The *Sinorhizobium meliloti* SyrM Regulon: Effects on Global Gene Expression Are Mediated by *syrA* and *nodD3*

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ABSTRACT

In *Sinorhizobium meliloti*, three NodD transcriptional regulators activate bacterial nodulation (*nod*) gene expression. NodD1 and NodD2 require plant compounds to activate *nod* genes. The NodD3 protein does not require exogenous compounds to activate *nod* gene expression; instead, another transcriptional regulator, SyrM, activates *nodD3* expression. In addition, NodD3 can activate *syrM* expression. SyrM also activates expression of another gene, *syrA*, which when overexpressed causes a dramatic increase in exopolysaccharide production. In a previous study, we identified more than 200 genes with altered expression in a strain overexpressing *nodD3*. In this work, we define the transcriptomes of strains overexpressing *syrM* or *syrA*. The *syrM*, *nodD3*, and *syrA* overexpression transcriptomes share similar gene expression changes; analyses imply that *nodD3* and *syrA* are the only targets directly activated by SyrM. We propose that most of the gene expression changes observed when *nodD3* is overexpressed are due to NodD3 activation of *syrM* expression, which in turn stimulates SyrM activation of *syrA* expression. The subsequent increase in SyrA abundance results in broad changes in gene expression, most likely mediated by the ChvI-ExoS-ExoR regulatory circuit.

IMPORTANCE

Symbioses with bacteria are prevalent across the animal and plant kingdoms. Our system of study, the rhizobium-legume symbiosis (*Sinorhizobium meliloti* and *Medicago* spp.), involves specific host-microbe signaling, differentiation in both partners, and metabolic exchange of bacterial fixed nitrogen for host photosynthate. During this complex developmental process, both bacteria and plants undergo profound changes in gene expression. The *S. meliloti* SyrM-NodD3-SyrA and ChvI-ExoS-ExoR regulatory circuits affect gene expression and are important for optimal symbiosis. In this study, we defined the transcriptomes of *S. meliloti* overexpressing SyrM or SyrA. In addition to identifying new targets of the SyrM-NodD3-SyrA regulatory circuit, our work further suggests how it is linked to the ChvI-ExoS-ExoR regulatory circuit.

The symbiotic soil alphaproteobacterium *Sinorhizobium meliloti* forms nitrogen-fixing nodules on the roots of leguminous plants such as *Medicago sativa* (alfalfa) and *Medicago truncatula* (barrel medic). The earliest stages of the symbiosis involve an exchange of molecular signals: plant roots exude compounds that induce transcription of bacterial *nod* genes, which encode enzymes that synthesize lipochitooligosaccharide Nod factors (NF) (1, 2). These bacterial NF trigger early plant responses such as root hair curling, calcium spiking, and root cortical cell divisions to form the nodule organ (2). Bacteria trapped in curled root hairs penetrate the root hair through an infection thread (IT), an ingrowth of plant cell membrane and wall (3). As the IT elongates into the root, the bacteria at the tip are actively dividing.

Bacterial polysaccharides, such as cyclic β -glucans and exopolysaccharide I (EPS-I; also known as succinoglycan), are essential for bacterial invasion of plant roots, but the precise mechanisms by which they act are unknown (4, 5). Most *S. meliloti* strains produce a second exopolysaccharide (EPS-II; also known as galactoglucan), but *S. meliloti* Rm1021 and other SU47-derived strains are defective in EPS-II production due to an insertion mutation in *expR*, encoding a positive regulator of the EPS-II synthesis genes (6). As infection threads reach infection-competent plant cells, the bacteria are released from the IT tip into vesicles of the plant-derived membrane (3). Bacterial polysaccharides, such as lipopolysaccharides (LPS), appear to be altered at later developmental stages and may play a role in suppressing plant defense responses and maintaining the symbiosis (5, 7). *S. meliloti* strains also pro-

duce the K-antigen capsular polysaccharide (KPS), but its requirement for root invasion varies among strains (7). Once inside the plant cell, the bacteria terminally differentiate into nitrogen-fixing bacteroids (8, 9). These bacteroids employ nitrogenase to reduce dinitrogen to ammonia, which supports plant growth, and in turn the plant provides bacteroids with carbon in the form of C₄-dicarboxylates, such as malate and succinate (10).

During this complex developmental process, both bacteria and plants undergo profound changes in gene expression (11). Relevant regulatory circuits are summarized in Fig. 1. In the early signal exchange, activation of bacterial *nod* gene expression occurs via NodD proteins (1). NodD proteins belong to the LysR family of transcriptional regulators and activate *S. meliloti* *nod* gene expression by binding to a conserved *nod* box sequence upstream of

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FIG 1 Diagram of the SyrM-NodD3-SyrA and ExoS-ChvI-ExoR regulatory circuits. Solid lines indicate positive (arrows) and negative (bars) genetic and physical interactions previously reported (12–16, 23, 25–27, 43, 46, 47, 57–59). Dashed lines indicate potential interactions discussed in this work.

each *nod* gene operon (*nodABCIJ*, *nodFEGPQ*, *nodH*, *nodMnolFGnodN*, *nodLnoeAB*) (12–14). *S. meliloti* has three NodD proteins (Fig. 1) (15, 16). NodD1 requires plant flavonoids, such as luteolin or methoxychalcone, to activate *nod* genes (17–19). NodD2 activates *nod* genes in the presence of plant betaines, such as trigonelline and stachydrine, and the flavonoid methoxychalcone (17, 20). NodD3 does not require specific plant compounds to activate *nod* gene expression; instead, another LysR family transcriptional regulator, SyrM, activates *nodD3* expression (21–23). In a strain overexpressing *nodD3*, more than 200 genes show up- or downregulated expression (24). In addition, SyrM and NodD3 form a self-amplifying circuit: NodD3 can activate *syrM* expression by binding to a degenerate Nod box sequence upstream of *syrM* (23, 25). SyrM also activates expression of another gene, *syrA*, which when overexpressed causes a dramatic increase in EPS-I production (26, 27). *nodD3* and *syrA* upstream regions each contain a conserved SyrM box motif (25). Therefore, at least when overexpressed, SyrM appears to coordinately control NF synthesis via *nodD3* and EPS-I production via *syrA*: in the absence of *nodD3* or *syrA*, *syrM* overexpression has no effect on NF synthesis or EPS-I production. Other regulators may play a part in the response of this circuit to nutritional and environmental conditions (28, 29).

In addition to being critical for host plant invasion, EPS-I production is stimulated by low nitrogen, high phosphate, and stresses such as acid shock, heat shock, osmotic stress, and antimicrobial peptides (30–36). EPS-I protects *S. meliloti* against H₂O₂-dependent damage (37) and may also play a role in direct signaling to the plant (3, 5). *exo* genes encode enzymes for biosynthesis and export of EPS-I (38). Regulation of *exo* gene expression is mediated primarily via the ChvI (response regulator)-ExoS (histidine kinase, also known as ChvG) two-component system

(Fig. 1) (39–45). ExoS autophosphorylates in response to an unknown signal and activates ChvI via transfer of the phosphate (41). *exoR* encodes a small periplasmic protein that suppresses ExoS activity, resulting in negative modulation of ChvI (Fig. 1) (40, 43, 46–48). ExoR autoregulates its own expression through ExoS-ChvI and may be subjected to regulatory proteolysis (47, 48). Mutations in *chvI*, *exoS*, and *exoR* cause severe and pleiotropic phenotypes, suggesting that, in addition to regulating EPS-I biosynthesis, this circuit affects symbiosis, motility, biofilm formation, cell envelope composition, EPS-II production, and central metabolism (39, 42–44, 46, 49–53). Other proteins affecting EPS-I production are MucR, which acts positively (54, 55), and ExoX, which acts negatively (56, 57).

Genes necessary for nitrogen fixation, a late stage of the symbiosis, are upregulated during bacteroid differentiation. A low oxygen concentration in the nodule triggers autophosphorylation of the bacterial histidine-kinase FixL and subsequent phosphate transfer to the response regulator FixJ, setting off a regulatory cascade required for activation of expression of nitrogenase and associated genes (58). FixJ activates expression of over 120 genes (59), including the critical regulatory loci, *nifA* and *fixK*. Transcription activation of genes encoding nitrogenase (*nifHDKE*) and a few other proteins is under the control of the alternative sigma factor, RpoN, in concert with the enhancer, NifA; most of the rest are dependent on the housekeeping sigma factor, RpoD, and the regulator, FixK (58, 59). Expression of *syra* is regulated differently in nitrogen-fixing bacteroids than in free-living bacteria: *syra* is not required in bacteroids. Instead, *syra* appears to be cotranscribed with upstream *nifHDKE* and, like them, requires a functional FixLJ regulatory cascade (Fig. 1) (26, 59).

To characterize the SyrM-NodD3-SyrA regulatory circuit in more detail, we defined transcriptomes of strains overexpressing *syrM* or *syrA*. We report here that the SyrM, NodD3, and SyrA transcriptomes share similar gene expression changes, that *syrM* overexpression activates modest *nod* gene expression independently of NodD proteins, and that *nodD3* and *syrA* may be the only targets directly activated by SyrM. We propose that most of the gene expression changes observed when *nodD3* is overexpressed are due to NodD3 activation of *syrM* expression, which in turn stimulates SyrM activation of *syrA* expression. The subsequent increase in SyrA abundance results in broad changes in gene expression, probably controlled through activity of the ChvI-ExoS-ExoR regulatory circuit.

MATERIALS AND METHODS

Strain and plasmid constructions. Strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in LB medium (60) at 37°C; *S. meliloti* strains were grown in TY (61), LB, or M9 sucrose medium (62) at 30°C. Antibiotics were used at the following concentrations: ampicillin (Ap), 50 to 100 µg ml⁻¹; chloramphenicol (Cm), 50 µg ml⁻¹; gentamicin (Gm), 5 µg ml⁻¹ for *E. coli* and 25 to 50 µg ml⁻¹ for *S. meliloti*; hygromycin (Hy), 25 to 50 µg ml⁻¹; kanamycin (Km), 25 µg ml⁻¹ for *E. coli*; neomycin (Nm), 50 to 100 µg ml⁻¹ for *S. meliloti*; spectinomycin (Sp), 50 µg ml⁻¹ for *E. coli* and 50 to 100 µg ml⁻¹ for *S. meliloti*; tetracycline (Tc), 10 µg ml⁻¹; and trimethoprim (Tm), 300 µg ml⁻¹. Triparental conjugations were performed using pRK600 as a helper plasmid (63). Marked insertions and deletions were transferred between *S. meliloti* strains using N3 phage transduction as previously described (64). Oligonucleotide primers used in this study are listed in Table S1 in the supplemental material.

nodD1, *nodD2*, *nodD3*, and *chvI* deletions marked with antibiotic re-

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference
<i>S. meliloti</i> strains		
A2102	Rm1021 <i>nodD1::Tn5 nodD2::Tm^r nodD3::Tn5-233</i> ; Sp ^r Tm ^r Nm ^r Gm ^r Sm ^r	16
A2105	Rm1021 <i>nodD1::Tn5 nodD2::Tm^r nodD3::Tn5-233 nodC::lacZ</i> ; Sp ^r Tm ^r Nm ^r Gm ^r Sm ^r	16
CL150	Rm1021 <i>pstC⁺ ecfR1⁺</i>	86
DW386	Rm1021 <i>nodF::uidA</i> ; Nm ^r Sm ^r	129
EC69	Rm1021 <i>chvI</i> K214T <i>hisB</i> ; Hy ^r Sm ^r	43
EC176	Rm1021 <i>hisB</i> ; Hy ^r Sm ^r	43
JM57	Rm1021 <i>nodC::lacZ</i> ; Sp ^r Sm ^r	18
JM61	Rm1021 <i>nodD1::lacZ</i> ; Sp ^r Sm ^r	18
MB669	Rm1021 <i>flaC::uidA</i> ; Sp ^r Sm ^r	80
MB670	Rm1021 <i>mcpX::uidA</i> ; Sp ^r Sm ^r	80
MB671	Rm1021 <i>mcpU::uidA</i> ; Sp ^r Sm ^r	80
MB672	Rm1021 SMc00888:: <i>uidA</i> ; Sp ^r Sm ^r	80
MB673	Rm1021 SMc00887:: <i>uidA</i> ; Sp ^r Sm ^r	80
MB705	Rm1021 Δ <i>syra</i> ; BglIII-Clal replaced with Nm ^r cassette; Nm ^r Sm ^r	94
MB709	Rm1021 Δ <i>syra</i> <i>exoR95::Tn5-233 pho-1</i> ; Nm ^r Sp ^r Gm ^r Sm ^r	This study
MB710	Rm1021 Δ <i>syra</i> <i>exoS96::Tn5-233 pho-1</i> ; Nm ^r Sp ^r Gm ^r Sm ^r	This study
MB735	Rm1021 Δ <i>nodD1</i> ; Nm ^r Sm ^r	This study
MB736	Rm1021 Δ <i>nodD2</i> ; Sp ^r Sm ^r	This study
MB771	Rm1021 Δ <i>nodD3</i> ; Hy ^r Sm ^r	This study
MB781	Rm1021 Δ <i>nodD1D2D3 nodC::lacZ</i> ; Hy ^r Nm ^r Sp ^r Sm ^r	This study
MB1002	CL150 Δ <i>nodD1D2D3</i> ; Hy ^r Nm ^r Sp ^r Sm ^r	This study
MB1003	CL150 Δ <i>nodD1D2D3 nodC::lacZ</i> ; Hy ^r Nm ^r Sp ^r Sm ^r	This study
MB1031	CL150 Δ <i>nodD3 nodD1::lacZ nodF::uidA</i> ; Hy ^r Nm ^r Sp ^r Sm ^r	This study
MB1035	CL150 Δ <i>syra</i> ; Nm ^r Sm ^r	This study
MB1058	Rm1021 with single crossover of pMB814; Gm ^r Nm ^r Sm ^r	This study
MB1061/pDW199	Δ <i>chvI</i> strain carrying pDW199; Tc ^r Nm ^r Sm ^r	This study
Rm1021	Wild-type SU47; Sm ^r	130
Rm7210	Rm1021 <i>exoY210::Tn5</i> ; Nm ^r Sm ^r	33
Rm7225	Rm1021 <i>exoH225::Tn5</i> ; Nm ^r Sm ^r	131
Rm8264	<i>exoA264::TnphoA</i> ; Nm ^r Sm ^r	89
Rm8270	<i>exoP270::TnphoA</i> ; Nm ^r Sm ^r	89
Rm8274	<i>exoT274::TnphoA</i> ; Nm ^r Sm ^r	89
Rm8286	<i>exoV2::Tn5</i> ; Nm ^r Sm ^r	87
Rm8295	Rm1021 <i>exoR95::Tn5-233 pho-1</i> ; Sp ^r Gm ^r Sm ^r	132
Rm8296	Rm1021 <i>exoS96::Tn5-233 pho-1</i> ; Sp ^r Gm ^r Sm ^r	132
Rm8332	<i>exoQ332::Tn5</i> ; Nm ^r Sm ^r	131
Rm8337	<i>exoU337::Tn5</i> ; Nm ^r Sm ^r	88
Rm8341	<i>exoZ341::Tn5</i> ; Nm ^r Sm ^r	133
Rm8369	<i>exoF369::TnphoA</i> ; Nm ^r Sm ^r	89
Rm8416	<i>exoN416::Tn5</i> ; Nm ^r Sm ^r	131
Rm8457	<i>exoM457::Tn5</i> ; Nm ^r Sm ^r	131
Rm8468	<i>exoP468::Tn5</i> ; Nm ^r Sm ^r	131
Rm8476	<i>exoK476::Tn5</i> ; Nm ^r Sm ^r	89
<i>E. coli</i> DH5 α	<i>endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1</i> Δ (<i>lacZYA-argF</i>)U169 <i>deoR</i>	128
Plasmids		
pBluescript SK (+/–)	ColE1; cloning vector; Ap ^r	134
pDW83	pMS03 <i>exoR</i> ; Sp ^r	43
pDW199	<i>PchvI-chvI</i> in IncP broad-host-range vector; Tc ^r	43
pE43	pTE3 <i>nodD1</i> ; Tc ^r	13
pE65	pTE3 <i>nodD3</i> ; Tc ^r	13
pJQ200SK	<i>sacB</i> vector; P15a <i>ori</i> , does not replicate in <i>S. meliloti</i> ; Gm ^r	66
pMB89	pTE3 <i>syra</i> ; Tc ^r	26
pMB90	pTE3 derivative; same as pMB89, except with insert in opposite orientation; Tc ^r	26
pMB387	Nm ^r and Km ^r in pBluescript SK(–); cassette excised as BamHI-HindIII	This study
pMB814	pJQ200SK Δ <i>chvI</i> ; Nm ^r Km ^r	This study
pMB763	pJQ200SK with Δ <i>nodD1</i> cloned in SpeI/XhoI sites; Nm ^r Km ^r	This study
pMB764	pJQ200SK with Δ <i>nodD2</i> cloned in XbaI/SpeI sites; Sp ^r	This study
pMB769	pJQ200SK with Δ <i>nodD3</i> cloned in ApaI/XbaI sites; Hy ^r	This study
pMS03	pBBR broad-host-range vector; <i>trp</i> promoter; Sp ^r	52
pRK290	IncP broad-host-range vector; Tc ^r	135
pRK600	ColE1; provides RK2 transfer functions; Cm ^r	63
pS73	pTE3 <i>syra</i> ; Tc ^r	23
pTE3	pRK290 with <i>trp</i> promoter; confers strong constitutive expression in <i>S. meliloti</i> ; Tc ^r	136
pVO122	Sp ^r in pBluescript SK(+); cassette excised as BamHI-XhoI	137
pVO205	Hy ^r cassette in in pBluescript SK(+); cassette excised as BamHI	137

sistance cassettes (Table 1) were constructed by PCR amplification of the flanking regions of the gene using Herculase II polymerase (Agilent Technologies), subcloning into pUC119 (65), sequencing to confirm that the amplified sequence was correct, digesting the pUC119 derivative clones with the appropriate restriction enzymes to release the insert, and ligating the flanking regions together with the antibiotic resistance cassette into the Gm^r *sacB* vector, pJQ200SK, which does not replicate in *S. meliloti* (66). The *nodD3* and *chvI* deletions completely remove each open reading frame (ORF), whereas the *nodD1* and *nodD2* deletions retain 5 nucleotides (nt) and 38 nt of the N-terminal coding sequence, respectively. The *nodD2* deletion was designed to preserve the divergently transcribed Nod box promoter that overlaps with the 5' untranslated region of the *nodD2* transcript. Each plasmid was conjugated into *S. meliloti*, selecting for Gm^r, Sm^r, and the resistance marker of the deletion to obtain single-crossover insertions of the plasmid into the genomic region corresponding to the cloned flanking regions. After colony purification, each single-crossover strain was grown overnight in LB medium lacking Gm. Cells were diluted and plated on LB agar plates containing 5% sucrose, Sm, and the antibiotic corresponding to the deletion marker. Sucrose-resistant colonies were rescreened on plates for sucrose resistance and Gm sensitivity. Putative sucrose-resistant, Gm^s double-crossover strains were checked by PCR and DNA sequencing to confirm proper integration of the marked deletion into the *S. meliloti* genome. Additionally, a primer pair that anneals to all three *nodD* genes was used to confirm their absence in the Δ *nodD1D2D3* strains (see Table S1 in the supplemental material).

Affymetrix symbiosis chip experiments. For microarray transcriptome analysis of *S. meliloti*, we used a custom dual-genome Affymetrix symbiosis chip with probe sets corresponding to *S. meliloti* genes and intergenic regions and *Medicago truncatula* expressed sequence tags (ESTs) (24). RNA purification, cDNA preparation, symbiosis chip hybridization, fluidics, scanning, and data analysis were performed as described previously (24). Three biological replicates were used for each strain analyzed.

Three different microarray experiments were performed in this study. For the SyrM transcriptome analysis, the experimental design was similar to that of our previous NodD1 and NodD3 transcriptome analyses (24). *S. meliloti* strain A2102/pS73 (*Ptp-syrM*) was compared to A2102/pTE3 (empty vector), with cells cultured in TY-Tc-Sm and harvested at an optical density at 600 nm (OD₆₀₀) of 0.59 to 0.67. For the SyrA transcriptome analysis, Rm1021/pMB89 (*Ptp-syrA*) was compared to Rm1021/pMB90 (a control plasmid that has the same insert as pMB89 but in the opposite orientation with respect to *Ptp*), with cells cultured in TY-Tc-Sm and harvested at an OD₆₀₀ of 0.57 to 0.64. For the *chvI* K214T/SyrA transcriptome analysis, four different strains were compared: EC176/pMB89 (wild type [WT]; *Ptp-syrA*), EC176/pMB90 (WT; control plasmid), EC69/pMB89 (*chvI* K214T mutant; *Ptp-syrA*), and EC69/pMB90 (*chvI* K214T mutant; control plasmid), with strains grown in LB-Tc-Hy-Sm and harvested at an OD₆₀₀ of 0.63 to 0.74. EC69, and its wild-type control strain, EC176, have an insertion bearing hygromycin resistance in the nearby *hisB* locus to facilitate transduction of the unmarked *chvI* K214T allele (42).

β -Glucuronidase and β -galactosidase assays. Assays were performed as described by Swanson et al. (23); 20 to 200 μ l of cultured cells was used, depending on the strain assayed. For experiments that included *Ptp-nodD1* strains, cultures were grown overnight and then diluted to an OD₆₀₀ of 0.02. Half of the *Ptp-nodD1* cultures were uninduced, and the rest were treated with 3 μ M luteolin. Cells were assayed after growth to mid-exponential phase (14 to 18 h of induction time).

Motility assays. We tested swimming motilities of the Rm1021, Rm7210, and Rm7225 strains by inoculating cells into TY 0.22% agar plates supplemented with Tc and Sm. The diameter of each colony was measured on day 3 after inoculation. Assays were performed in triplicate. The motilities of the EC69 and EC176 strains were tested similarly, except that LB agar medium supplemented with 2.5 mM calcium chloride, Tc, Hy, and Sm was used.

Nodulation assays. Assays for nodulation ability on *M. sativa* cv. AS13 (alfalfa) and *M. truncatula* cv. Jemalong were performed as described before (67) with the following modifications: Tween 20 was omitted during seed surface sterilization, plants were grown in large (500-cm²) square plates (Thermo Scientific Nunc), and aminoethoxyvinylglycine (AVG) was added to a concentration of 0.1 μ M to inhibit ethylene production and allow better plant growth. Seedlings were inoculated 2 days after planting on the square plates, by adding 1.5 μ l of bacteria (washed and resuspended in 10 mM MgSO₄ to an OD₆₀₀ of 0.1) to each root tip. Plant appearance, nodule number, and nodule color were monitored for 6 weeks.

Microarray data accession number. Transcriptome data have been deposited in GEO (68) under the accession number GSE61524.

RESULTS

NodD3, SyrM, and SyrA transcriptomes share similar gene expression changes. As part of our previous work on the nodulation and exopolysaccharide (EPS-I) production regulatory circuits, we characterized the NodD1 and NodD3 transcriptomes by overexpressing *nodD1* and *nodD3* from the strongly constitutive *Salmonella enterica* serovar Typhimurium *trp* promoter (*Ptp*) in *S. meliloti* strain A2102 with mutations in *nodD1D2D3* (16, 24). *Ptp-nodD1* and its inducer luteolin caused increased expression of *nod* genes. *Ptp-nodD3* increased the expression of the *nod* genes and caused ~200 additional changes, including increased expression of *exo* genes and decreased expression of motility and chemotaxis genes (24). We hypothesized that many of these genes were not direct NodD3 targets but were regulated indirectly because NodD3 activates *syrM* expression, which in turn activates *syrA* expression, and the A2102 strain used is wild type for *syrM* and *syrA*.

To further characterize the SyrM-NodD3-SyrA regulatory circuit, we tested the effects of *Ptp-syrM* and *Ptp-syrA* on global gene expression using Affymetrix symbiosis chips (Materials and Methods). To detect gene expression changes that were dependent on *syrM* overexpression alone, and not on *nodD3*, *Ptp-syrM* was tested in the same A2102 strain background that we used for our *Ptp-nodD3* analyses. We tested *Ptp-syrA* in the wild-type Rm1021 background because it was previously shown that *syrA* overexpression does not affect expression of *syrM* or the *nodD* genes (21, 27).

We compared gene expression changes when *syrM*, *syrA*, or *nodD3* were overexpressed (Fig. 2) (24). More than half of the genes regulated by *syrM*, *syrA*, or *nodD3* were regulated by at least one of the other two regulators. Changes common to all three transcriptomes include increased expression of the *exo* genes, *sinI* N-acyl-L-homoserine lactone synthetase, *eglC* glycanase, and genes of unknown function (see Data set S1 in the supplemental material). *Ptp-syrA* was previously shown to increase expression of *exoY*-, *exoH*- and *exoF-uidA* gene fusions (69). In *S. meliloti* strains bearing an intact *expR*, *sinI* is required for quorum sensing via long-chain acyl homoserine lactones; *sinI* mutants do not show swarming motility and are less efficient at forming nodules than the wild type (70, 71). *EglC* plays a role in depolymerization of EPS-I, and *eglC* mutants are more symbiotically efficient than the wild type (72). Genes showing decreased expression include motility and chemotaxis genes and two regulatory genes encoding a diguanylate cyclase/phosphodiesterase (SMc00887) and a histidine kinase (SMc00888).

To confirm our array data, we assayed β -glucuronidase (GUS) activity for *uidA* fusions to several motility and regulatory genes

FIG 2 Venn diagram of genes whose expression changed when *syrM*, *syrA*, or *nodD3* was overexpressed. *syrM*- and *syrA*-dependent gene expression changes were identified in this study; *nodD3*-dependent changes were previously published (24). Arrows indicate the direction of the change: upward arrows correspond to genes whose expression increased with overexpression of the regulator; downward arrows correspond to genes whose expression decreased. Genes included in the diagram showed expression changes of at least 2-fold in one or more of the overexpression strains.

when *syrA* was or was not overexpressed. These assays showed that *syrA* strongly reduced expression, in agreement with our symbiosis chip data (Fig. 3). We had previously shown that *Ptpr-nodD3* strains are less motile (60 to 75%) than the wild type (24). Given the strong decrease of motility and chemotaxis genes in the *Ptpr-syrA* strain, we tested its swimming motility on soft agar plates and found it to be nonmotile (Fig. 4). Wondering if the nonmotile phenotype might be due to the abundant EPS-I produced by the

FIG 3 Expression of motility and regulatory gene fusions to *uidA* is decreased when *syrA* is overexpressed. β -Glucuronidase assays were performed as described in Materials and Methods. Cells were grown in TY-Tc-Sp-Sm.

FIG 4 A *Ptpr-syrA*-dependent decrease in motility is independent of EPS-I production. Swimming motility was tested by inoculating cells into TY 0.22% agar plates supplemented with tetracycline and streptomycin. Strains containing *Ptpr-syrA* (pMB89) are shown on the left side, and strains with pMB90 negative-control plasmid are on the right side.

Ptpr-syrA strain, we tested the plasmid in *exoY* and *exoH* mutant strains: *exoY* mutants are blocked at the first committed step of EPS-I biosynthesis and do not synthesize EPS-I; *exoH* mutants are blocked at the ninth step and make only biologically inactive high-molecular-weight EPS-I lacking the succinyl modification (73). Both strains were nonmotile with *Ptpr-syrA* and motile with the control plasmid (Fig. 4), showing that the nonmotile phenotype can be uncoupled from the EPS phenotype, a behavior similar to that of certain *exoR* and *exoS* mutants (74).

We asked whether the nonmotile phenotype could be due to decreased expression of the two regulatory genes mentioned above (SMc00887 and SMc00888), especially since diguanylate cyclases and phosphodiesterases are often involved in regulating the switch between motility and biofilm formation (75). Indeed, in *S. meliloti* global expression studies, these genes showed decreased expression under other conditions in which motility and chemotaxis gene expression was decreased, suggesting that these genes may play a role in motility regulation. When we deleted both of these genes, the resulting mutant was motile, showed calcofluor brightness similar to that of the wild type, and expressed *flaC*-, *mcpU*-, and *mcpX-uidA* fusions at normal levels (M. Barnett, unpublished data). We conclude that SMc00887 and SMc00888 are not essential for swimming motility on soft agar plates.

In general, the magnitude of the changes for genes shared by all three was strongest with the *Ptpr-syrA* strain and weakest with the *Ptpr-syrM* strain, corresponding to the degree of mucoid and motility phenotypes of the three strains (*Ptpr-syrA* > *Ptpr-nodD3* > *Ptpr-syrM*). Moreover, within functional classes common to the three transcriptomes, there were some genes that showed no altered expression in the *Ptpr-syrM* strain; for example, 22 motility and chemotaxis genes (decreased) and six *exo* genes (increased) were changed only in the *Ptpr-nodD3* or *Ptpr-syrA* strain (*exoH*, *exoM*, *exoO*, *exoU*, *exoZ*, and SMb20950). Likewise, of four genes in an operon predicted to be involved in the production of a novel glycan (76, 77), two had increased expression in all three transcriptomes (SMb21190 and *msbA2*) and two in the *Ptpr-syrA* and *Ptpr-nodD3* strains only (SMb21188 and SMb21189). We suggest

that SyrM regulates Smb21188 and Smb21189 as well, but because of the relative magnitude of SyrM effects on gene expression, their changes were not detectable above the background. Other cell surface-related genes found only in the *Ptp-syrA* and *Ptp-nodD3* groups include *kpsF1*, Smb20810, Smb21506, *pssF*, and *lpsS*; *lpsS* encodes an LPS sulfotransferase previously shown to increase expression when *syrA* was overexpressed (69). Thus, the SyrM-NodD3-SyrA regulatory circuit appears to influence key cellular processes, such as motility and production of cell surface components.

Given the apparent bias in the SyrM-NodD3-SyrA data set toward genes involved in motility and cell surface components, we compared our set of unique gene changes ($n = 370$) to the most recent subcellular localization predictions for the *S. meliloti* genome using PSORTdb version 3, which is optimized for use with bacterial and archaeal genomes (78). We found a strong bias against proteins with a predicted cytoplasmic location and toward proteins with predicted membrane, periplasmic, and extracellular locations (see Fig. S1 in the supplemental material). Such biases have been observed in other *S. meliloti* strains with increased EPS-I production and decreased motility, such as *cbrA*, *podJ*, *exoR*, and *exoS* mutants (see Fig. S1) (43, 79, 80), and suggest that remodeling of the cell envelope occurs with overexpression of *syrM*, *nodD3*, or *syrA*. *CbrA* is a histidine kinase involved in cell cycle regulation and is required for an effective symbiosis (81–83). *PodJ* is a polar localization factor that is dispensable for symbiosis (79). *cbrA* and *podJ* mutants have envelope defects and are sensitive to the detergent deoxycholate (DOC) (79, 81, 83). The *Ptp-syrA* strain (Rm1021/pMB89) grew as well as the wild type (Rm1021/pMB90) on LB agar plates supplemented with 0.01% SDS or 0.7% DOC, whereas an envelope-defective mutant, *lpsB*, did not (Barnett, unpublished). Therefore, any alterations in the cell envelope of the *Ptp-syrA* strain are not as severe as the alterations in *cbrA* and *podJ* mutants.

***syrM* overexpression activates low-level *nod* gene expression independently of NodD proteins.** Among the genes common to only the *nodD3* and *syrM* transcriptomes, all but three are *nod* genes (Sma1957, SMC01713, and SMC02656, encoding hypothetical proteins). We were surprised to find that overexpressing *syrM* in the absence of *nodD3* induces *nod* gene expression, because whether SyrM activates *nod* gene expression directly has been the subject of some debate (21–23). We previously proposed that *syrM* does not directly activate *nod* gene expression but exerts effects on *nod* gene expression indirectly by activating *nodD3* expression, which in turn activates *nod* gene expression (23). The magnitude of expression increases was lower with *Ptp-syrM* than with *Ptp-nodD3*, raising concerns that the increased expression might be due to residual NodD activity in the A2102 *nodD1D2D3* mutant: the *nodD1* and *nodD3* genes contain transposon insertions; the exact location of the *nodD3* insertion was not determined but was placed in or very near *nodD3* by restriction mapping (16). Therefore, we constructed null deletion mutants for *nodD1*, *nodD2*, and *nodD3* (see Materials and Methods) and made triple *nodD* deletion mutants in both Rm1021 and CL150 (Rm1021 *pstC⁺ecfR1⁺*) backgrounds (Table 1). Testing these mutants for *nodC::lacZ* expression confirmed the symbiosis chip results for *nodC* expression (see Data set S1 in the supplemental material) and showed that the A2102 *nodD1D2D3* mutant behaves like our new deletion mutants for *nodC::lacZ* expression: *Ptp-syrM* confers a 5- to 7-fold increase in *nod* gene expression

FIG 5 *Ptp-syrM* induces *nod* gene expression in the absence of all three NodD proteins. *nodD1D2D3 nodC::lacZ* mutant strains overexpressing *syrM*, *nodD1*, or *nodD3* from *Ptp* were assayed for β -galactosidase as described in Materials and Methods. A2105 and MB781 are derived from Rm1021, and MB1003 is derived from CL150 (Rm1021 *pstC⁺ecfR1⁺*). Cells were grown in TY-Tc-Sp-Sm. The *Ptp-nodD1* strain was assayed $\pm 3 \mu\text{M}$ luteolin and after 18 h of induction.

that is independent of activation by NodD (Fig. 5). This is much lower than the 60- to 143-fold increases observed with *Ptp-nodD1* with luteolin or *Ptp-nodD3* but greater than those for *Ptp-nodD1* without luteolin or the empty vector control (Fig. 5). Inducing the *Ptp-syrM nodC::lacZ* strains with 3 μM luteolin or *M. sativa* and *M. truncatula* seed exudates had no additional effects on *nodC::lacZ* expression (Barnett, unpublished). To test a different *nod* gene, we assayed the effect of *Ptp-syrM* on the expression of a *nodF::uidA* fusion: this showed a 17-fold increase in expression, compared to 163- and 296-fold for *Ptp-nodD3* and *Ptp-nodD1* with luteolin (see Fig. S2 in the supplemental material).

Since plasmid-borne *nodD1*, *nodD2*, or *nodD3* can suppress the nodulation defects of a *nodD1D2D3* triple mutant (this study) (16, 21, 22), we tested if *Ptp-syrM* could also suppress its Nod[−] phenotype on *M. sativa* or *M. truncatula*. The $\Delta nodD1D2D3$ strain carrying *Ptp-nodD1*, *Ptp-nodD2*, or *Ptp-nodD3* formed Nod⁺ Fix⁺ nodules, but the $\Delta nodD1D2D3$ strain carrying *Ptp-syrM* was completely Nod[−] (Barnett, unpublished). An Rm1021 strain carrying *Ptp-syrM* nodulated like wild-type Rm1021; therefore, the Nod[−] phenotype of the $\Delta nodD1D2D3$ *Ptp-syrM* strain is unlikely to result from *syrM* overexpression (Barnett, unpublished). Apparently, the level of *nod* gene induction when *syrM* is overexpressed is insufficient to support nodulation.

***nodD3* and *syrA* are the main targets directly activated by SyrM.** Consistent with SyrM's ability to activate expression of

FIG 6 Most of the genes whose expression changes in a *Ptrp-syrA* strain compared to a control strain showed altered expression in the *exoR95::Tn5* and *exoS96::Tn5* mutants (43). Arrows indicate the direction of the change: upward arrows correspond to genes whose expression increased in the *Ptrp-syrA* strain (Rm1021/pMB89 versus Rm1021/pMB90) or in the *exoR95::Tn5* and *exoS96::Tn5* mutants compared to that of the wild type, and downward arrows correspond to genes whose expression decreased. Genes included in the diagram showed expression changes of at least 2-fold in one or more of the strains. The total number of genes changed with *Ptrp-syrA* is different here than in Fig. 2, because a different set of genes was used for comparison.

nodD3 and *syrA*, each of these two target genes has a conserved sequence upstream of its transcription start site, the “SyrM box” (see Fig. S3 in the supplemental material) (25, 26, 84). With the *syrM*, *nodD3*, and *syrA* transcriptome data in hand, we hypothesized that direct targets were most likely to be found among those genes whose expression changed in *Ptrp-syrM* strains only or in the unique intersecting set of *Ptrp-syrM* and *Ptrp-nodD3* strains (because *syrM* expression is activated by *Ptrp-nodD3*, and because *S. meliloti* A2102 contains a wild-type *syrM* gene, some SyrM direct targets may be represented in the *Ptrp-nodD3* transcriptome). Beyond the *nod* genes, this set includes genes encoding a sugar transporter, lysine/ectoine/asparagine transporter, aminopeptidase, aminohydrolase, dehydrogenases, and proteins of unknown function (see Data set S1 in supplemental material). Using the SyrM box consensus sequence as the query, we failed to find SyrM box-like sequences upstream of any of the *syrM*-dependent genes.

We searched with subregions of the SyrM box (GCAT-N₃-GGGAT, GCAT-N₁₄-TTTGCAT, GCAT-N₁₇-GCAT, TTTGCATG) and with GGGAT-N₆-TTTGCAT motifs that are also conserved upstream of SyrM2-activated genes in the closely related *Sinorhizobium fredii* NGR234 (see Fig. S3 in the supplemental material) (85). We found a match to GCAT-N₁₇-GCAT >200 nt upstream of SMb20892 and a match to the GCAT direct repeat

motif with an 18-nt spacer upstream of SMA1957 (see Fig. S3). Failing to find many conserved motifs upstream of genes whose expression changed with *Ptrp-syrM*, we searched the *S. meliloti* upstream genome, including sequences 400 bp upstream of all open reading frames and 100 bp upstream of all mRNA transcription start sites (86). We failed to find any full-length matches to the SyrM box in this genome-wide search but found 18 matches to subregions (see Fig. S3), most to the GCAT-N₁₇-GCAT motif. Because *S. fredii* NGR234 SyrM2 activates a *nodD* gene, we carefully examined the sequence upstream of *S. meliloti nodD1* and *nodD2* and found a weak match to the SyrM box (see Fig. S3), 462 bp upstream of the known *nodD2* transcription start site (Barnett, unpublished) (86). In sum, the bioinformatics analyses indicate that *nodD3* and *syrA* are the main direct targets for SyrM activation and are likely responsible for all the broader effects of SyrM on gene expression.

***Ptrp-syrA* suppresses phenotypes of a *chvI* mutation, and strains overexpressing *syrA* share some phenotypes with the *exoR95::Tn5* and *exoS96::Tn5* mutants.** In comparing our new transcriptome data sets to other global studies, we noted that the *Ptrp-syrA* data set was similar to that of the *exoR95::Tn5* and *exoS96::Tn5* transcriptomes (Fig. 6; see also Data set S1 in the supplemental material). ExoS is a histidine kinase that activates the ChvI response regulator; ExoR is a periplasmic negative regulator of ExoS activity (Fig. 1) (41–43, 46).

While published null *chvI* mutants cannot grow in rich medium (49, 53) or, in the case of the null insertion mutant, liquid medium (49), a point mutation (K214T) in the putative DNA binding domain of ChvI confers partial loss of function: the ChvI K214T strain produces little EPS-I, fails to grow on TY-rich medium, grows slowly on LB-rich medium, shows decreased expression of ChvI target genes, is symbiotically defective (42, 43), and grows poorly when selecting for Tc^r plasmids (69). As discussed above, *Ptrp-syrA* and *exoR95* and *exoS96* strains share two key phenotypes: nonmotility and EPS-I overproduction. The *exoR95::Tn5* (and some *exoS* alleles) suppress phenotypes of the *chvI* K214T mutant, presumably by increasing ChvI activity and expression of ChvI target genes (41–43, 46). We tested a *Ptrp::syrA chvI* K214T strain to see if overexpressing *syrA* would suppress any of the *chvI* K214T phenotypes listed above. We found that the *Ptrp-syrA chvI* K214T strain grows on TY medium and has a more calcofluor bright and less dry colonial morphology than does the *chvI* K214T strain bearing the control plasmid (Table 2). The *chvI* K214T strain overexpressing *syrA* is also more effective in symbiosis with *M. sativa* and *M. truncatula* than is the *chvI* K214T strain bearing a control plasmid: its host plants are less chlorotic and

TABLE 2 Phenotypes of *Ptrp-syrA* strains

Strain	Description	Motility ^a	EPS-I ^b	Growth on TY	Symbiosis ^c	<i>Ptrp-exoR</i> compatible ^d
Rm1021/pMB89	WT; <i>Ptrp-syrA</i>	—	++	+	+	+
Rm1021/pMB90	WT; control plasmid	+	+	+	+	—
EC69/pMB89	<i>chvI</i> K214T mutant; <i>Ptrp-syrA</i>	+/—	+	+	+	+
EC69/pMB90	<i>chvI</i> K214T mutant; control plasmid	+	—	—	+/—	—

^a Swimming motility assayed as described in Materials and Methods. Swim motility colony diameter of EC69 pMB89 was ~55% of that of EC69 pMB90. —, nonmotile; +, motile.

^b Brightness on LB agar plates containing 0.02% calcofluor white and *exo* gene expression in arrays. ++ indicates both increased brightness and *exo* gene expression compared to the wild type (+), and — indicates both decreased brightness and *exo* gene expression compared to the wild type.

^c *Medicago sativa* and *M. truncatula* overall effectiveness of symbiosis based on the number of nodules and plant appearance. —, ineffective symbiosis; +, effective symbiosis.

^d Ability to introduce the *Ptrp-exoR* plasmid (pDW83) into the strain. The empty vector (pMS03) was successfully used as a positive control for conjugations of pDW83 into *S. meliloti* strains containing pMB89 or pMB90. Rm1021/pMB89 and Rm1021/pDW83 and EC69/pMB89 and EC69/pDW83 strains were mucoid. —, not compatible; +, compatible.

form more pink nodules, indicating more effective nitrogen fixation (Table 2; see also Fig. S4 in the supplemental material). In contrast to a previous report, we found that the *chvI* K214T strain bearing the control plasmid grows on LB with 10 $\mu\text{g ml}^{-1}$ tetracycline, although the same strain carrying *P**trp-syrA* grows even better, consistent with the previous results (69). The motility of the *P**trp-syrA* *chvI* K214T strain was reduced compared to that of the *chvI* K214T control plasmid strain, suggesting that SyrA partially suppresses motility, even with a less active ChvI protein. Therefore, overexpressing SyrA affects *chvI* K214T mutant phenotypes similar to the *exoR95::Tn5* mutation.

Previous studies showed that the *exoR95::Tn5* and *exoS96::Tn5* alleles confer lethality in strains with certain blocks in EPS-I biosynthesis: mutations in *exoL*, *exoM*, *exoP*, *exoQ*, *exoT*, *exoU*, *exoV*, or *exoW* likely accumulate toxic lipid-linked intermediates (87–89). We tested conjugation of plasmid-borne *P**trp-syrA* into strains bearing insertions of *exoA*, *exoF1*, *exoH*, *exoK*, *exoM*, *exoN1*, *exoP*, *exoQ*, *exoT*, *exoU*, *exoV*, *exoY*, and *exoZ*, using pMB90 as a positive control. We could conjugate *P**trp-syrA* into all of these except the *exoM* strain (Rm8457). We note that the *exoA* (Rm8264) and *exoT* (Rm8293) strains grew more slowly when *syrA* was overexpressed (Barnett, unpublished). Thus, SyrA overexpression does not result in the accumulation of toxic intermediates in *exo* mutants to the extent that the *exoR95::Tn5* and *exoS96::Tn5* alleles do.

It had been previously shown that a plasmid overexpressing *exoR* (*P**trp-exoR*) could not be introduced into wild-type strains (43). Since ExoR inhibits activity of the ExoS histidine kinase and, hence, subsequent activation of ChvI, a *P**trp-exoR* strain is expected to behave like a *chvI*-null mutant. For example, a *P**trp-exoR* strain is not viable on LB medium unless the *exoS96::Tn5* allele that confers increased ExoS activity is present (43, 46). Because *P**trp-syrA* suppressed known ChvI K214T phenotypes, we asked whether it would allow conjugation of *P**trp-exoR* into a wild-type strain. We performed conjugations of *P**trp-exoR* or the control empty vector into strains containing either *P**trp-syrA* or a control plasmid (Table 2). We could introduce *P**trp-exoR* into a wild-type strain when it contained *P**trp-syrA* but not when it contained the empty vector. We were surprised to find that we could also introduce *P**trp-exoR* into the *chvI* K214T mutant strain containing *P**trp-syrA*, indicating that SyrA overexpression is sufficiently strong to suppress the lethal effects of *P**trp-exoR*, even in a mutant with only partially active ChvI. In summary, the *P**trp-syrA* strain behaves similarly to the *exoS96::Tn5* strain in that it allows introduction of a plasmid overexpressing *exoR*.

***P**trp-syrA* suppresses altered gene expression of the *chvI* K214T mutant.** Given our results that *P**trp-syrA*-containing strains have phenotypes similar to those of *exoR95::Tn5* and *exoS96::Tn5* mutants and that *syrA* can suppress phenotypes of a *chvI* K214T mutant, we wished to determine how these *P**trp-syrA*-dependent behaviors affected global transcription changes in the *chvI* K214T mutant. We used our symbiosis chip to probe expression in four strains: the wild type with and without *P**trp-syrA* and the *chvI* K214T mutant with and without *P**trp-syrA*. The results are provided in Data set S2 in supplemental material.

Some pairwise comparisons of this experiment are analogous to comparisons of earlier experiments (e.g., the wild type with and without *P**trp-syrA* of this study and the wild type versus the *chvI* K214T mutant published by Chen et al. [42]) and generally agree with each other, although some differences were expected due to

the use of different strains and growth conditions. One notable difference is that 13 chemotaxis/motility-related genes showed increased expression in the *chvI* K214T mutant compared to that in the wild type, whereas these genes were unchanged in the previous study (42). Our motility tests (see above) showed that the *chvI* K214T mutant was only slightly more motile than the wild type, although this difference may be obscured by the much slower growth of the *chvI* K214T mutant than the wild type on LB medium. Our data are consistent with the previous observation that a *chvI*-null mutant is hypermotile compared to the wild type on M9 succinate minimal medium (53).

Comparison of the *chvI* K214T *P**trp-syrA* strain (EC69/pMB89) and the wild-type control strain (EC176/pMB90) is most relevant to this study. Given *P**trp-syrA*'s suppression of *chvI* K214T mutant phenotypes, we suspected that the two transcriptomes might be similar: i.e., we predicted that overexpressing SyrA would compensate for altered *chvI* function. We found that only 41 genes showed expression changes of ≥ 2 -fold in EC69/pMB89 compared to in EC176/pMB90; nearly half encode proteins lacking predicted functions (Table 3). Expression of EPS-I biosynthesis genes did not significantly differ between the two strains, indicating that *P**trp-syrA* complements the *chvI* K214T mutant's *exo* gene expression defect. Seven motility genes show slightly decreased ($< 2.5\times$) expression in EC69/pMB89, consistent with its slight motility defect on soft agar plates (see above) and transcriptome data showing 48 chemotaxis/motility-related genes with decreased expression in EC69/pMB89 versus EC69/pMB90. Thus, our data show that abundant SyrA can counteract the transcription effects of the *chvI* K214T mutation, resulting in cells whose transcriptome is very similar to that of the wild type.

The *chvI* K214T mutant fails to grow on TY medium and grows poorly on LB medium (43). We sought transcriptome clues as to whether this might relate to specific metabolic pathways, to energy use, or to cell envelope changes. Previous studies showed that a *chvI*-null mutant failed to grow on some carbon sources and that uracil or proline supplementation could generally improve its growth, suggesting that the ExoS-ChvI circuit affects carbon metabolism (49, 90). Analysis of metabolic gene expression in the transcriptomes did not provide any obvious explanation for why the *P**trp-syrA* plasmid enables the *chvI* K214T mutant to grow better on rich media. We find that *S. meliloti* swimming motility is greater on TY and LB medium than on M9 minimal medium, with calcium-rich TY conferring the most motility. Perhaps growth of a hypermotile *chvI* mutant is too energetically costly on rich medium, especially because the *chvI* mutant may be less effective at using some amino acids as carbon sources (49). By reducing motility, *P**trp-syrA* may allow a metabolically defective *chvI* K214T mutant to grow. Alternatively, poor growth of the *chvI* K214T mutant on rich medium may not be related to its nutritional requirements but instead results from defects in cell envelope structure. Since an *exoY* mutant that fails to synthesize EPS-I grows well on rich media (56, 91), it is unlikely that the EPS-I defect of the *chvI* K214T mutant is the sole cause of its poor growth on rich media. Thus, if cell envelope defects are indeed responsible for poor growth of *chvI* mutants on rich media, other less well-studied components, or synergistic effects of multiple envelope components, may be responsible.

The production of cyclic β -glucans, which are essential for nodule invasion, is also regulated by a two-component system, FeuP (response regulator)-FeuQ (histidine kinase). FeuP activates

TABLE 3 Genes whose expression was changed ≥ 2 -fold in *chvI* K214T *Ptp-syrA* (EC69/pMB89) strains compared to that in wild-type (EC176/pMB90) strains

Gene name ^b	Description of gene product	Avg SLR	SD
SMA0840 ^a	NodD3, transcriptional regulator	-2.49	0.34
SMB21269	ABC transporter, ATP-binding/permease protein	-1.13	0.39
SMB21440	Hypothetical protein	-2.66	0.21
SMc00084	Hypothetical protein	-0.99	0.14
SMc00404	Hypothetical protein	-1.12	0.25
SMc01163	Oxidoreductase, essential for growth with scyllo-inositol	-1.69	1.11
SMc01165	IolC, sugar kinase, inositol metabolism	-2.14	1.24
SMc01580	Hypothetical transmembrane protein	-1.56	0.22
SMc01581	Hypothetical transmembrane protein	-2.11	1.25
SMc01855	Membrane-bound lytic transglycosylase	-1.02	0.17
SMc02317	Hypothetical signal peptide protein	-0.96	0.12
SMc03005	Conserved hypothetical protein, required for motility	-1.28	0.17
SMc03021	FlhM, flagellar motor switch transmembrane protein	-1.07	0.15
SMc03022	MotA, chemotaxis/motility protein A	-0.99	0.25
SMc03023	Conserved hypothetical protein	-1.01	0.14
SMc03030	FlgG, flagellar basal-body rod protein	-1.09	0.14
SMc03033	MotE, chaperone for MotC folding and stability	-1.19	0.14
SMc03050	FlaF, flagellin synthesis regulator	-1.04	0.07
SMc03071	Hypothetical protein	-1.20	0.18
SMA0835	Hypothetical 10.5-kDa Orf	3.38	0.35
SMA0838	SyrA, periplasmic protein involved in EPS-I production	5.57	0.14
SMA2297	Hypothetical protein	1.68	0.13
SMB20838	Secreted calcium-binding protein	1.37	0.17
SMc00065	FeuN, modulator of FeuPQ two-component system	1.07	0.21
SMc00092	CysH, phosphoadenosine phosphosulfate (PAPS) reductase	1.12	0.38
SMc00141	Hypothetical protein	1.64	0.21
SMc00198	Hypothetical protein	2.01	0.26
SMc00252	Hypothetical signal peptide protein	1.28	0.27
SMc00712	Conserved hypothetical transmembrane protein	1.27	0.26
SMc00809	Hypothetical signal peptide protein	1.40	0.39
SMc01016	Hypothetical protein	1.46	0.14
SMc01216	Hypothetical protein	1.50	0.35
SMc01557	Hypothetical signal peptide protein	1.26	0.30
SMc01586	Hypothetical transmembrane protein	1.49	0.17
SMc02171	FrcB, fructose ABC transporter, periplasmic binding protein	2.21	1.31
SMc02388	Hypothetical transmembrane protein	1.46	0.16
SMc02389	Hypothetical transmembrane protein	1.84	0.50
SMc02669	rRNA 5S	1.52	0.20
SMc03900	NdvA, cyclic beta-1,2-glucan ABC transporter	1.03	0.29
SMc04336	Hypothetical transmembrane protein	2.03	0.44
SMc04434	RpmH, 50S ribosomal protein L34	0.96	0.43

^a Decrease in *nodD3* expression may be due to deletion of 404 bp of the C-terminal end of the *nodD3* ORF on the *syrA* plasmids (26), resulting in a truncated *nodD3* mRNA being transcribed from *Ptp* on pMB90.

^b Genes with names in bold belong to the FeuPQ regulon (77). Genes with names in italics belong to the motility and chemotaxis regulon.

transcription of *ndvA*, encoding a cyclic β -glucan exporter, and at least 13 other genes (77). An intriguing observation from comparison of the *chvI* K214T *Ptp-syrA* mutant to the wild type is the increased expression of 12 of the 14 genes under the control of the response regulator FeuP (77). Examination of the fold change values suggests that *Ptp-syrA* only partially suppresses the high expression of these genes in a *chvI* K214T mutant (Table 3; see also Data set S2 in the supplemental material).

SyrA overexpression fails to allow construction of a *chvI* deletion mutant. A previous report suggested that *syrA* overexpression from plasmid pMB89 did not allow construction of null mutations in *chvI* (69). To confirm this, we constructed a complete *chvI* deletion in a *sacB* vector (see Materials and Methods) and introduced it into Rm1021 by single crossover. Then into this single-crossover strain, we mated plasmids with *chvI* (positive control) or *Ptp-syrA* or control plasmids (pMB90 or pRK290) and selected for the double crossover on either LB-rich solid medium or M9 minimal solid medium, each containing 5% sucrose. Only the *chvI* plasmid could support viability of the double crossover, suggesting that *chvI* is essential for growth under our conditions, and that *syrA*, even when overexpressed, fails to substitute for *chvI* in supporting growth.

DISCUSSION

What is the role of the SyrM-NodD3-SyrA regulatory circuit?

Our SyrM and SyrA transcriptome data agree with previous studies showing that SyrM and NodD3 form a self-amplifying circuit that leads to increased expression of *nod* and *exo* genes and decreased expression of motility and chemotaxis genes (21–24, 26, 27, 69). Our results extend these studies by identifying additional gene expression changes: for example, SyrM activates low-level *nod* gene expression, and the vast majority of SyrA-mediated gene expression changes may be due to SyrA interacting with the ExoR-ExoS-ChvI regulatory circuit.

syrM is required for efficient nodulation of *M. sativa* and *M. truncatula* (21, 67, 92). Additionally, *syrM* and *nodD3* by themselves are insufficient for *M. truncatula*: another *nodD* is necessary for optimal nodulation (67). The *syrM* requirement of *M. truncatula* appears to be in sustaining nodulation, because *syrM* mutants induced nodule organogenesis, but nodule development was aborted early (67). Our results further demonstrate that while SyrM activates low-level *nod* gene expression, *syrM* cannot complement the inability of a triple *nodD* mutant to initiate nodules, even when strongly expressed. This raises the question of why SyrM shows low-level activation of *nod* gene promoters. Because a single genomic copy of *syrM* fails to activate *nodC::lacZ* or *nodF::uidA* expression above background levels, their activation in *Ptp-syrM* strains could be artifactual, due to extremely high levels of SyrM produced via the *trp* promoter plasmid. Alternatively, SyrM activation of *nod* gene expression may be important later in nodule development, perhaps during invasion, bacterial release, or bacteroid differentiation. This explanation is difficult to test, because an early block in nodule initiation precludes progression to subsequent development. However, in support of a later role, *syrM* expression is strongly induced in bacteroids (11, 23), *syrM* is required for sustaining nodule development on *M. truncatula* (67), and strains with mutations in *syrM* or the SyrM target gene, *nodD3*, are attenuated in symbiosis on alfalfa (92). Thus, the cumulative data suggest that *syrM* is not required for nodule initiation; its mechanism for sustaining nodule development remains

unknown. Previous work showed that either *syrM* or *nodD3* independently stimulated synthesis of Nod factors (NF) acylated by (ω -1)-hydroxylated fatty acids (HFAC), perhaps by activating genes that increase the HFAC pool or that encode acyltransferases that carry out transfer of HFAC (93). Purified NF containing HFAC induced normal root hair deformation on *M. sativa* but fewer nodules than wild-type NF. Previous work did not test the effects of *syrA* on HFAC NF production, but because either *syrM* or *nodD3* overexpression was sufficient to increase HFAC, and because SyrM, but not NodD3, activates *syrA* expression, it seems unlikely that SyrA modulates HFAC synthesis. However, two genes encoding proteins with similarity to acyltransferases show increased expression in *Ptp-syrA* strains (SMb21188 and SMb20810).

Since *syrA* is expressed strongly in nodule bacteria, another possibility is that the requirement for *syrM* in *M. truncatula* symbiosis is due to its positive effects on *syrA* expression. However, *syrA* expression in planta requires FixJ, which activates expression of nitrogen fixation genes (26, 59), not SyrM. That *syrA* expression is high in nodules and coregulated with nitrogen fixation genes suggests that SyrA may play a role in modulating the ExoR-ExoS-ChvI regulatory circuit in bacteroids. Because *syrA* appears dispensable for symbiosis of alfalfa (26, 94) and *M. truncatula* (Barnett, unpublished), it is more likely to be involved in fine-tuning circuit activity as proposed for its role in free-living bacteria.

syrM, *nodD3*, and *syrA* appear to be universal in the *Sinorhizobium* genus based on published genome sequences (95–104). Among *S. meliloti* and *S. medicae* strains, the orthologous proteins are nearly identical, and the gene arrangement is syntenic to *S. meliloti* Rm1021 (Fig. 1). In *S. fredii*, *Sinorhizobium saheli*, and *Sinorhizobium teranga* strains, SyrM, NodD3, and SyrA are ~70%, ~75%, and 46 to 52% identical, respectively, to their *S. meliloti* orthologs, and the gene arrangement differs. Unlike *S. meliloti* and *S. medicae*, the other *Sinorhizobium* species encode only two *nodD* genes and, in the case of some *S. fredii* strains, encode two *syrM* genes. Regulatory circuits for *S. fredii* NGR234 *nodD1*, *nodD2*, *syrM1*, and *syrM2* have been characterized: NodD1 with a flavonoid inducer activates *nod* gene expression, SyrM2 activates *nodD2* expression, NodD1 activates *syrM2* expression (85), NodD2 represses *nodD1* expression (105), and *syrM1* regulates Nod factor sulfation (106). Because *syrM2* expression is regulated mainly by NodD1, it is unlikely that SyrM2 and NodD2 form a positive regulatory loop similar to that of SyrM and NodD3 in *S. meliloti* (107). *syrA* has not been studied in other *Sinorhizobium* species, but its *S. fredii* NGR234 ortholog (y4xQ/NGR_00380) is located downstream of a putative SyrM binding site, suggesting that its expression may be activated by SyrM1 or SyrM2 (107). An explanation for this complexity of *nod* gene regulatory circuits in *Sinorhizobium* remains elusive and cannot be explained strictly by host range, because *S. meliloti* nodulates a relatively small number of genera (*Medicago*, *Trigonella*, and *Melilotus*) compared to *S. fredii* NGR234, which nodulates at least 112 genera (108).

When discussing possible roles of the SyrM-NodD3-SyrA regulatory circuit, it is important to note that our experiments were performed in a well-studied laboratory strain, Rm1021, which may behave differently than more wild isolates. Rm1021 lacks functional genes encoding NolR, a *nod* gene repressor (109), ExpR, an LuxR-family global regulator of population-based behavior (6), EcfRI, which regulates sulfite metabolism (110), PstC,

involved in high-affinity phosphate transport (111), and perhaps others (99, 102). NolR of *S. meliloti* Rm41 represses expression of *nodD1* and *nodD2* but probably not *nodD3* and *syrM* (112); NolR may also regulate genes involved in bacterial growth, survival, and conjugative plasmid transfer (113). ExpR, whose activity depends on *N*-acyl-homoserine lactones (AHLs), regulates expression of nearly 9% of *S. meliloti* genes, including those important for quorum sensing, motility, and EPS biosynthesis (114–116). ExpR⁺ strains are difficult to cultivate and manipulate in the lab because of their extremely mucoid colonial morphology and negative selection acting against a functional *expR* gene (117). Global analyses of ExpR function suggest that ExpR does not regulate SyrM, NodD3, or SyrA expression (70, 115, 116). On the other hand, our results show that expression of *sinI*, encoding the AHL synthase, is increased when *syrM*, *nodD3*, or *syrA* is overexpressed; thus, further investigation may be needed to discover possible connections between the SyrM-NodD3-SyrA and ExpR-SinI regulatory circuits.

Does SyrA suppress *chvI* K214T mutant phenotypes via the ExoR-ExoS-ChvI regulatory circuit? Strains overexpressing *syrA* share some phenotypes with the *exoR95::Tn5* (loss of function) and *exoS96::Tn5* (gain of function) mutants. Our work shows that *Ptp-syrA* suppresses phenotypes of the *chvI* K214T mutant, including its altered gene expression. Also, similar to what was reported for the *exoS96::Tn5* allele (43), *Ptp-syrA* allows introduction of a plasmid overexpressing *exoR*. Because *syrA* overexpression suppresses phenotypes of the *chvI* K214T mutant but not a *chvI*-null mutant, it is likely that SyrA does not act downstream of ChvI. One potential mechanism for *Ptp-syrA* suppression of *chvI* K214T phenotypes parallels the one hypothesized for the *exoR95::Tn5* and *exoS96::Tn5* mutants: in the absence of repression mediated by ExoR, or with constitutively active ExoS, suppression occurs by increasing the activity of the ExoS-ChvI circuit, so that even though ChvI K214T binds DNA poorly, there is more active ChvI available for binding (41, 43). Another possibility is that SyrA acts separately from the ExoS-ChvI circuit, in a parallel pathway that can compensate for *chvI* K214T mutant phenotypes independent of ChvI.

Because SyrA is not similar to any known transcriptional regulators, we propose that *Ptp-syrA* suppresses *chvI* K214T mutant phenotypes by increasing ChvI activity via protein-protein interactions, perhaps by contact with either ExoR or ExoS in the periplasm or with ExoS in the cytoplasm or cytoplasmic membrane. SyrA is predicted by the membrane protein structure prediction programs TMpred (118) and TopPred (119) to be a cytoplasmic membrane protein with its N and C termini in the cytoplasm, two transmembrane domains (amino acids 6 to 24 and 36 to 55), and a small periplasmic loop. The signal peptide prediction program, SignalP 4.1 (120), does not predict a cleavable signal peptide sequence in SyrA. An earlier study showed that the first 31 amino acids of SyrA conferred phosphatase activity to truncated PhoA lacking its signal peptide, suggesting that the SyrA N terminus is secreted to the periplasm (69). Because transmembrane helices may serve as translocation signals (121), and because its first 31 amino acids is predicted to have a transmembrane domain, it is unclear whether the N terminus or another region of SyrA (such as the predicted small loop, amino acids 25 to 35) is periplasmic.

There are several possible models for how SyrA may interact with players in the ChvI regulatory circuit (Fig. 1). By direct pro-

tein-protein interaction, SyrA could inhibit ExoR repression of ExoS activity, and thus activation of ChvI or SyrA could stimulate ExoS activity by direct interaction, leading to increased activation of ChvI. Alternatively, SyrA could influence ExoR or ExoS activity through an as-yet-identified intermediate protein that interacts with ExoR, ExoS, or ChvI. For example, since ExoR undergoes periplasmic proteolysis (48), one possibility for an indirect effect of SyrA on ExoR would be to modulate a protease. Genetic screens for suppression of *chvI* K214T mutant phenotypes identified mutations in only the *exoR* or *exoS* genes (46). While suggestive that additional interacting proteins in the ExoS-ChvI circuit were unlikely, such screens failed to identify *syrA*, probably because of the low probability that a spontaneous mutation could increase SyrA to sufficiently high levels. When we tested if *syrA* deletion suppressed phenotypes of the *exoR95::Tn5* or *exoS96::Tn5* mutants, the phenotypes of the resulting strains were similar to those of their parents: they were nonmotile, mucoid, and, in the case of the *exoR95::Tn5* Δ *syrA* mutant, defective for symbiosis (Barnett, unpublished). Therefore, while *syrA* overexpression may increase ChvI activity, *syrA* deletion fails to abrogate ExoS-mediated activation of ChvI, suggesting that rather than being a core component of the regulatory circuit, SyrA may instead act to fine-tune it.

Although *syrA* overexpression suppressed many of the gene expression changes observed in the *chvI* K214T mutant, *Ptp-syrA* only partially suppressed the increased expression of FeuP regulon genes. It is not known why expression of FeuP regulon genes is increased in the *chvI* K214T mutant. While FeuP-activated genes showed increased expression in the *chvI* K214T mutant versus the wild type here and in our previous study (42), they are probably not direct ChvI targets, because their expression was unchanged with the constitutively active ChvI D52E mutant (42). *feuP* mutants fail to invade nodules and grow poorly under hypoosmotic conditions (77). These phenotypes can be suppressed by ectopic expression of *ndvA*, which encodes a cyclic β -glucan exporter whose expression is activated by FeuP (77). FeuP is phosphorylated to its active form by the FeuQ histidine kinase (77), and FeuN is a small periplasmic modulator of FeuQ activity (122); most of the other FeuP regulon members are predicted to be secreted or membrane associated. The precise environmental signaling events leading to activation of FeuP are unknown. In addition to the *chvI* K214T mutant, expression of most FeuP-activated genes is also increased in *cbrA* (13/14 genes), *podJ* (13/14), and *hfq* (12/14) mutants. Therefore, activation of FeuP regulon genes may correlate with alterations in the cell envelope.

The expression of FeuP regulon genes also increases upon exposure of free-living bacterial cells to sublethal concentrations of two different nodule-induced cysteine-rich (NCR) peptides (35, 123). These plant NCR peptides are critical for an effective symbiosis because they modulate bacteroid differentiation (124, 125). The FeuP regulon also showed elevated expression in certain *M. truncatula* Fix[−] mutants compared to that of the wild type (C. Lang, unpublished data). Since *feuP*, *cbrA*, *hfq*, and *chvI* mutants have symbiotic defects, understanding what leads to increased expression of the FeuP regulon genes in these mutants may be helpful in understanding how NCR peptides exert their effects during symbiosis. Determining why increased FeuP regulon gene expression in the *chvI* K214T mutant is not completely suppressed by *Ptp-syrA*, while many other gene expression changes appear to be

suppressed, may help us understand how SyrA affects the ExoR-ExoS-ChvI regulatory circuit.

Our work raises the question: are there additional candidates for interaction with the ExoR-ExoS-ChvI regulatory circuit? ExoX, conserved in all *Sinorhizobium* genomes sequenced to date, shares amino acid similarity and a predicted secondary structure with SyrA, yet it exerts opposite effects on EPS-I abundance: strains null for *exoX* or overexpressing *syrA* both increase EPS-I abundance (26, 56, 57). Similarly, strains null for *syrA* appear to produce less EPS-I, whereas strains overexpressing *exoX* are defective in EPS-I production and symbiosis (26, 56, 57). Unlike *Ptp-syrA* strains, however, EPS-I overproducing *exoX* mutants are as motile as the wild type (Barnett, unpublished). ExoX has been postulated to modulate EPS-I production by interacting with ExoY, which catalyzes the first committed step of EPS-I biosynthesis (57). Another possibility is that ExoX regulates EPS-I production by negatively regulating the activity of the ExoR-ExoS-ChvI regulatory circuit, either directly by interacting with one of its core components or indirectly by interacting with ExoY, SyrA, or an as-yet-identified protein.

Concluding remarks. While the expression of regulatory proteins from the ectopic *trp* promoter may complicate interpreting the biological relevance of our results, we chose this approach because *syrM* and *syrA* are very weakly expressed from their own promoters in free-living cells yet strongly induced in nodule bacteria (23, 24, 26, 126, 127). By using ectopic expression to increase SyrM, NodD3, or SyrA levels in free-living bacteria, we strove to define as many gene expression changes as possible, some of which may be similarly changed in the nodule environment. Our identification of a genetic connection of SyrA to the ExoR-ExoS-ChvI regulatory circuit, which is critical for symbiosis, justifies this as a valid approach.

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