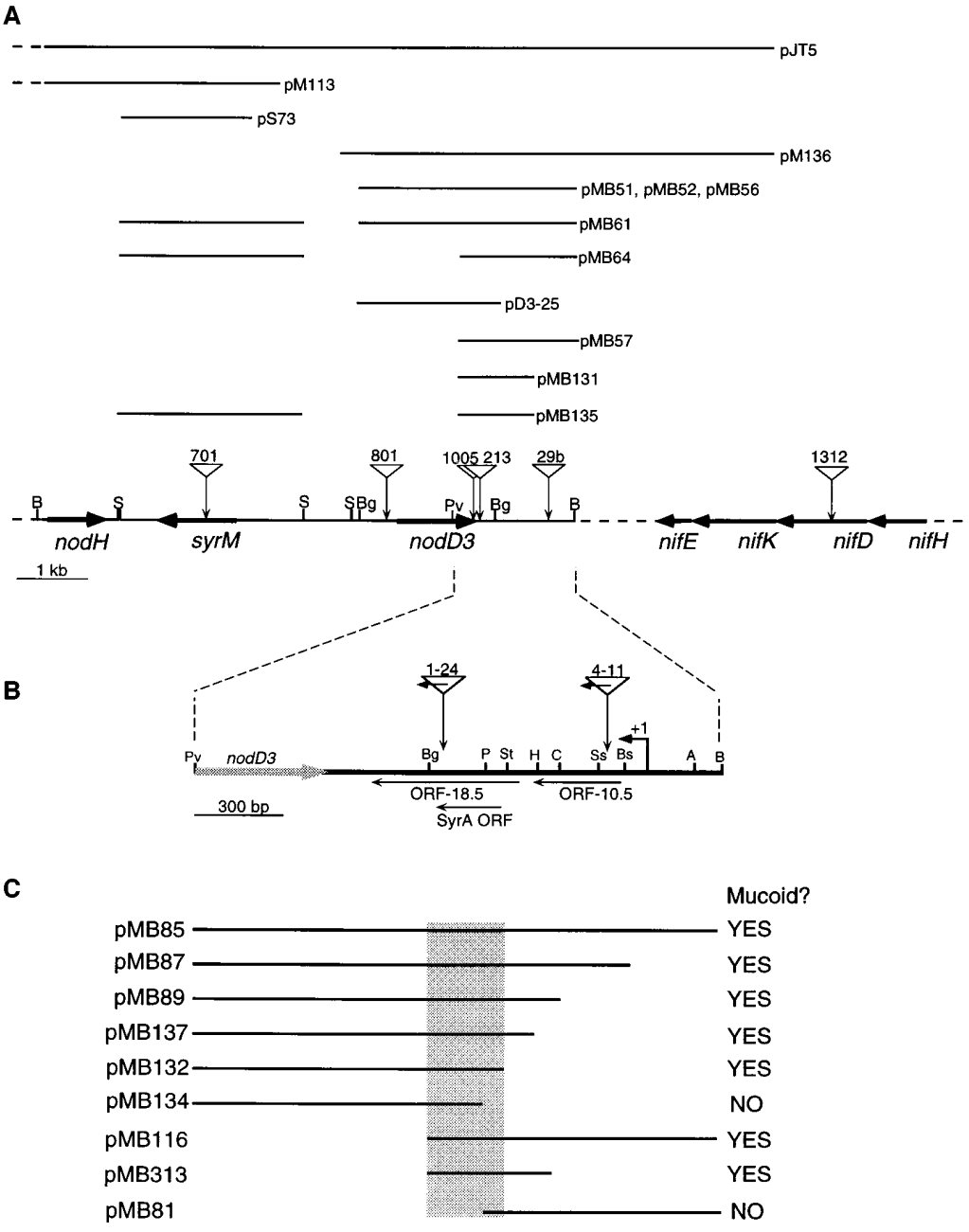

Figure 1.—Physical map of the *syrA* region and clones used for genetic analyses. (A) Restriction map of *nod*, *syr* and *nif* region of pSymA. Extent of each plasmid insert is represented by a line above the restriction map. pJT5 and pM113 contain additional sequence upstream of *nodH* not shown on map (indicated by dashes). Locations of relevant transposon insertions are marked with arrowed triangles. (B) Restriction map of the 1.8–kb *Pvu*II-*Bam*HI fragment containing *syrA*. Potential ORFs are marked with thin arrows. *syrA-gusA* insertions are represented by arrowed triangles; horizontal arrows within each triangle indicate the direction of *gusA* transcription. (C) Deletion derivatives of the 1.8–kb *Pvu*II-*Bam*HI fragment shown in (B). The extent of each plasmid insert relative to the map in (B) is shown along with the corresponding mucoid phenotype. Each clone was tested in both orientations with respect to the exogenous *trp* promoter as described in materials and methods. The shaded region denotes the smallest region able to confer the mucoid phenotype as defined by these deletion derivatives. Abbreviations: S, *Sph*I; Bg, *Bgl* II; Pv, *Pvu*II; B, *Bam*HI; P, *Pst*I; St, *Sty*I, H, *Hpa*I; C, *Cla*I; Ss, *Sst*I; Bs, *Bst*EII; A, *Apa*I.

TABLE 2

β-glucuronidase activities of *syrA-gusA* fusion strains

Plasmid	Description	Mucoid phenotype	JAS303 *syrA-gusA* 1–24	JAS304 *syrA-gusA* 4–11
1. none		−	9	38
2. pJT5	20–kb cosmid clone	+	884	1273
3. pS701	pJT5, *syrM*::Tn*5*	−	25	68
4. pS801	pJT5, *nodD3*::Tn*5*	+	1303	1066
5. pM111	pJT5, *syrA*::Tn*5*	−	1399	1053
6. pMB64	*syrM, syrA*	+	315	709
7. pM113	*syrM*	−	450	1327
8. pS73	*trp* p.o.-*syrM*	−	813	2540
9. pD3–25	*nodD3*	−	36	28
10. pE65	*trp* p.o.-*nodD3*	−	420	1053
11. pM136	*nodD3, syrA*	−	n.t.	n.t.
12. pMB51	*trp* p.o.-*nodD3, syrA*	+	n.t.	n.t.
13. pMB52	*trp* p.o.-*syrA, nodD3,*	+	n.t.	n.t.
14. pMB56	*lac* p.o.-*nodD3, syrA*	+	353	1729
15. pMB57	*syrA*	−	15	44
16. pMB131	*syrA* coding region	−	n.t.	n.t.
17. pMB135	*syrM, syrA* coding region	−	n.t.	n.t.
18. pJWR127	*exoD*	−	5.1	5.2

sumed to be responsible for the mucoid phenotype since genes in the EPS I synthesis pathway are required (Mulligan and Long 1989).

We defined the requirements for the mucoid phenotype by constructing plasmids containing various combinations of *syrA*, *syrM* and *nodD3* (Figure 1; Table 2). Any plasmid conferring a mucoid colony morphology that was qualitatively similar to that conferred by pJT5 was scored as positive. pS701 (pJT5, *syrM*::Tn*5*), pS303 (pJT5, *nodD3*::Tn*5*) and pM111 (pJT5, *syrA*::Tn*5*) were sufficient to confer a mucoid phenotype as reported previously (Mulligan and Long 1989). pMB61, which contains only *syrM*, *nodD3* and *syrA*, also conferred a mucoid phenotype. *nodD3* is not required for the mucoid phenotype: strains containing plasmids with only *syrM* and *syrA* were still mucoid (pMB64). *syrA* alone on a plasmid (pMB57) was not sufficient to confer the mucoid phenotype. Likewise, *syrM* alone on pM113 or pS73 did not confer a mucoid phenotype.

We tested a possible role for *nodD3* in determining the mucoid phenotype using pMB51, pMB52 and pMB56, which each contain *nodD3* and *syrA* on a 3.1–kb *Bgl*II-*Bam*HI insert expressed from different exogenous promoters (see materials and methods). All of these plasmids confer a mucoid phenotype to 1021 (Figure 1; Table 2, lines 12–14), but only in strains containing a normal genomic copy of *syrM* (data not shown). Therefore, we conclude the mucoid phenotype conferred by these plasmids is an indirect result of *nodD3* increasing expression of *syrM*. This is supported by the observation that neither, pM136, which contains *nodD3* and *syrA* but no exogenous promoter, nor pE65, which contains only *nodD3* strongly expressed from a vector promoter, was able to confer a mucoid phenotype (Table 2, lines 10 and 11).

We determined whether the *syrM-syrA* mucoid phenotype was dependent on the presence of the *syrA* upstream region. A strain containing a plasmid-born *syrA* but with a deletion from the *Sty*I site to *Bam*HI site was

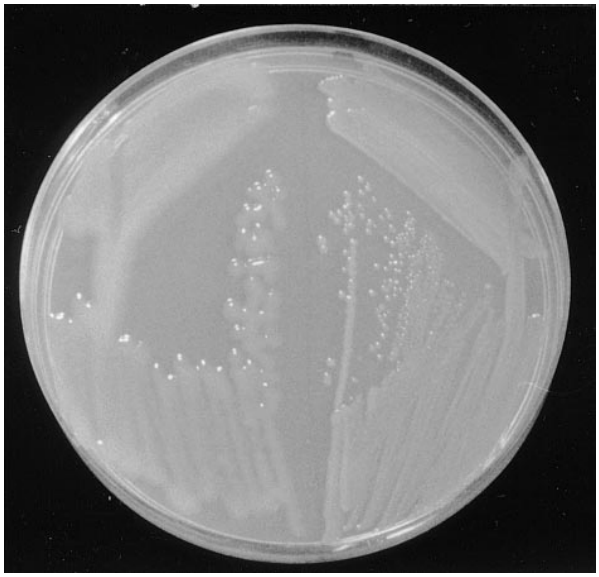

Figure 2.—Plate assay of the *syrA* mucoid phenotype. The photograph shows the mucoid phenotype of *R. meliloti* strain Rm1021 containing either pMB89 (left) or pMB90 (right). pMB89 contains the *Cla*I-*Pvu*II fragment (Figure 1, B and C) oriented such that *syrA* is overexpressed via the exogenous *trp* promoter. pMB90 contains the same insert in the opposite orientation.

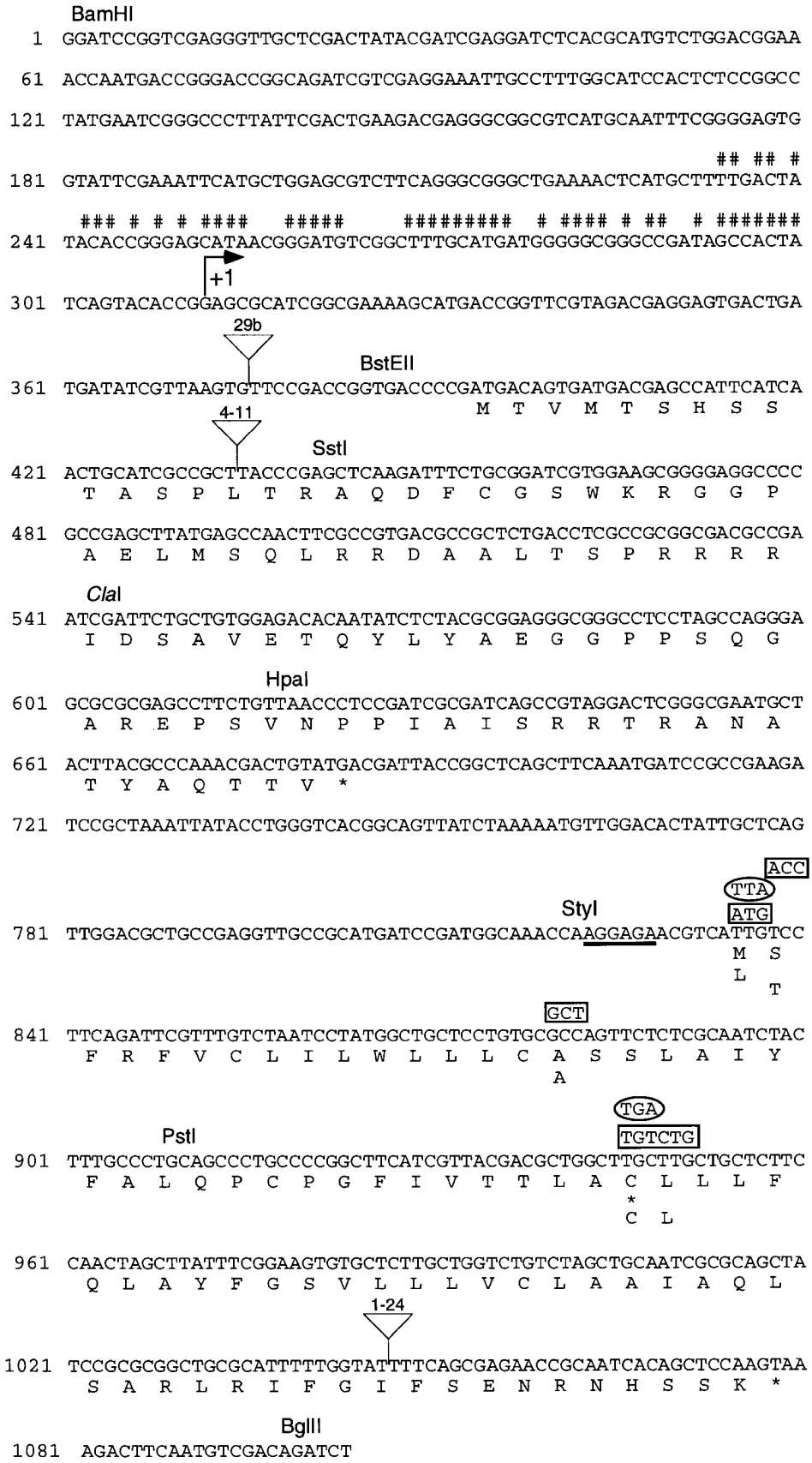

Figure 3.—Sequence of 1.1–kb *Bam*HI-*Bgl* II fragment containing the *syrA* locus (Genbank accession U90221). Restriction sites are those shown in Figure 1A. The amino acid sequences of the ORF encoding a putative 10.5 kD protein and of *SyrA* are shown below the nucleotide sequence. Transposon insertion points determined by DNA sequencing are shown with triangles. A potential ribosome binding sequence for *syrA* is underlined. A region upstream of the 10.5 kD ORF similar to a region upstream of *nodD3* is marked with # symbols. Each codon containing a point mutation is shown above the nucleotide sequence line. The corresponding amino acid change in SyrA is shown below the amino acid sequence line. Mutations represented by circled codons abolished the mucoid phenotype. Boxed codons represent mutations that had no effect on the mucoid phenotype. All point mutations were created as stated in materials and methods and were confirmed by DNA sequencing.

not mucoid (Table 2, line 16). Cloning *syrM* into this plasmid failed to restore the mucoid phenotype (Table 2, line 17). But, when *syrM* is cloned into a plasmid containing the intact *syrA* upstream region a mucoid phenotype is seen (Table 2, lines 6 and 15), indicating the importance of a *cis*-acting region upstream of *syrA*.

The requirement for plasmid-born copies of *syrM* could be circumvented by overexpressing *syrA* from the *trp* promoter. We took advantage of this *syrM*-independent expression to define the *syrA* locus further. Deletion derivatives of the 1.8–kb *Bam*HI-*Pvu*II DNA were cloned in both orientations into the *trp* promoter expression vector pTE3 (Egelhoff and Long 1985; Figure 1). These plasmids were conjugated into *R. meliloti* and scored for their mucoid phenotype.

An example of such a screen for pMB89 is shown in Figure 2. pMB89 contains the *Cla*I-*Pvu*II fragment of Figure 1B oriented such that the transcription from the *trp* promoter proceeds in the direction from the *Cla*I site to the *Pvu*II site. *R. meliloti* colonies containing this plasmid are extremely mucoid. pMB90, which contains the insert in the opposite orientation, confers no mucoid phenotype. The combined results presented in Figure 1C indicate that the region contained within a 280 bp *Sty*I-*Bgl* II fragment, shown by shading, is sufficient to confer mucoid colony morphology when expressed from the *trp* promoter of pTE3.

Nucleotide sequence of *syrA*: The 1.1–kb *Bam*HI-*Bgl* II fragment shown in Figure 1B is adjacent to the *nodD3* sequence reported earlier by Rushing *et al.* (1991). Results from the genetic analyses described above, and from gene fusion data, indicated that *syrA* was probably expressed from right to left as shown in Figure 1B.

Three potential open reading frames (ORFs) inferred from the sequence are shown by thin arrows in Figure 1B. The ORF closest to the *Bam*HI site encodes a polypeptide of 10.5 kD and has an *R. meliloti*-like codon usage pattern. Tn*5* #29b, which abolishes the mucoid phenotype, is located just upstream, and Tn*3* #4–11 is located in the N-terminal region of this ORF (Figure 3). However, a region containing this ORF is not sufficient to confer a mucoid phenotype (Figure 1C, pMB81), nor does its removal abolish the mucoid phenotype (Figure 1C, pMB137 and pMB132). *In vitro* transcription / translation experiments failed to detect a protein corresponding to this ORF. A larger ORF encoding a potential protein of 18.5 kD spans a region outside of the 280–bp *Sty*I-*Bgl* II segment. Since only the N-terminal half of this protein would be contained within the 280–bp segment, this putative protein is not a likely candidate for SyrA.

Contained entirely within the 280–bp *Sty*I-*Bgl* II fragment is a smaller ORF that encodes a putative protein of 9 kD. This ORF corresponds to the region whose overexpression is responsible for the *syrA* phenotype. This 81-amino acid ORF begins with a TTG (leucine) start codon. Upstream lies a good match with a consensus ribosome binding sequence (underlined in Figure 3). We obtained a Tn*3*::*gusA* fusion, #1–24, that is located in the carboxy terminus of the protein product of this ORF.

Site-directed mutagenesis of *syrA*: Because this putative ORF begins with a nonconventional start-codon and lacks a strong *R. meliloti* codon usage pattern, we sought to confirm that this ORF encodes SyrA. We used site-directed mutagenesis to introduce point mutations into the *syrA* region as described in materials and methods. These mutated DNAs were cloned in vector pTE3 and assayed for mucoid phenotype as described earlier. The location of each introduced change is shown in Figure 3. Mutations that abolish the mucoid phenotype are circled; those that do not are boxed.

One such mutation, a C to T change at nucleotide 882, introduces a stop codon in frame with the ORF encoding the 18.5-kD protein, but is silent with respect to the 9-kD ORF. A *syrA* plasmid containing this mutation still confers the mucoid phenotype. This confirms that the 18.5 kD ORF is not required for the mucoid phenotype.

Another mutation (948 C to A) introduces a stop codon in the 9-kD ORF and abolishes the mucoid phenotype. To confirm that this phenotypic change results from a truncation of the SyrA protein and not some other reason, such as an alteration of a binding site or a mutation of a nontranslated RNA, we made another mutated derivative which has a CT to TC change at this same position (948-949), but which is silent at the amino acid level. The mucoid phenotype was observed in this mutant.

Although no candidate ORFs were found in the third reading frame, we nonetheless tested it by the introduction of a stop codon (837 G to A). This mutation introduces a conservative serine to threonine change in the 9-kD ORF and a nonconservative valine to glutamate change in the 18.5-kD ORF. The plasmid carrying this mutation still confers a mucoid phenotype to *R. meliloti*.

The above results strongly support the hypothesis that the 9-kD ORF encodes SyrA. We created two additional mutations to confirm that this ORF begins at the TTG codon. First, we changed the TTG leucine codon of the putative *syrA* ORF to a TTA leucine codon (Figure 3). TTA codons specify leucine, but have never been shown to be translational start codons. The mucoid phenotype was lost with a plasmid (pMB372) carrying this mutation. We also made a mutation in which SyrA starts with an ATG instead of a TTG codon. pMB374 confers a mucoid phenotype to *R. meliloti* similar to that of a plasmid carrying wild-type *syrA*. These results confirm that SyrA initiates translation from a TTG codon.

SyrA has features similar to ExoX proteins: A TFASTA search of the Genbank database showed SyrA is 34% identical to ExoX from the broad host range *Rhizobium*

```
          1                                                  50
SyrA      .....LSFRF VCLILWLLLC ASSLAIYFAL QPCPGFIVTT LACLLLFQLA
Rm ExoX   ....MFAPRV F.LSMIGALA AFAVATYYLN GSLASTAIQT LICAVLIQVG
NGR ExoX  ....MFAPRF V.VSMLGALA AFAIATYFLT GSIASTAVQT LLCAVLIQVG
Psi       VHQRCFGLRA S.LSIFKAFA V......... .........T LAASVFLQVV

          51                                                 100
SyrA      YFGSVLLLVC LAAIA..QLS ARLRIFGIFS ENRNHSSK*. ..........
Rm ExoX   YFIAVLFLVW KEARERRRLS SQKQFMTAEA ANDEKQPG.. ....KVSLRR
NGR ExoX  YFLAVLFLVW KEARDRRKLS PGQ..LPADP TNDEKQTG.. ....KLSLRR
Psi       YFLSLLFMSF RPTRESDRSI HSGTRQADQP QKRDRDKTEQ SNVPKLDPRR

          101    110
SyrA      ..........
Rm ExoX   LNRPHHLNS*
NGR ExoX  LNRPPHFNS*
Psi       KRRTP*....
```

Figure 4.—Amino acid sequence alignment of *SyrA* protein. The amino acid of *SyrA* is aligned with the amino acid sequences of *Rhizobium meliloti* ExoX (Reed *et al.* 1991), *Rhizobium* NGR234 ExoX (Gray *et al.* 1990a), and *R. leguminosarum* bv. *phaseoli* Psi (Borthakur and Johnston 1987). Amino acids of the ExoX and Psi proteins identical or conserved in *SyrA* are shown shaded. Conserved groupings used in the alignment are as follows: Leu, Val, Ile; His, Lys, Arg.

sp. NGR234 (Gray *et al.* 1990), 26% identical to ExoX from *R. meliloti* (Reed *et al.* 1991a), and 33% identical to Psi from *R. leguminosarum* biovar *phaseoli* (Borthakur and Johnston 1987) (Figure 4). All of these proteins have in common a hydrophobic N-terminal region and a hydrophilic C-terminus.

Determination of transcription start site: We mapped the transcription start site as described in materials and methods. Our data reveal a single start site 522 nucleotides upstream of the TTG translation start site (Figures 3 and 5). The location of this start site may mean that the 10.5-kD ORF upstream of *syrA* is cotranscribed with *syrA*. Alternatively, *syrA* may share a feature with *syrM* and *nodD3*: there is no evidence for the existence of ORFs upstream of *syrM* and *nodD3*; yet, these genes possess long leader sequences (Barnett *et al.* 1996).

Immediately upstream of the start site is a 68 bp region that is 67.6% identical to a region upstream of the *nodD3* start site (marked with # symbols in Figure 3). Because expression of both *nodD3* and *syrA* is affected by *syrM*, this sequence may be required for interaction of SyrM with these promoters. Additional experiments are necessary to determine if this sequence represents a SyrM binding site.

***syrM, nodD3*, and *syrA*: effects on *syrA-gusA* reporter gene fusions:** We used the *syrA-gusA* gene fusions described above to assay effects of activators carried in *trans* (Table 2). Both of the *syrA-gusA* fusions have basal levels of GUS activity that are unaffected by mutation of *syrM* (Barnett 1994). Plasmid pJT5 greatly enhanced expression of these *syrA* fusions, and plasmid copies of *syrM*, but not *nodD3* or *syrA*, were required for pJT5 to confer these high levels of GUS activity (Table 2, lines 3–5).

Smaller plasmids containing *syrM* also activated *syrA* expression (Table 2, plasmids 6–8). *nodD3* increased activity of the *syrA* fusions, but only when expressed in certain contexts. *nodD3*, expressed from its own promoter, failed to activate *syrA* expression (Table 2, line 9). In pE65, *nodD3* is expressed from the *trp* promoter and in pMB56 *nodD3* is expressed from the *lac* promoter (Table 2, lines 10 and 14). *syrA-gusA* gene fusion strains containing these plasmids had high GUS activity. As was the case with the mucoid phenotype, *nodD3* appears to enhance *syrA* expression indirectly via *syrM*. pE65 and pMB56 do not increase activity of the *syrA* reporter fusions when assayed in strains containing a *syrM* insertion (Barnett 1994).

It was previously reported that a plasmid expressing *syrA* can suppress the calcofluor-dim phenotype of an *exoD* mutant (Reed and Walker 1991a). We found that a *syrA* plasmid does not affect expression of *exoD*::*lac*-

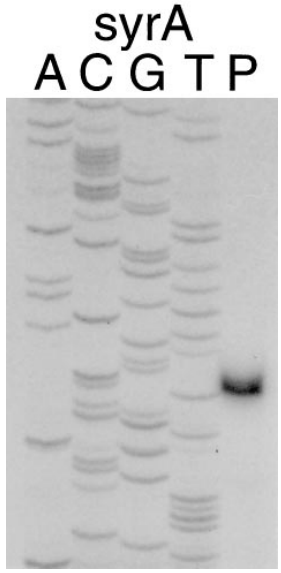

Figure 5.—*syrA* transcription initiation site. Autoradiogram of primer extension products (P) and sequencing reactions (A, C, G and T, respectively). The extension product shown here was obtained using a primer complementary to a region 108 nucleotides downstream of the *syrA* start site (materials and methods). pM149 (J. Mulligan, unpublished results) was used as a template for double-stranded sequencing using the same primer as for the primer extension.

ZYA gene fusions. However, *exoD* may have subtle effects on *syrA* expression. We found that a plasmid containing *exoD* causes about a sevenfold reduction in expression of the 4–11 *syrA* fusion, but not the 1–24 fusion (Table 2, line 18).

syrA does not appear to affect its own expression as strains containing *syrA* alone on a plasmid showed basal levels of expression (Table 2, pMB57). Additional evidence for lack of *syrA* autoregulation is the observation that a *syrA*::Tn*5* insertion on plasmid pJT5 did not adversely effect *syrA* expression (Table 2, line 5).

Symbiotic expression of *syrA-gusA* fusions: *syrA* mutants form normal nitrogen-fixing nodules on alfalfa. We tested the 1–24 and 4–11 *syrA-gusA* fusions for *in planta* β-glucuronidase (GUS) activity. Alfalfa plants were inoculated with strains containing these fusions. Nodules were harvested at various time points, sectioned, stained for GUS activity and observed by mi-

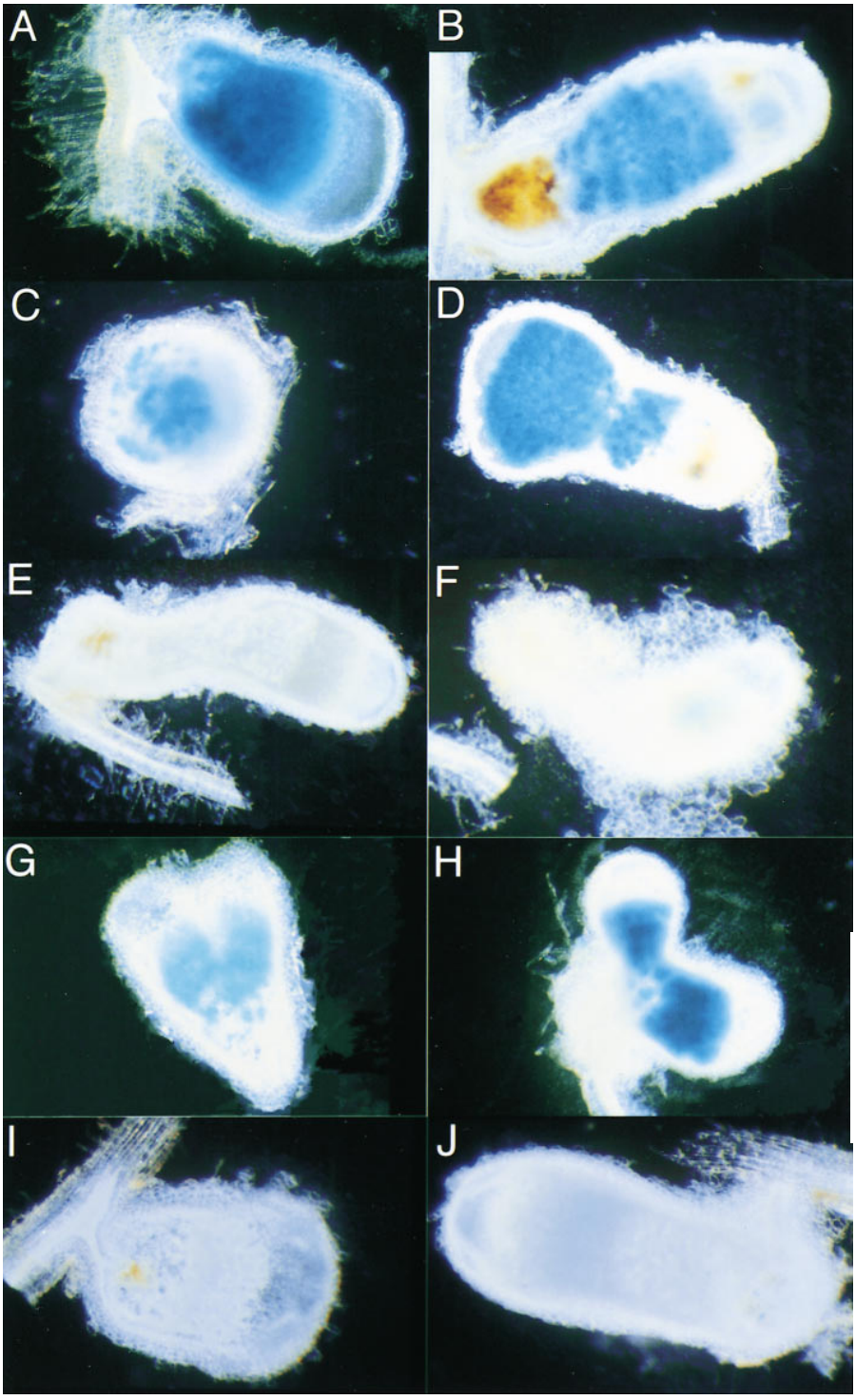

Figure 6.—Symbiotic expression of *syrA-gusA* gene fusions. (A–J) Photomicrographs of hand-sectioned nodules stained for β-glucuronidase activity (see materials and methods). (A) JAS303 (Tn*3-gusA* 1–24) 22 days post inoculation (dpi), (B) JAS304 (Tn*3-gusA* 4–11) 23 dpi, (C) JAS142 (Tn*3-gusA* 1–24, *syrM*::Tn*5*) 12 dpi, (D) JAS143 (Tn*3-gusA* 4–11, *syrM*::Tn*5*) 25 dpi, (E) JAS144 (Tn*3-gusA* 1–24, *ntrA*::Tn*5*) 38 dpi, (F) JAS145 (Tn*3-gusA* 4–11, *ntrA*::Tn*5*) 38 dpi, (G) MB310 (Tn*3-gusA* 1–24, *fixH*::Tn*5*–233) 22 dpi, (H) MB309 (Tn*3-gusA* 4–11, *fixH*::Tn*5*–233) 22 dpi, (I) MB304 (Tn*3-gusA* 1–24, *nifD*::Tn*5*–233) 20 dpi, (J) MB303 (Tn*3-gusA* 4–11, *nifD*::Tn*5*–233) 20 dpi.

croscopy. For the strains containing a *syrA* fusion in an otherwise wild type background, we observed intense staining in bacteroid-containing cells, shown in Figure 6, A and B. To our surprise, we still observed high levels of Gus staining when a mutation was introduced in *syrM* (Figure 6, C and D). This is contrary to the situation in free-living cells where *syrM* is required for activation of *syrA* (Table 2).

We wondered if perhaps an additional mechanism for activation of *syrA* occurs in the symbiotic state. NtrA is necessary for the activation of diverse functions including nitrogen fixation, dicarboxylic acid metabolism, and growth on nitrate (Ronson *et al.* 1987). An *ntrA* mutation had no effect on *syrA* activity when assayed in culture (data not shown). However, when nodules inhabited by *syrA-gus* strains containing a mutation in *ntrA* were assayed, no visible staining was detected (Figure 6E) with one exception: in less than 10% of the cases we observed faint clusters of stained cells in young nodules inhabited by 4–11 fusion strains. One such nodule is shown in Figure 6F. FixL is required for expression of *nifA* which in turn acts with NtrA to activate symbiotic promoters. A *fixL* insertion had a similar phenotype as the *ntrA* insertion except that we never observed even faint or patchy staining (data not shown). This supports the conclusion that a component of the NtrA-NifA circuit is necessary for symbiotic expression of *syrA*.

Several models can account for the lack of *syrA* expression in an *ntrA* mutant background. Although $NtrA^-$ bacteria are capable of invasion, they form Fix^- nodules and senesce prematurely. It is possible that *syrA* is only active in mature, nitrogen-fixing bacteroids and that NtrA is not directly required for *syrA* activation. On the other hand, NtrA may be more directly involved either by acting on the *syrA* promoter itself or by acting on an intermediate, which then acts on *syrA*. Another possibility is that *syrA* is transcribed from a different, NtrA-dependent promoter in bacteroids.

In order to test this hypothesis, we transduced a *fixH* insertion into *syrA*::*gusA* strains. *fixH* is located on pSyma, about 200 kb from *syrA*. The *fixGHIS* operon is proposed to encode a redox-coupled cation pump (Kahn *et al.* 1989). *fixH* mutants form ineffective nodules: the bacteroids are not able to fix dinitrogen and senesce prematurely (Vasse *et al.* 1990). In nodules formed by these *syrA-gus*A, *fixH* mutant strains, we observed a GUS^+ phenotype (Figure 6, G and H). Appearance of this phenotype was developmentally delayed by several days compared to the $fixH^+$ nodules and was qualitatively less intense. These differences may reflect a decreased ability to invade, or the premature senescence of the *fixH* mutated bacteria.

The above results indicate that *syrA* expression does occur in the absence of nitrogen fixation. As a first step in dissecting the pathway of *syrA* activation, we tested the effect of an insertion in the *nifD* locus (Ruvkun *et al.* 1982). *NifD* is part of the NtrA-activated *nifHDKE* operon and encodes the MoFe protein subunit α of nitrogenase. $NifD^-$ strains are Fix^- (Ruvkun and Ausubel 1981), but nodules induced by these strains more closely resemble wild-type than those induced by NtrA mutants (Hirsch *et al.* 1983). We chose a *nifD* mutation because of the proximity of the *nifHDKE* operon to *syrA* (Figure 1A). The entire nucleotide sequence of this operon has not been reported, but genetic and physical analyses indicate that *nifHDKE* is transcribed in the same direction as *syrA* and may end 1–2 kb upstream of *syrA*. Staining of nodules inhabited by the NifD mutant strains was similar to that of NtrA mutant strains (Figure 6, I and J). No staining was observed except for the occasional faint staining of young nodules containing *nifD*::Tn*5*-233, *syrA*::*gusA* 4–11 strains. Similar to the *ntrA* insertion, the *nifD* insertion had no effect on expression of *syrA* in free-living bacteria (data not shown).

DISCUSSION

We identified the *syrA* locus by the phenotype it confers when present on a plasmid: bacterial strains with multiple copies of *syrA* form mucoid colonies on agar plates (Mulligan and Long 1989). Mutations in *exoA* abolish the ability of SyrA to confer a mucoid phenotype (Mulligan and Long 1989). This provides indirect evidence that the mucoid colonies seen with *syrA* overexpression are overproducing EPS I. A single Tn*5* insertion located downstream from *nodD3* abolishes the *syrA* phenotype (Mulligan and Long 1989).

Here we report the identification and characterization of the *syrA* ORF, and analyze expression using *syrA-gusA* gene fusions. Our results confirm that SyrM activates *syrA* expression resulting in a mucoid colony phenotype. In addition, we showed that *nodD3* is not required for the activation of *syrA* by SyrM. Plasmids containing only *syrM*, activated expression of the *syrA-gusA* fusions 30- to 100-fold above background levels, but failed to confer a mucoid phenotype when tested in a wild-type background. Only those strains containing multiple copies of *syrA* were mucoid. This may indicate that an excess of SyrA is needed before EPS I abundance increases.

The need for SyrM in conferring the *syrA*-mediated mucoid phenotype can be overcome if *syrA* is expressed from an exogenous promoter. We tested various segments of DNA for their ability to confer a mucoid colony morphology in *R. meliloti*, and found that a 280–bp piece of DNA was sufficient. Using site-directed mutagenesis, we confirmed that an ORF, which begins with a TTG start codon, expresses a 9-Kd protein responsible for the mucoid colony morphology. It is estimated that ~9% of all known prokaryotic ORFs begin with start codons other than ATG, and that ~10% of these begin with TTG (Gualerzi and Pon 1990). It has been postulated

that rare initiation codons are targets for regulatory mechanisms directed at select genes (Gualerzi and Pon 1990). Whether the TTG initiation codon of the *syrA* gene serves as such a target remains to be proven.

We mapped the transcription start site of *syrA* using RNA prepared from cells overexpressing *syrM*, *nodD3* and *syrA*. Our data show a single start site located 522 nucleotides upstream of the *syrA* TTG codon. All insertions that abolish the *syrA* phenotype, as well as the 10.5 kD ORF, are located downstream of the start site. This biochemical data is consistent with our genetic data: both *gusA* insertions in the *syrA* region are activated by SyrM. Upstream of the start is a region with similarity to the *nodD3* promoter region (Barnett *et al.* 1996). This conservation suggests these *cis* sequences are important for control by *syrM*.

SyrA is similar to other proteins involved in exopolysaccharide production: ExoX of *R. meliloti* (Reed *et al.* 1991a) and *Rhizobium* sp. NGR234 (Gray *et al.* 1990), and Psi from *R. leguminosarum* bv. *phaseoli* (Borthakur and Johnston 1987). ExoX and Psi are hypothesized to be membrane proteins (Borthakur and Johnston 1987; Gray *et al.* 1990; Latchford *et al.* 1991). The predicted hydropathy of SyrA is greater than either ExoX or Psi. The SyrA protein contains only 7 charged residues, all but one of which are confined to the last 17 residues.

ExoX and Psi inhibit EPS synthesis, an effect opposite that of SyrA (Borthakur *et al.* 1985; Gray *et al.* 1990; Reed *et al.* 1991a). The effects of ExoX and Psi on EPS I synthesis are thought to occur posttranslationally (Borthakur *et al.* 1988; Gray and Rolfe 1992; Latchford *et al.* 1991). SyrA does not significantly alter expression of two genes in the EPS I synthesis pathway, *exoF* and *exoP* (Barnett 1994). Therefore, it seems likely that SyrA effects on EPS synthesis are posttranslational as well.

Reed and Walker (1991a) showed that the calcofluor-dim phenotype of an *exoD* mutant is suppressed when *syrM* and *syrA* are present on a plasmid. The mechanism by which this suppression occurs is unknown. ExoD mutants produce EPS identical in structure to EPS produced by wild-type strains, but in lower amounts (Reed and Walker 1991a). Even though *exoD* is probably not directly involved in EPS synthesis, *exoD* appears to be necessary for the *syrA*-mediated mucoid phenotype: an *exoD* mutant containing a plasmid overexpressing *syrA* was calcofluor-bright, but not mucoid (data not shown). *exoD* only slightly affects *syrA* expression, as tested using *syrA-gusA* fusion strains, and overexpression of *syrA* has no effect on expression of an *exoD*::*lacZ* fusion (data not shown). Therefore, if there is an interaction between *exoD* and *syrA*, it may occur posttranslationally.

We found that *syrM* is not required for symbiotic expression of *syrA*. Instead, *ntrA* and *nifD* are necessary. The observations that a *nifD* mutation abolishes *syrA* expression, whereas a *fixH* mutation does not, support the hypothesis that something specific in the NtrA circuit is required for *syrA* expression rather than nitrogen fixation per se. Our observation that an insertion in *nifD* reduces expression of *syrA*, weakens the case for direct activation of *syrA* by NtrA. Moreover, sequence analysis of an 830 bp region upstream of *syrA* failed to reveal an *ntrA-ntrC*/*ntrA-nifA* consensus sequence or a *nifA* upstream activating sequence (Gussin *et al.* 1986). It is possible that the insertion in *nifD* has polar effects on *syrA* transcription. The exact distance between the end of the *nifHDKE* operon and the translational start of *syrA* is not known, but is approximately 2.5-kb and other genes may map to this region. Preliminary sequence analysis has identified an ORF with similarity to a ferredoxin-like protein from *Rhodobacter capsulatus* located about 1 kb from the *syrA* translational start (data not shown). In *R. capsulatus* this ferredoxin is transcribed with the *nifENX* operon (Moreno-Vivian *et al.* 1989). The proximity of the *nif* operon to *syrA* is the basis for another model which explains the *nifD*-dependence of *syrA* expression. In this model, *syrA* can be expressed via two promoters, a *syrM*-dependent promoter located upstream of *syrA* and the *ntrA-nifA*-dependent promoter upstream of the *nifHDKE* operon. In the free-living state, expression occurs when *syrM* is overexpressed. In the symbiotic state expression occurs via transcriptional read-through from the *nif* promoter. Because NtrA is necessary for activation of the *nifHDKE* promoter (Ronson *et al.* 1987), mutations in both *nifD* and *ntrA* would abolish transcription of the *nif* operon and therefore of *syrA*. A similar situation has been observed for the expression of NifA in *R. meliloti*: *nifA* can be expressed from its own promoter as well as from the promoter of the upstream *fixABC* operon (Kim et al. 1986). Additional experiments are required to determine if *syrA* is expressed from a *nif* promoter and, if so, to determine the functional consequence of coupling expression of a gene involved in EPS production to expression of those involved in nitrogen fixation.

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