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Sinorhizobium meliloti SyrA Mediates the Transcriptional Regulation of Genes Involved in Lipopolysaccharide Sulfation and Exopolysaccharide Biosynthesis[▽]

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***Sinorhizobium meliloti* is a gram-negative soil bacterium found either in free-living form or as a nitrogen-fixing endosymbiont of leguminous plants such as *Medicago sativa* (alfalfa). *S. meliloti* synthesizes an unusual sulfate-modified form of lipopolysaccharide (LPS). A recent study reported the identification of a gene, *lpsS*, which encodes an LPS sulfotransferase activity in *S. meliloti*. Mutants bearing a disrupted version of *lpsS* exhibit an altered symbiosis, in that they elicit more nodules than wild type. However, under free-living conditions, the *lpsS* mutant displayed no change in LPS sulfation. These data suggest that the expression of *lpsS* is differentially regulated, such that it is transcriptionally repressed during free-living conditions but upregulated during symbiosis. Here, I show that the expression of *lpsS* is upregulated in strains that constitutively express the symbiotic regulator SyrA. SyrA is a small protein that lacks an apparent DNA binding domain and is predicted to be located in the cytoplasmic membrane yet is sufficient to upregulate *lpsS* transcription. Furthermore, SyrA can mediate the transcriptional upregulation of *exo* genes involved in the biosynthesis of the symbiotic exopolysaccharide succinoglycan. The SyrA-mediated transcriptional upregulation of *lpsS* and *exo* transcription is blocked in mutants harboring a mutation in *chvI*, which encodes the response regulator of a conserved two-component system. Thus, SyrA likely acts indirectly to promote transcriptional upregulation of *lpsS* and *exo* genes through a mechanism that requires the ExoS/ChvI two-component system.**

When nitrogen is limiting, leguminous plants enter into symbioses with members of the genera *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Azorhizobium*, and *Sinorhizobium* (collectively called rhizobia) resulting in the formation of nodules. Within the nodule, differentiated cytoplasmic rhizobia called bacteroids reduce molecular dinitrogen to ammonia. Bacterial colonization of the nodule requires morphological alteration of epidermal cells called root hairs, resulting in the formation of a curled structure referred to as a shepherd's crook. Shepherd's crook formation is followed developmentally by the formation of an infection thread, a tubular ingrowth of the root hair that penetrates the plant. The infection thread is occupied by the bacteria, allowing their entry into the plant interior. Finally, the rhizobia are released from the infection thread into the plant cytoplasm, where they differentiate into nitrogen-fixing bacteroids (7, 8, 24, 30, 54, 63).

Symbiosis between rhizobia and legumes is dependent on bacterial synthesis of Nod factor, a lipochitooligosaccharide composed of β-(1,4)-linked *N*-acetylglucosamine residues and *N*-acylated at the nonreducing end (13, 14, 18, 23). The Nod factor produced by *S. meliloti* carries a 16:2 *N*-acyl group and 6-*O*-acetyl group at the nonreducing end of the molecule. It also carries a 6-*O*-sulfate modification at the reducing end (34), which is essential for its biological activity. Nod factor biosynthesis is dependent upon *nod* genes, the transcription of which is upregulated during interaction with the plant host. Upregulation of *nod* gene transcription requires three LysR family members: NodD1, NodD2, and NodD3. NodD1 and NodD2 activate transcription of *nod* genes in response to plant-derived compounds such as luteolin (49) and betaines such as trigonelline and stachydrine (50). NodD3 does not appear to require a coinducer for transcriptional activation (31, 37, 46). Transcription of *nodD3* is dependent on SyrM, a LysR family transcriptional activator (37, 60). Interestingly, NodD3 can activate the transcription of *syrM* (60). Thus, these proteins are proposed to participate in a self-amplifying loop (60).

Colonization of alfalfa nodules by *S. meliloti* also requires biosynthesis of succinoglycan, an acidic exopolysaccharide. Succinoglycan biosynthesis is dependent on the products of *exo* genes, which are transcriptionally upregulated in the *exoR*::Tn*5* and *exoS*::Tn*5* mutants. The *exoS* gene encodes the sensor kinase of a two-component system, with *chvI* encoding its cognate response regulator (10, 17). The *exoR* gene encodes a negative regulator of *exo* gene transcription (17, 52). Succinoglycan biosynthesis is also increased in strains that overexpress *syrA*, which encodes a small (9,002 Da), basic protein (pI 9.07) (1). SyrA has been reported to upregulate succinoglycan biosynthesis in a posttranscriptional manner (1). Like *nodD3*, *syrA* transcription is regulated by SyrM. Thus, SyrA serves to link the biosynthesis of the two critical symbiotic polysaccharides in *S. meliloti*.

Cell surface polysaccharides such as such as lipopolysaccharide (LPS) and capsular polysaccharide (K-antigen) are also required for optimum symbiosis. In *S. meliloti*, the LPS undergoes an unusual covalent modification by sulfate (9, 29). Al-

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though common in mammalian cells, sulfated carbohydrates appear to be rare in bacteria, having only been reported in *S. meliloti* (9), *Mycobacterium* (45, 55), *Mesorhizobium loti* (62), and *Pseudoalteromonas* (56) to date. The physiological function of these sulfated molecules remains obscure, although mutants of *S. meliloti* and *M. loti* with decreased polysaccharide sulfation exhibit alterations in symbiosis (11, 62; D. H. Keating, G. R. O. Campbell, and G. C. Walker, submitted for publication). A recent publication reported the identification of a gene, *lpsS*, which encodes an LPS sulfotransferase activity in *S. meliloti* (11). Mutants bearing disrupted forms of *lpsS* produce nearly equivalent amounts of sulfated LPS as wild-type cells under free-living conditions. However, the *lpsS* mutant showed an altered symbiosis with alfalfa, eliciting the formation of nitrogen-fixing nodules at a greater rate than wild type (11).

The difference between the free-living and symbiotic phenotypes could be explained by repressed *lpsS* expression under laboratory conditions and upregulated expression during symbiosis. Here, I report that transcription of *lpsS* (as well as *exo* genes involved in biosynthesis of succinoglycan) is increased in strains that constitutively express the symbiotic regulator SyrA. Surprisingly, the ability of SyrA to mediate transcriptional upregulation of *lpsS* and *exo* genes is blocked in mutants that affect the ExoS/ChvI two-component system.

MATERIALS AND METHODS

Bacterial strains and media. All strains used are derivatives of *S. meliloti* Rm1021 (41) and are described in Table 1. All strains were grown in LB (12), tryptone yeast extract (TY) (3), or M9 (38) medium with antibiotic concentrations as previously described (48).

Strain construction. Plasmids were introduced into *S. meliloti* by triparental mating as described previously (16). Strain DKR396 was constructed by introduction of the plasmid pDKR396 (which harbors an internal fragment of *lpsS*) into Rm1021 and selection for neomycin-resistant colonies. pDKR396 cannot replicate within *S. meliloti*; thus, neomycin-resistant colonies arise from recombination events that integrate the plasmid into the genome at the *lpsS* locus, disrupting the *lpsS* gene. The insertion events were then confirmed by PCR. Strains DKR395 and DKR405 were constructed in the same manner. Strains DKR400 and DKR402 were constructed by transduction of the *nodD3*::pVO155 from strain DKR395 into DKR340 and DKR361, respectively. Strains DKR408 and DKR409 were constructed by transduction of *syrM*::pVO155 from strain DKR405 into strains DKR340 into DKR361, respectively. Strains DKR403 and DKR404 were constructed by transduction of the *chvI*::pDW33 (K214T) allele from strain EC69 to strains DKR398 and DKR399, respectively. Strains DKR411 and DKR412 were constructed by transduction of the *chvI*::pDW33 (K214T) allele from strain EC69 to strains DKR342 and DKR385, respectively. Strains DKR413 and DKR414 were constructed by transduction of the *chvI*::pDW33 (K214T) allele from strain EC69 to strains DKR420 and DKR421, respectively.

Plasmid construction. Plasmid pDKR396 was constructed by amplifying an internal fragment of *lpsS* from Rm1021 chromosomal DNA using the primers 5′-AGGTCGACGGAAGGGATTTCATTCA-3′ and 5′-TCGGATCCGCGGCGAGCTCCTCGTA-3′. This fragment was then cloned into plasmid pCR2.1 (Invitrogen), and its presence was verified by colony PCR and restriction enzyme digestion. The *lpsS*-containing fragment was then isolated from the pCR2.1 plasmid by restriction enzyme digestion with BamH1 and Sal1 and ligated into pVO155 (48), digested with the same enzymes.

Plasmid pDKR395 was constructed by amplifying *nodD3* via PCR using the primers 5′-AGGTCGACGAGCGCGTGGCTCGGGA-3′ and 5′-TGGGATCCAACATGCCCCATCGACA-3′. The fragments were then cloned into plasmid pVO155 by the method described for plasmid pDKR396.

Plasmid pDKR405 was constructed using the primers 5′-ACGCGAGTCGACAGATGATGAACCT-3′ and 5′-AAGGATCCGAGCGGAGCGGCGCCCA-3′ to amplify *syrM*. The fragments were then cloned into plasmid pVO155 by the method described for plasmid pDKR396.

Plasmid pDKR452 was constructed using primers 5′-CTATCGATCAGTTGGACGCTGCCGA-3′ and 5′-GACATATGATTGCGGTTCTCGCTGA-3′ to amplify the *syrA* open reading frame. The PCR product was then cloned into pCR2.1. The fragment was then cloned into plasmid pCR2.1 and verified by colony PCR and restriction enzyme digestion.

Plasmid pDKR453 was constructed using primers 5′-CTATCGATCAGTTGGACGCTGCCGA-3′ and 5′-GCGTCGTAACCATATGGCCGGGGCAGGGCT-3′ to amplify an N-terminal portion of the *syrA* open reading frame (residues 1 to 31). The PCR product was then cloned by the method described for plasmid pDKR452.

Plasmid pDKR454 was constructed using primers 5′-CACATATGCCTGTTCTGGAAAACCGGGCTG-3′ and 5′-CGGTACCGAAAATTCACTGCCGGGC-3′ to amplify the C-terminal portion of the *phoA* open reading frame (residues 24 to the C terminus [$phoA_{24\text{-}C\ terminus}$]). The PCR product was then cloned by the method described for plasmid pDKR452.

Plasmid pDKR451 was constructed by ligating the Xba1/Nde1 fragment from plasmid pDKR453 with the BamH1/Nde1 fragment from plasmid pDKR454. The fragment was then ligated into plasmid pRF771 (64) digested with BamH1 and Xba1 and verified by colony PCR and restriction enzyme digestion.

Plasmid pDKR482 was constructed by ligating the Xba1/Nde1 fragment from plasmid pDKR452 with the BamH1/Nde1 fragment from plasmid pDKR454 and cloning into pRF771 in the manner described for plasmid pDR451.

Preparation of extracts for LPS analysis. Extracts were prepared according to Reuhs et al. (53), as modified by Cronan et al. (11). The pellet was resuspended in 50 μl of sample loading buffer, and the polysaccharides were fractionated by Tris-Tricine-polyacrylamide gel electrophoresis (PAGE) as described previously (47). The polysaccharides were then visualized by silver staining (Bio-Rad).

In vivo labeling of LPS. Wild-type and *lpsS* mutants were cultured in TY medium containing 5 μCi of $Na_2{}^{35}SO_4$ (ICN) as described previously (11). The LPS was then extracted as described above and fractionated by Tris-Tricine-PAGE (47). The PAGE gel was then silver stained (Bio-Rad) to determine the relative amount of extracted LPS and dried, and the incorporated $^{35}SO_4$ was visualized by autoradiography and quantified by phosphorimaging (Amersham Pharmacia).

Preparation of cell surface protein extracts. Extracts were prepared as described previously (29). The resulting pellet was resuspended in 100 μl of buffer A (0.05 M Na_2HPO_4, 0.005 M EDTA; pH 7), and protein concentration was determined by a modified Bradford assay (Bio-Rad).

In vitro cell surface sulfation assay. In vitro LPS sulfation was assayed as described previously (29). A total of 0.25 to 1 μg of a particulate extract was combined with 1 μl of *S. meliloti* LPS (which was added as a sulfate acceptor), 5 μCi of $^{35}SO_4$-labeled PAPS (3′-phosphoadenosine-5′-phosphosulfate) prepared as described previously (20, 35, 58), and 2 μl of 5× buffer B (50 mM Tris-HCl [pH 8], 30 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol) in a total reaction volume of 10 μl. The mixture was then allowed to incubate for 30 min at 30°C, and the reaction was stopped by incubation for 2 min at 95°C. The samples were then heated at 95°C for 5 min in sodium dodecyl sulfate sample buffer and fractionated on a 12.5% sodium dodecyl sulfate-PAGE gel. The gel was dried, and the incorporation of $^{35}SO_4$ into LPS in the particulate fraction was measured using a phosphorimager (Amersham Pharmacia).

PAPS analysis by thin-layer chromatography. PAPS was analyzed as described previously (4), as modified by Townsend et al. (62). Briefly, wild-type strains harboring vector control, multicopy *syrA*, or multicopy *nodPQ*, were cultured in 1 ml of TY medium with $Na_2{}^{35}SO_4$ to saturation. The cells were centrifuged at 8,000 × *g*, resuspended in 1 ml of water, and centrifuged again at 8,000 × *g*. The resulting cell mass was resuspended in 0.2 ml of water, and 20 μl of 11 N formic acid was added. The mixture was mixed by vortexing, incubated on ice for 30 min, and centrifuged at 8,000 × *g* for 10 min. Fifty microliters of each supernatant was then spotted on a polyethyleneimine (PEI)-cellulose thin-layer chromatography (TLC) plate (Baker), the plate was immersed in methanol, and allowed to dry before being placed in a TLC chamber containing 100 ml of 0.9 M $LiCl_2$. After the solvent front reached the top of the TLC plate, the plate was again immersed in methanol for 2 min and allowed to dry. The $^{35}SO_4$ incorporation was visualized by autoradiography and quantified by phosphorimaging.

Reverse transcription (RT)-PCR assay. *S. meliloti* strains were cultured to stationary phase in LB medium (optical density at 600 nm [OD_{600}] of 2.5). The cells were then washed with LB and frozen at −20°C. RNA was extracted from the pellets via modified phenol-chloroform extraction (Trizol). Dilutions of the RNA were then used as templates for the synthesis of cDNA (First-Strand cDNA synthesis kit; Fermentas). Two microliters of cDNA from each dilution was used to amplify DNA via PCR. The DNA was then fractionated on a 1% agarose gel, and the DNA was detected with ethidium bromide staining, followed by fluorescent imaging (Typhoon-Amersham).

Alkaline phosphatase assay. Plasmids containing *syrA-phoA* translational fusions were introduced into strain Rm8002 (which exhibits greatly reduced alkaline phosphatase activity [36]) by triparental mating. The plasmid-bearing strains

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristic(s)[a]	Reference or source
S. meliloti strains		
Rm1021	Str^r SU47	41
Rm8002	Rm1021; *phoA* mutant; Str^r	36
DKR153	*lpsS*::pDW33	11
DKR192	Rm1021/pTE3	This study
DKR381	Rm1021/pTE3::*syrA*	This study
DKR340	*lpsS*::pDW33/pTE3	This study
DKR341	*lpsS*::pDW33/pRmJT5	This study
DKR360	*lpsS*::pDW33/pTE3::*syrM*	This study
DKR361	*lpsS*::pDW33/pTE3::*syrA*	This study
DKR475	*lpsS*::pDW33/pMS03/TE3	This study
DKR477	*lpsS*::pDW33/pMS03::*nodPQ*/pTE3	This study
DKR476	*lpsS*::pDW33/pMS03/pTE3::*syrA*	This study
DKR478	*lpsS*::pDW33/pMS::*nodPQ*/pTE3::*syrA*	This study
DKR395	*nodD3*::pVO155	This study
DKR400	*lpsS*::pDW33 *nodD3*::pVO155/pTE3	This study
DKR402	*lpsS*::pDW33 *nodD3*::pVO155/pTE3::*syrA*	This study
DKR405	*syrM*::pVO155	This study
DKR408	*lpsS*::pDW33 *syrM*::pVO155/pTE3	This study
DKR409	*lpsS*::pDW33 *syrM*::pVO155/pTE3::*syrA*	This study
DKR396	*lpsS*::pVO155	This study
DKR398	*lpsS*::pVO155/pTE3	This study
DKR399	*lpsS*::pVO155/pTE3::*syrA*	This study
EC69	*chvI*::pDW33 encoding K214T mutation	D. H. Wells, E. J. Chen, and S. R. Long
DKR403	*lpsS*::pVO155 *chvI*::pDW33 (K214T)/pTE3	This study
DKR404	*lpsS*::pVO155 *chvI*::pDW33 (K214T)/pTE3::*syrA*	This study
DKR373	*lpsS*::pDW33 *exoR*::Tn*5*	28
DKR375	*lpsS*::pDW33 *exoS*::Tn*5*	28
DKR497	*lpsS*::pDW33 *exoS*::Tn5/pTE3	This study
DKR498	*lpsS*::pDW33 *exoS*::Tn5/pTE3::*syrA*	This study
DKR494	*exoS*::Tn5/pMS03	This study
DKR495	*exoS*::Tn5/pMS03::*nodPQ*	This study
DKR342	*exoY*::pVO155/pTE3	This study
DKR385	*exoY*::pVO155/pTE3::*syrA*	This study
DKR411	*exoY*::pVO155 *chvI*::pDW33 (K214T)/pTE3	This study
DKR412	*exoY*::pVO155 *chvI*::pDW33 (K214T)/pTE3::*syrA*	This study
DW223	*exoH*::pVO155	64
DKR420	*exoH*::pVO155/pTE3	This study
DKR421	*exoH*::pVO155/pTE3::*syrA*	This study
DKR451	Rm8002/pDKR451 $syrA_{1\text{-}31}$::$phoA_{24\text{-C terminus}}$	This study
DKR482	Rm8002/pDKR451 $syrA_{\text{full-length}}$::$phoA_{24\text{-C terminus}}$	This study
DKR457	Rm8002/pTE3	This study
DKR458	Rm8002/pTE3::*syrA*	This study
Plasmids		
pTE3	pLAFR harboring serovar Typhimurium *trp* promoter	19
pRF771	pLAFR harboring serovar Typhimurium *trp* promoter and an improved multicloning site	64
pVO155	Insertional activation plasmid Nm^r	48
pDW33	Insertional activation plasmid Hyg^r	11
pMS03	Broad-host-range vector high-copy-number derivative of pMB393 containing the *trp* promoter from serovar Typhimurium	62
pRmJT5	pLAFR harboring 20-kb fragment with host specific *nod* genes	61
pS73	pTE3::*syrM*	61
pMB89	pTE3::*syrA*	1
pDKR395	pVO155::*nodD3*	This study
pDKR396	pVO155::*lpsS*	This study
pDKR405	pVO155::*syrM*	This study
pDKR451	pRF771::$syrA_{1\text{-}31}$::$phoA_{24\text{-C terminus}}$	This study
pDKR482	pRF771::$syrA_{\text{full-length}}$::$phoA_{24\text{-C terminus}}$	This study
pDKR453	pCR2.1::$syrA_{1\text{-}31}$	This study
pDKR454	pCR2.1::$phoA_{24\text{-C terminus}}$	This study
pDKR452	pCR2.1::$syrA_{\text{full-length}}$	This study
pGTO101	pMS03::*nodPQ*	62

[a] All strains are derived from strain *S. meliloti* Rm1021.

were then streaked onto LB plates containing 60 μg/ml 5-bromo-4-chloro-3-indolyl phosphate. The plates were grown for 5 days and then photographed.

β-Glucuronidase assay. Cells were grown to stationary phase ($OD_{600}$of 2.5) and then harvested. β-Glucuronidase activity was assayed under free-living conditions according to Jefferson et al. (27).

RESULTS

Expression of the *lpsS* gene is upregulated by plasmid pRmJT5, which encodes the symbiotic regulators SyrM, NodD3, and SyrA. Mutants bearing disrupted forms of *lpsS* elicit increased numbers of nitrogen-fixing nodules on the plant host alfalfa, compared to wild-type *S. meliloti* (11). However, under free-living conditions, *lpsS*::pDW33 mutants display no change in growth rate, LPS structure, or LPS sulfation compared to wild type (11). I hypothesized that the difference in observed phenotypes between free-living growth and growth in planta could result from differential expression of *lpsS*, such that it is transcribed at a low level under free-living conditions but transcriptionally upregulated during symbiosis. To test this hypothesis, I utilized a previously constructed *lpsS*::pDW33 mutant, which results in a transcriptional fusion of *lpsS* to *uidA* (11) encoding β-glucuronidase. Using this transcriptional fusion, I examined *lpsS* transcription under laboratory conditions in strains that constitutively express known symbiotic regulatory genes. The regulation of many symbiotic genes in *S. meliloti* involves three transcriptional activators: NodD1, which promotes transcription of *nod* genes in the presence of the flavonoid luteolin (49); NodD2, which promotes transcription in response to betaines such as trigonelline and stachydrine (50); and NodD3/SyrM, which upregulates *nod* genes (and other symbiotically relevant genes) during symbiosis (2, 60).

A previous report detected no changes in *lpsS* transcription in cells cultured in the presence of luteolin (11); therefore, I examined the effect of NodD3/SyrM on expression of *lpsS*. The genes *nodD3* and *syrM* are expressed at a very low level during free-living growth (60) but are upregulated during symbiosis. While the regulatory mechanism of *nodD3* and *syrM* in planta is only incompletely understood, the expression levels of *nodD3* and *syrM* are known to be increased when placed on the low-copy-number plasmid pRmJT5 (61), which contains *nodD3*, *syrM*, and *syrA*, as well as several other host-specific *nod* genes. Introduction of pRmJT5 increased transcription of the *lpsS*::*uidA* fusion by 2.8-fold compared to plasmid pTE3 (Fig. 1A), which I employed as a vector control. Therefore, the expression of the *lpsS*::*uidA* fusion is upregulated by a gene or genes present on plasmid pRmJT5.

Upregulation of expression of *lpsS* is dependent upon the symbiotic regulator *syrA*. Having shown that plasmid pRmJT5 increases the expression of *lpsS*::*uidA* transcriptional fusion, I then sought to determine whether SyrM and/or NodD3 was responsible for this transcriptional upregulation. I utilized plasmid pS73 (61), a derivative of plasmid pTE3 containing the *syrM* gene placed downstream of the *Salmonella enterica* serovar Typhimurium *trp* promoter (which leads to constitutive expression in *S. meliloti* [19]). I found that multicopy *syrM* resulted in a 1.7-fold increase in *lpsS*::*uidA* transcription, compared to the vector control (Fig. 1A). Due to the presence of the strong *trp* promoter, plasmid pS73 would be expected to produce SyrM at a higher level than plasmid pRmJT5, suggesting that SyrM might be operating in an indirect manner to promote expression of *lpsS*. SyrM has been reported to directly activate transcription of only two genes: *nodD3* and *syrA* (1, 46). NodD3 upregulates transcription by binding to a conserved 5′ region of *nod* genes called a *nod* box. Since the *lpsS* gene does not contain an apparent *nod* box, it seemed unlikely that NodD3 was responsible for the increase in *lpsS* transcription. Plasmid pRmJT5 also contains the gene *syrA*, which encodes a protein that leads to increased biosynthesis of the exopolysaccharide succinoglycan (1). I introduced the plasmid pMB89 (1) that places *syrA* under control of the *trp* promoter and measured expression of the *lpsS*::*uidA* fusion. Expression of the *lpsS*::*uidA* fusion was upregulated fourfold in the presence of multicopy *syrA* (Fig. 1A) compared to the vector control. Analysis of steady-state RNA levels by RT-PCR also showed an increase in *lpsS* transcription in the presence of multicopy *syrA* (Fig. 1B). Thus, overexpression of *syrA* results in an increase in *lpsS* transcription.

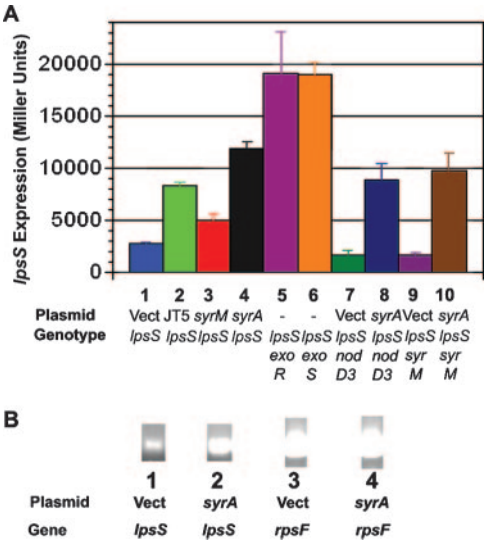

FIG. 1. *lpsS* expression is affected by plasmids overexpressing SyrA. (A) Expression of *lpsS*::*uidA* transcriptional fusions. Plasmids harboring *S. meliloti* host-specific *nod* genes were introduced into the *lpsS*::pDW33 insertion (which results in a transcriptional fusion of *lpsS* to the *uidA* gene). In addition, the *exoR*::Tn*5*, *exoS*::Tn*5*, *syrM*::pVO155, and *nodD3*::pVO155 regulatory mutations were introduced into *lpsS*::pDW33 by transduction. The strains were then grown to stationary phase (OD_{600} of 2.5) and assayed for β-glucuronidase activity as described in Materials and Methods. Activity is in Miller units. Error bars represent standard deviations of experiments carried out in triplicate. Strains lacking the *lpsS*::pDW33 showed background levels of β-glucuronidase activity (≤50 Miller units). Lane 1, *lpsS*::pDW33/pTE3 (vector); lane 2, *lpsS*::pDW33/pRmJT5 (which contains a 20-kb fragment of pSymA with host-specific *nod* genes); lane 3, *lpsS*::pDW33/pTE3::*syrM*; lane 4 *lpsS*::pDW33/pTE3::*syrA*; lane 5, *lpsS*::pDW33 *exoR*::Tn*5*; lane 6, *lpsS*::pDW33 *exoS*::Tn*5*; lane 7, *lpsS*::pDW33 *nodD3*::pVO155/pTE3; lane 8, *lpsS*::pDW33 *nodD3*::pVO155/pTE3::*syrA*; lane 9, *lpsS*::pDW33 *syrM*::pVO155/pTE3; lane 10, *lpsS*::pDW33 *syrM*::pVO155/pTE3::*syrA*. (B) Measurement of *lpsS* expression by RT-PCR. *S. meliloti* strains were cultured in LB medium. RNA was extracted from the pellets and cDNA was prepared and used as template for DNA amplification by PCR as described in Materials and Methods. Lane 1, cDNA prepared from wild type/pTE3, amplified with *lpsS*-specific primers; lane 2, cDNA prepared from wild type/pTE3::*syrA*, amplified with *lpsS*-specific primers; lane 3, cDNA prepared from wild type/pTE3, amplified with *rpsF*-specific primers; lane 4, cDNA prepared from wild type/pTE3::*syrA*, amplified with *rpsF*-specific primers.

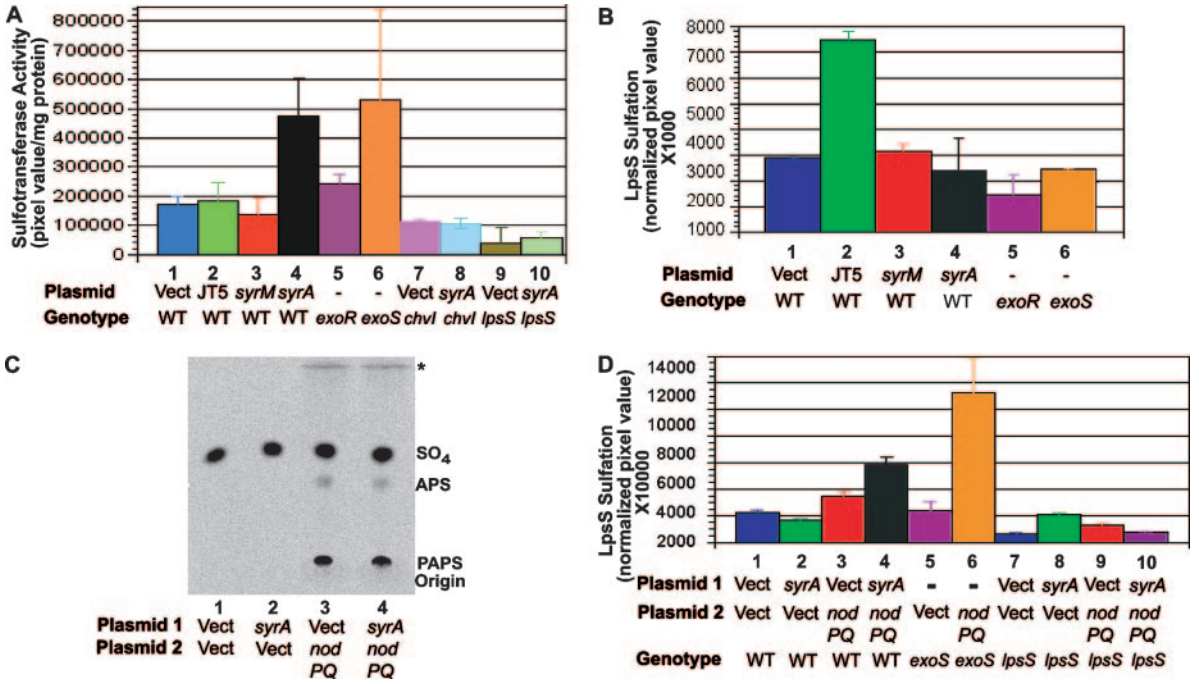

FIG. 2. Increased LpsS activity in strains overexpressing SyrA. Plasmids harboring *S. meliloti* host-specific *nod* genes were introduced into Rm1021 (wild type). In addition, the *exoR*::Tn*5* and *exoS*::Tn*5* regulatory mutations were introduced into Rm1021 by transduction. (A) LPS sulfotransferase activity. The strains were grown to saturation (OD_{600} of 2.5) and extracts were assayed for LPS sulfotransferase activity as described in Materials and Methods. LPS sulfotransferase activity represents sulfate incorporated into LPS (as measured by phosphorimaging)/mg of protein. Lane 1, Rm1021 (wild type)/pTE3 (vector); lane 2, Rm1021/pRmJT5 (which contains a 20-kb fragment of pSymA with host-specific *nod* genes); lane 3 Rm1021/pTE3::*syrM*; lane 4, Rm1021/pTE3::*syrA*; lane 5, *exoR*::Tn*5*; lane 6, *exoS*::Tn*5*; lane 7, *chvI*(*K214T*)/pTE3; lane 8, *chvI*(*K214T*)/pTE3::*syrA*; lane 9, *lpsS*::pDW33/pTE3; lane 10, *lpsS*::pDW33/pTE3::*syrA*. Error bars represent standard deviations of experiments carried out in triplicate. (B) LPS sulfation in strains overexpressing SyrA. Strains were grown to saturation (OD_{600} of 2.5) in the presence of $Na_2{}^{35}SO_4$ (ICN). Cell surface polysaccharides were then extracted and fractionated by Tris-Tricine-PAGE, and the incorporation of sulfate was measured by phosphorimaging as described in Materials and Methods. Lane 1, Rm1021 (wild type)/pTE3 (vector); lane 2, Rm1021/pRmJT5 (which contains a 20-kb fragment of pSymA with host-specific *nod* genes); lane 3, Rm1021/pTE3::*syrM*; lane 4, Rm1021/pTE3::*syrA*; lane 5, *exoR*::Tn*5*; lane 6, *exoS*::Tn*5*. (C) Measurement of PAPS biosynthesis. Strains were cultured in the presence of $^{35}SO_4$, and PAPS and APS (adenosine-5′-phosphosulfate, a derivative of PAPS) was recovered by formic acid extraction as described in Materials and Methods. The formic acid extracts were then subjected to fractionation on PEI-cellulose, and the radioactive material was detected by phosphorimaging. Migration of PAPS and APS was determined by comparison to labeled standards (not shown). The asterisk signifies a high mobility spot that did not comigrate with any of the standards. Lane 1, Rm1021 (wild type) containing pTE3 (Vect) and pMS03 (Vect); lane 2, Rm1021 containing pTE3::*syrA* and pMS03; lane 3, Rm1021 containing pTE3 and pMS03::*nodPQ*; lane 4, Rm1021 containing pTE3::*syrA* and pMS03::*nodPQ*. (D) Overexpression of *nodPQ* results in a SyrA-dependent increase in LPS sulfation. Either pMS03 (vector control) or pMS03 containing *nodPQ* from *M. loti* was introduced into wild-type strains harboring either pTE3 or pTE3::*syrA*. The incorporation of sulfate was then measured as described in panel B. Lane 1, Rm1021 (wild type), containing pTE3 (Vect) and pMS03 (Vect); lane 2, Rm1021 containing pTE3::*syrA* and pMS03; lane 3, Rm1021 containing pTE3 and pMS03::*nodPQ*; lane 4, Rm1021 containing pTE3::*syrA* and pMS03::*nodPQ*; lane 5, *exoS*::Tn*5* containing pMS03; lane 6, *exoS*::Tn*5* containing pMS03::*nodPQ*; lane 7, *lpsS*::pDW33 containing pTE3 and pMS03; lane 8, *lpsS*::pDW33 containing pTE3::*syrA* and pMS03; lane 9, *lpsS*::pDW33 containing pTE3 and pMS03::*nodPQ*; lane 10, *lpsS*::pDW33 containing pTE3::*syrA* and pMS03::*nodPQ*. Error bars represent standard deviations of experiments carried out in triplicate.

***lpsS*-dependent sulfotransferase activity is increased in SyrA overexpressing backgrounds.** Transcription of *lpsS* is elevated in the presence of multicopy *syrA*, which would be expected to increase the steady-state levels of LpsS. Antisera directed against LpsS was not available; thus, LpsS protein levels could not be measured directly. However, increased steady-state levels of LpsS protein would be expected to result in an increase in LPS sulfotransferase activity. Thus, I measured LPS sulfotransferase activity in extracts derived from cells overexpressing *nodD3*, *syrM*, and *syrA*. Extracts from strains bearing either plasmid pRmJT5 or overproducing SyrM did not exhibit a significant increase in LPS sulfotransferase activity (Fig. 2A). The reason for the lack of an increase in LPS sulfotransferase activity in the strain containing pRmJT5 is not known but may result from an increase in intracellular PAPS in the extract (which competes with the sulfate donor in the in vitro assay). However, extracts prepared from strains overexpressing *syrA* showed a twofold increase in sulfotransferase activity (Fig. 2A). Mutants that lack a functional copy of *lpsS* but overexpress *syrA* showed only 20% of the sulfotransferase activity observed in wild type (Fig. 2A), demonstrating that the increase in LPS sulfotransferase activity is LpsS dependent. Therefore, multicopy *syrA* upregulates the transcription of the *lpsS* gene and results in increased LpsS activity.

Although overexpression of *syrA* resulted in a measurable increase in LpsS activity, it was unclear whether this would contribute to a change in overall LPS sulfation. Thus, I measured the incorporation of radiolabeled sulfate into LPS in vivo

in the presence and absence of pRmJT5 or multicopy plasmids bearing *syrM* and *syrA* (Fig. 2B). Plasmid pRmJT5 resulted in a 2.5-fold increase in LPS sulfation compared to the vector control, but overexpression of *syrM* or *syrA* did not result in a measurable increase in LPS sulfation (Fig. 2B). A similar result was observed previously in strains with multicopy forms of *lpsS* (11). These strains showed increased LPS sulfotransferase activity but did not display an increase in LPS sulfation. However, strains harboring multicopy forms of *lpsS* did show increased LPS sulfation compared to vector controls when luteolin was added to the culture. Addition of luteolin also led to increased LPS sulfation in strains overexpressing *syrA*, compared to vector control strains (my unpublished results). Luteolin is known to induce the transcription of several classes of genes including *nodPQ*. The *nodPQ* genes catalyze the synthesis of PAPS, the activated form of sulfate used by LpsS and other sulfotransferases. Plasmid pRmJT5 encodes the *nodPQ* genes, as well as the regulators *syrM*, *syrA*, and *nodD3*. NodD3 can activate transcription of both the plasmid and genomic copies of *nodPQ* and would be expected to result in elevated biosynthesis of PAPS (60). Thus, it seemed possible that the differences in LPS sulfation between strains harboring multicopy *syrA* and pRmJT5 might result from differences in intracellular PAPS concentration. I measured the PAPS biosynthesis of a strain carrying pRmJT5 and observed an increase in the intracellular PAPS concentration with respect to strains carrying vector alone (my unpublished results). Therefore, I hypothesized that plasmid pRmJT5 led to an increase in LPS sulfation for two reasons: the ability to increase *lpsS* expression and the ability to increase PAPS concentration resulting from upregulation of *nodPQ* transcription. Conversely, overexpression of *syrA* increased *lpsS* expression, leading to elevated levels of LpsS-dependent sulfotransferase activity, but did not increase LPS sulfation because of limiting PAPS. To test whether LPS sulfation was limited by the internal PAPS concentration in strains overexpressing *syrA*, I utilized a plasmid (62) that places the *nodPQ* genes from *M. loti* under control of the *trp* promoter in the plasmid pMS03. PAPS was undetectable in strains harboring vector alone, suggesting that PAPS is limiting for LPS sulfation in *S. meliloti*. However, overexpression of *nodPQ* from this plasmid in a wild-type background resulted in an increase in PAPS as measured by TLC on PEI-cellulose (Fig. 2C) and a 1.5-fold increase in LPS sulfation (Fig. 2D). However, when *nodPQ* and *syrA* were both overexpressed, I observed a 2.3-fold increase in LPS sulfation (Fig. 2D). Therefore, *syrA* is capable of increasing LPS sulfation, provided that sufficient PAPS is present in the cell.

Expression of exopolysaccharide biosynthetic genes is upregulated by the symbiotic regulator *syrA*. The surprising finding that *lpsS* transcription was affected by SyrA led to a reexamination of the role of SyrA in the regulation of symbiotically important polysaccharides. A previous study had shown that overexpression of SyrA resulted in increased production of the symbiotic polysaccharide succinoglycan but did not detect increased expression of transcriptional fusions to the succinoglycan biosynthetic genes *exoP* and *exoF* (1). I introduced plasmid pMB89 harboring *syrA* into previously constructed single-copy *exoY*::*uidA* and *exoH*::*uidA* transcriptional fusions (64) (Fig. 3A). Expression of the *exoY*::*uidA* fusion was increased 2.6-fold in the presence of multicopy *syrA*, while expression of the

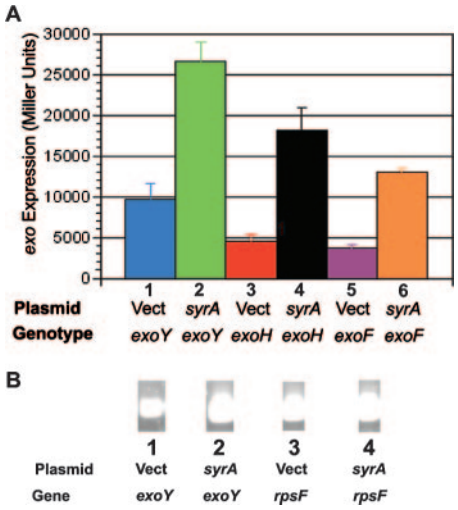

FIG. 3. Increased *exo* gene transcription in strains overexpressing *syrA*. (A) Expression of *exo*::*uidA* transcriptional fusions. Plasmids harboring *S. meliloti syrA* were introduced into the *exoY*::pVO155, *exoH*::pVO155, and *exoF*::pVO155 mutants (which result in transcriptional fusions to the *uidA* gene). The strains were then grown to saturation and assayed for β-glucuronidase activity as described in Materials and Methods. Error bars represent standard deviations of experiments carried out in triplicate. Activity is in Miller units. Lane 1, *exoY*::pVO155/pTE3 (vector); lane 2, *exoY*::pVO155/pTE3::*syrA*; lane 3, *exoH*::pVO155/pTE3; lane 4, *exoH*::pVO155/pTE3::*syrA*; lane 5, *exoF*::pVO155/pTE3; lane 6, *exoF*::pVO155/pTE3::*syrA*. (B) Measurement of *exoY* expression by RT-PCR. Purified RNA was used as template for RT-PCR. Lane 1: cDNA prepared from wild type/pTE3, amplified with *exoY*-specific primers; lane 2, cDNA prepared from wild type/pTE3::*syrA*, amplified with *exoY*-specific primers; lane 3, cDNA prepared from wild type/pTE3, amplified with *rpsF*-specific primers; lane 4, cDNA prepared from wild type/pTE3::*syrA*, amplified with *rpsF*-specific primers.

exoH::*uidA* fusion was increased fourfold in the presence of multicopy *syrA* (Fig. 3A), compared to vector alone. Utilizing RT-PCR, I also demonstrated an upregulation of *exoY* expression (Fig. 3B). Because the previous analysis of *syrA*-mediated expression had used a transcriptional fusion to *exoF*, I also constructed a single-copy *exoF*::*uidA* transcriptional fusion and measured the expression of this fusion in the presence and absence of multicopy *syrA*. The *exoF*::*uidA* fusion was upregulated threefold in cells harboring multicopy *syrA* (Fig. 3A) compared to vector alone. Thus, overexpression of *syrA* upregulates expression of multiple genes involved in succinoglycan biosynthesis.

The *nod3* and *syrM* genes are not required for SyrA-mediated transcriptional regulation of *lpsS*. The finding that overexpression of *syrA* can influence transcription was surprising, in that SyrA shows no sequence identity to known transcriptional regulators (1). Furthermore, analysis of its sequence predicted the presence of a cleavable signal sequence and a transmembrane region, which would be expected to direct the protein to a location in the cytoplasmic membrane. While membrane-associated transcriptional regulators are not unprecedented (44), it seemed likely that SyrA affected transcription in an indirect manner. One possibility was that SyrA might somehow affect the expression or activity of either NodD3 or SyrM, which would subsequently upregulate transcription. Therefore,

I examined the effect of SyrA on expression of the *lpsS*::*uidA* transcriptional fusion in backgrounds inactivated for either *nodD3* or *syrM*. Inactivation of either *nodD3* or *syrM* did not affect the ability of overexpressed *syrA* to mediate an increase in transcription of *lpsS*::*uidA* (Fig. 1A). Therefore, SyrA upregulates transcription of *lpsS* and *exo* genes through a mechanism independent of SyrM and NodD3 (although SyrM and NodD3 are clearly required for the symbiotic expression of *syrA* [1, 46]).

Expression of *lpsS* is upregulated in *exoR*::Tn*5* and *exoS*::Tn*5* mutants. The finding that SyrA could influence the expression of the *lpsS*, *exoY*, *exoH*, and *exoF* fusion suggested that SyrA upregulates transcription of *lpsS* and *exo* genes through a common mechanism. The *exo* genes are known to be transcriptionally upregulated in two mutants: *exoR*::Tn*5*, which encodes a poorly understood negative regulator of transcription (17, 52), and *exoS*::Tn*5*, which affects the sensor kinase of the ExoS/ChvI two-component regulator system (10, 17). As reported previously (28), expression of the *lpsS*::*uidA* fusion was upregulated in both the *exoS*::Tn*5* and *exoR*::Tn*5* mutants (Fig. 1A) compared to wild type. The *exoS*::Tn*5* and *exoR*::Tn*5* mutants also exhibited a 3-fold and 1.5-fold increase in LPS sulfotransferase activity, respectively (Fig. 2A). The modest increase in LPS sulfotransferase activity was surprising, given the large increase in expression of the *lpsS*::*uidA* transcriptional fusion. The reason for this is unknown but may result from the massive amounts of succinoglycan (which has an overall inhibitory effect on the in vitro sulfotransferase assay) that accumulate in the *exoR*::Tn*5* and *exoS*::Tn*5* strains. No increase in LPS sulfation was observed in vivo in either the *exoR*::Tn*5* or *exoS*::Tn*5* mutants (Fig. 2B). In order to test whether the *exoS*::Tn*5* mutant also failed to upregulate LPS sulfation due to a limitation for PAPS, I introduced plasmid pGTO101 harboring *nodPQ* into the *exoS*::Tn*5* mutant. Introduction of plasmid pGTO101 into the *exoS*::Tn*5* mutant resulted in a 4.6-fold increase in LPS sulfation compared to strains harboring vector alone (Fig. 2D). Thus, limiting PAPS appeared to prevent an increase in LPS sulfation from being observed in the *exoS*::Tn*5* mutant background. Interestingly, I was unable to introduce the plasmid pGTO101 into the *exoR*::Tn*5* strain for reasons that are unknown.

Transcriptional upregulation of *lpsS* by *SyrA* requires wild-type *chvI*. The *exoS*::Tn*5* mutant shows upregulation of *lpsS* and *exo* genes, suggesting the possibility that ExoS/ChvI and SyrA cooperate to regulate transcription of *lpsS* and *exo* genes. I thus asked whether the ExoS/ChvI two-component system is required for SyrA-mediated upregulation of *lpsS* and *exo* gene transcription. Null mutations in *exoS* or *chvI* have not been reported (10), likely because both genes are essential for the growth of *S. meliloti* in laboratory medium (the *exoS*::Tn*5* mutation discussed above is an insertion at the N terminus of the protein which results in an N-terminal truncation of the protein and what is believed to result in a constitutively active form of the ExoS/ChvI two-component system [10]). However, a recent study led to the identification of a mutation (D. H. Wells, E. J. Chen, and S. R. Long, submitted for publication), which encodes a K214T change in the ChvI protein. The K214T mutation affects the proposed DNA binding domain of ChvI and has been hypothesized to alter its ability to function as a transcriptional regulator (Wells et al., submitted). Utilizing a derivative of the plasmid pDW33 (Wells et al., submitted) I introduced the *chvI*(*K214T*) mutation into the genome of *S. meliloti* and asked whether it affected the ability of SyrA to upregulate transcription of *lpsS*::*uidA* and *exoY*::*uidA* fusions. Expression of *lpsS*::*uidA* in the *chvI*(*K214T*) mutant harboring the vector control pTE3 showed a slight increase with respect to the *lpsS*::*uidA* in a wild-type background (Fig. 4A). The reason for this increase is not known. However, expression of

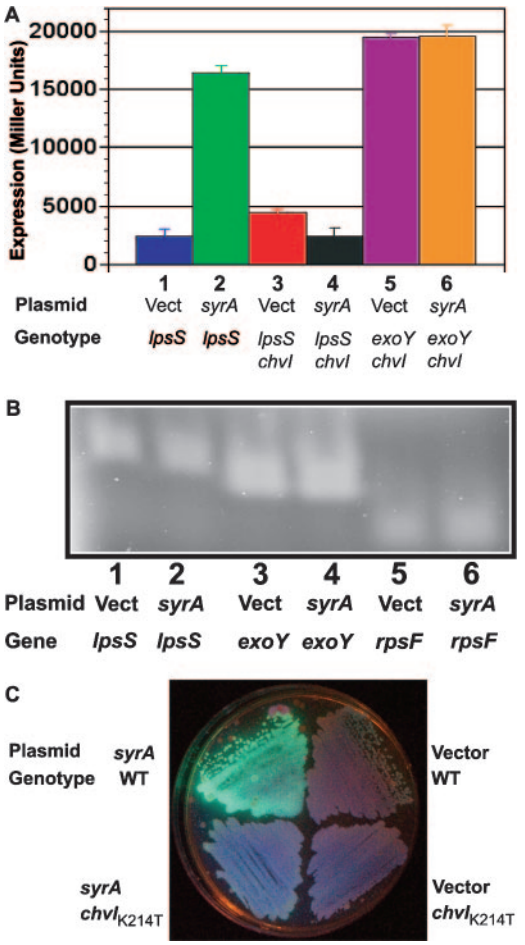

FIG. 4. Upregulation of *lpsS* and *exo* genes by SyrA requires wild type *chvI*. A K214T mutation in *chvI* was introduced by transduction into *lpsS*::pVO155 strains (which contain an *lpsS*::*uidA* fusion) harboring vector alone or overexpressing SyrA. (A) Expression of *lpsS*::*uidA* fusion. Strains were grown to saturation (OD_{600} of 2.5) and assayed for β-glucuronidase activity as described in Materials and Methods. Error bars represent standard deviations of experiments carried out in triplicate. Activity is in Miller units. Lane 1, *lpsS*::pVO155/pTE3 (vector); lane 2, *lpsS*::pVO155/pTE3::*syrA*; lane 3, *lpsS*::pVO155 *chvI*(*K214T*)/pTE3; lane 4, *lpsS*::pVO155 *chvI*(*K214T*)/pTE3::*syrA*; lane 5, *exoY*::pVO155 *chvI*(*K214T*)/pTE3; lane 6, *exoY*::pVO155 *chvI*(*K214T*)/pTE3::*syrA*. (B) Measurement of expression by RT-PCR. Lane 1, cDNA prepared from *chvI*(*K214T*)/pTE3, amplified with *lpsS*-specific primers; lane 2, cDNA prepared from *chvI*(*K214T*)/pTE3::*syrA*, amplified with *lpsS*-specific primers; lane 3, cDNA prepared from *chvI*(*K214T*)/pTE3, amplified with *exoY*-specific primers; lane 4, cDNA prepared from *chvI*(*K214T*)/pTE3::*syrA*, amplified with *exoY*-specific primers; lane 5, cDNA prepared from *chvI*(*K214T*)/pTE3, amplified with *rpsF*-specific primers; lane 6, cDNA prepared from *chvI*(*K214T*)/pTE3::*syrA*, amplified with *rpsF*-specific primers. (C) Upregulation of succinoglycan production by SyrA requires wild-type *chvI*. Strains were streaked out on plates containing calcofluor to determine succinoglycan production and photographed under UV light.

the *lpsS*::*uidA* fusion in the *chvI*(*K214T*) mutant did not increase and, in fact, decreased 55% in the presence of multicopy *syrA* (Fig. 4A). RT-PCR analysis of mRNA from strains carrying the *chvI*(*K214T*) mutation showed no upregulation of *lpsS* or *exoY* in the presence of multicopy *syrA* (Fig. 4B). I also measured LPS sulfotransferase activity in the presence of the *chvI*(*K214T*) mutation and observed no increase in LPS sulfotransferase activity in the presence of multicopy *syrA* (Fig. 2A).

I also tested the effect of the *chvI*(*K214T*) mutation on the transcriptional upregulation of *exo* genes. The *chvI*(*K214T*) mutation prevented an increase in *exo* expression (as judged by *exoY*::*uidA* fusions) (Fig. 4A) in the presence of multicopy *syrA*, which was confirmed by RT-PCR (Fig. 4B). It should be noted that the overall level of expression of the *exoY*::*uidA* fusions was higher in strains carrying the *chvI*(*K214T*) compared to the *exoY*::*uidA* fusion in a wild-type background. This apparent increase in expression likely results from the reduced amount of tetracycline in the medium used for the *chvI*(*K214T*) mutant strains (see below). The lack of transcriptional upregulation by SyrA in the presence of the *chvI*(*K214T*) mutation was also reflected in reduced upregulation of succinoglycan biosynthesis, as judged by a lack of mucoidy (my unpublished data) and decreased fluorescence when cultured on medium containing calcofluor (which fluoresces in the presence of succinoglycan [21, 33]) (Fig. 4C). Therefore, upregulation of *lpsS* and *exo* gene transcription by overexpressed SyrA is greatly reduced in strains harboring a mutant version of *chvI*.

As discussed above, the *chvI* gene is believed to be essential for growth of *S. meliloti*, and, not surprisingly, the *chvI*(*K214T*) mutant shows a number of growth phenotypes. For example, the *chvI*(*K214T*) mutant harboring the vector control pTE3 (which encodes tetracycline resistance) is unable to grow on LB plates containing 10 μg/ml tetracycline (although it will grow at 2 μg/ml tetracycline). Introduction of multicopy *syrA* restored the ability of the *chvI*(*K214T*) mutant to grow on medium containing 10 μg/ml tetracycline (Fig. 5A). Thus, overexpression of *syrA* can suppress the growth phenotype of the *chvI*(*K214T*) mutant.

The *exoS*::Tn*5* mutation prevents upregulation of *lpsS* by SyrA. The *chvI*(*K214T*) mutant prevents transcriptional regulation by SyrA, and overexpression of *syrA* can suppress the growth phenotype of the *chvI*(*K214T*) mutation. Therefore, SyrA could function through manipulation of the functionality of the ExoS/ChvI two-component system. If SyrA affects the activity of the ExoS/ChvI two-component system, then constitutively active forms of the ExoS/ChvI two-component system would be expected to be unable to carry out SyrA-mediated upregulation of *lpsS* and *exo* gene expression. I introduced a vector alone and a multicopy form of *syrA* into the *exoS*::Tn*5* mutant and measured their ability to upregulate expression of the *lpsS*::*uidA* transcriptional fusion. Although expression of the *lpsS*::*uidA* fusion was elevated in the *exoS*::Tn*5* mutant background with respect to the wild type, the overall level of expression was lower than was observed in the absence of plasmids. The reason for this is unknown but may result from the effect of tetracycline (required for retention of the plasmids) in the medium. More importantly, the expression of *lpsS*::*uidA* did not increase in the presence of multicopy *syrA* (Fig. 5B). Therefore, the *exoS*::Tn*5* mutant appears to prevent upregulation of transcription by SyrA.

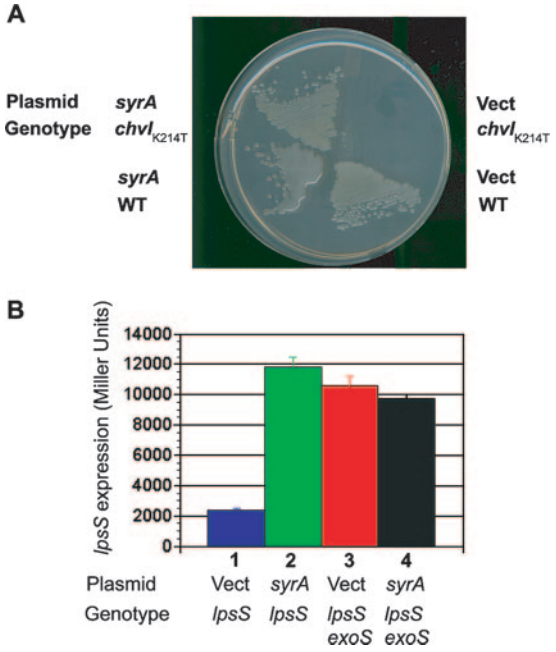

FIG. 5. Phenotypes of *chvI exoS*::Tn*5* strains overexpressing *syrA*. (A) Overexpression of *syrA* restores resistance of *chvI*(*K214T*) mutants to high concentrations of tetracycline. Wild type, strains carrying the *chvI*(*K214T*) mutation, and either vector control or vector carrying *syrA* were cultured on LB medium in the presence of 10 μg/ml tetracycline, and the plates were then photographed. (B) The *exoS*::Tn*5* mutation prevents upregulation of the *lpsS*::*uidA* transcriptional fusion in the presence of overexpressed *syrA*. Vector control and vector containing *syrA* were introduced into wild-type *S. meliloti* and the *exoS*::Tn*5* mutation containing the *lpsS*::DW33 insertion (which results in an *lpsS*::*uidA* transcriptional fusion). The extracts were then assayed for β-glucuronidase activity as described in Materials and Methods. Lane 1, *lpsS*::pDW33 harboring pTE3; lane2, *lpsS*::pDW33 harboring pTE3::*syrA*; lane 3, *lpsS*::pDW33 *exoS*::Tn*5* harboring pTE3; lane 4, *lpsS*::pDW33 *exoS*::Tn*5* harboring pTE3::*syrA*.

The N terminus of SyrA contains a signal sequence. As mentioned above, inspection of the SyrA amino acid sequence strongly suggested the presence of a N-terminal signal sequence (residues 1 to 31) and a transmembrane sequence. Thus, the protein would be expected to be located in the cytoplasmic membrane. I constructed a translational fusion of a fragment of *syrA* encoding residues 1 to 31 to a deleted form of *phoA* that encodes a mutant alkaline phosphatase lacking the N terminus (residues 1 to 24) (25, 26, 39, 40, 42, 43). The truncated N-terminal region of PhoA removes the leader peptide that directs delivery to the periplasm. Accordingly, the leader peptide mutant of PhoA would be expected to remain cytoplasmic and would be inactive (15). If the N terminus of SyrA contains a signal sequence, it would be expected to facilitate delivery of the leaderless PhoA out of the cytoplasm, resulting in active alkaline phosphatase. The translational fusion containing residues 1 to 31 ($SyrA_{1-31}$) with the leaderless PhoA resulted in alkaline phosphatase activity (as judged by blue color on medium containing 5-bromo-4-chloro-3-indolyl phosphate, which was not seen in the strains overexpressing *syrA* or the vector alone) (Fig. 6). Thus, the N terminus of SyrA encodes a functional signal sequence. I also constructed a translational fusion of the leaderless *phoA* to full-length *syrA*.

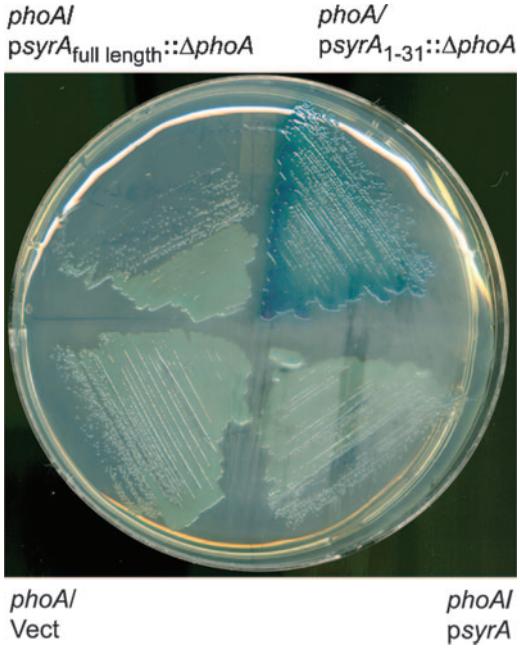

FIG. 6. The N terminus of SyrA contains a signal sequence. Either wild-type *syrA* or a fragment of *syrA* encoding residues 1 to 31 was translationally fused to a mutant form of *phoA* lacking residues 1 to 23 of the open reading frame. The plasmids were then introduced into *S. meliloti* strain Rm8002 (which shows greatly reduced alkaline phosphatase activity), streaked onto plates containing 5-bromo-4-chloro-3-indolyl phosphate), and photographed.

In contrast to the fusion to residues 1 to 31 of SyrA, this fusion did not result in alkaline phosphatase activity, as evidenced by a lack of blue color on medium containing 5-bromo-4-chloro-3-indolyl phosphate (Fig. 6). Because it was possible that the fusion resulted in an unstable form of the protein, I assayed the ability of the full-length *syrA*::*phoA* translational fusion to upregulate transcription of *lpsS*. The fusion was active as a transcriptional activator (my unpublished results). Therefore, these data suggest that the N terminus is capable of mediating the translocation of a heterologous protein from the cytoplasm, but the C terminus of SyrA remains cytoplasmic.

DISCUSSION

S. meliloti lpsS mutants exhibit an altered symbiosis, suggesting that *lpsS* is necessary under symbiotic conditions and that its expression might be symbiotically regulated. Using a combination of transcriptional fusions, RT-PCR, and assays of enzyme activity, I have shown that transcription of *lpsS* is upregulated by the symbiotic regulator SyrA. SyrA also upregulates the transcription of multiple *exo* genes, suggesting that it is a global regulator of gene transcription in *S. meliloti*. SyrA-mediated upregulation of *lpsS* and *exo* genes was prevented in strains harboring the *chvI*(*K214T*) mutation or in strains harboring the *exoS*::Tn*5* mutation, implying an involvement of the global ExoS/ChvI two-component system in transcriptional upregulation by SyrA.

The observation that the expression of *lpsS* was increased by multicopy SyrA was surprising, in that SyrA had not previously been implicated as a transcriptional regulator. The *syrA* gene was originally identified through its ability to upregulate the biosynthesis of succinoglycan (1, 46). Analysis of its open reading frame predicted the presence of a leader peptide, a transmembrane region, and the lack of a DNA binding domain. Furthermore, the authors of the previous study did not observe a change in expression of *exoP* and *exoF* transcriptional fusions and therefore concluded that SyrA upregulated succinoglycan biosynthesis via a posttranscriptional mechanism (1). I demonstrated an increase in expression of *exoY*::*uidA*, *exoH*::*uidA*, and *exoF*::*uidA* transcriptional fusions in the presence of multicopy *syrA*. Although these data differ from previous results regarding the expression of the *exo* genes (1), the clear effect of multicopy *syrA* on expression of *exo* fusions, steady-state levels of mRNA, and succinoglycan production leads to the interpretation that *syrA* affects transcription of *exo* genes. Furthermore, the finding that regulation via SyrA requires the ExoS/ChvI two-component system (a known transcriptional regulator of *exo* genes [10, 17]) further strengthens this conclusion. A recent study of global transcription in *S. meliloti* reported that overexpression of *nodD3* resulted in increased transcription of *syrM* and *syrA*, as well as *exo* genes and *lpsS* (2). In fact, overexpression of *nodD3* was shown in this study to upregulate >70 genes, and repress ca. 100 genes through what the authors suggest is an indirect mechanism. The elevated levels of SyrA observed under conditions of *nodD3* overexpression would be expected to mimic the situation observed in strains that overexpress *syrA*, leading to increased transcription of *exo* genes and *lpsS*. Thus, it seems possible that SyrA was responsible for the indirect global regulation observed in strains that overexpress *nodD3*.

Although I observed an increase in *lpsS* transcription and LPS sulfotransferase activity in strains harboring multicopy *syrA*, this increased activity was not reflected in a detectable increase in sulfated LPS. However, increased LPS sulfation was detected in strains that overexpressed *syrA* as well as *nodPQ*, which is responsible for the biosynthesis of the activated sulfate donor (PAPS) (57–59). Measurements in wild-type *S. meliloti* showed that the intracellular PAPS concentration was below the limit of detection, suggesting that limiting PAPS prevents an increase in LPS sulfation under conditions of SyrA overexpression. During symbiosis, *syrA* transcription is predicted to be upregulated as part of a regulatory loop that also leads to an increase in *nodD3* transcription (60). NodD3 can activate transcription of the *nodPQ* (one of two copies of the *nodPQ* genes) genes, which encode the enzymes necessary to synthesize PAPS (2, 46). Thus, *syrA* transcription in planta would be expected to result in an increase in LpsS activity, an elevated intracellular pool of PAPS, and increased LPS sulfation.

Examination of the primary sequence of SyrA predicts that the N terminus contains a cleavable signal peptide (residues 1 to 21) and a transmembrane sequence (residues 40 to 62). Thus, cleavage of the signal peptide upon translocation would result in a periplasmic N terminus (residues 22 to 39), followed by a transmembrane region (residues 40 to 62), and a cytoplasmic C terminus (residues 63 to 81). The alkaline phosphatase data presented here are consistent with the predicted topology. While membrane-associated transcriptional regulators have been described previously (44), SyrA is a very small protein (ca. 9 kDa) that shows no similarity to known DNA binding

proteins. Furthermore, these studies show that SyrA functionality requires the ExoS/ChvI two-component system. The *exoS*::Tn*5* mutant (which is believed to result in a constitutively active form of ExoS/ChvI [10]) has recently been reported to affect the expression of ca. 250 genes, both positively and negatively (65). Thus, it was not surprising that *lpsS* transcription was upregulated in the *exoS*::Tn*5* mutant. It was surprising however to find that the *chvI*(*K214T*) mutant prevented transcriptional upregulation via SyrA. Although the mechanism of upregulation remains unclear, several lines of evidence suggest that SyrA could function via manipulation of the activity of the ExoS/ChvI two-component system. First, although only a subset of genes has been tested, all genes that are regulated by SyrA are also controlled by ExoS/ChvI. Second, the *exoS*::Tn*5* mutation is unable to undergo SyrA-mediated upregulation of *lpsS*. Third, SyrA-mediated upregulation of *lpsS* is blocked in the *chvI*(*K214T*) mutant. Fourth, overexpression of *syrA* allows growth of the *chvI*(*K214T*) mutant on LB medium containing 10 μg/ml tetracycline. However, overexpression of SyrA will not allow the construction of null mutations in *chvI*, suggesting that overexpression of *syrA* cannot functionally replace ExoS/ChvI (my unpublished results). Collectively, the data presented here are consistent with a model where SyrA alters the functionality of the ExoS/ChvI two-component system.

Finally, although it does not resemble known transcriptional regulators, SyrA bears significant sequence identity to ExoX from *S. meliloti* (36% identity over the N-terminal half of the protein) (51, 66) and strain NGR234 (22), as well as Psi (42% identity over the central part of the protein) from *Rhizobium etli* (5, 6, 32). Interestingly, ExoX was also identified as a regulator of succinoglycan biosynthesis. A previous study reported that ExoX did not affect the expression of a translational fusion to the *exoP* gene, implying a posttranslational mechanism of regulation (66). However, considering the sequence similarities between ExoX and SyrA and the complex regulation of *exo* genes, it seems possible that ExoX could operate through a similar mechanism. Experiments to address this possibility are under way.

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