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The LuxR Homolog ExpR, in Combination with the Sin Quorum Sensing System, Plays a Central Role in *Sinorhizobium meliloti* Gene Expression†

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Quorum sensing, a population density-dependent mechanism for bacterial communication and gene regulation, plays a crucial role in the symbiosis between alfalfa and its symbiont *Sinorhizobium meliloti*. The Sin system, one of three quorum sensing systems present in *S. meliloti*, controls the production of the symbiotically active exopolysaccharide EPS II. Based on DNA microarray data, the Sin system also seems to regulate a multitude of *S. meliloti* genes, including genes that participate in low-molecular-weight succinoglycan production, motility, and chemotaxis, as well as other cellular processes. Most of the regulation by the Sin system is dependent on the presence of the ExpR regulator, a LuxR homolog. Gene expression profiling data indicate that ExpR participates in additional cellular processes that include nitrogen fixation, metabolism, and metal transport. Based on our microarray analysis we propose a model for the regulation of gene expression by the Sin/ExpR quorum sensing system and another possible quorum sensing system(s) in *S. meliloti*.

The gram-negative soil bacterium, *Sinorhizobium meliloti*, fixes atmospheric nitrogen in symbiotic association with its host plant, *Medicago sativa* (alfalfa). This relationship involves a series of intricate signaling events between the host and the symbiont (26, 34, 58). Initially, alfalfa releases flavonoids that attract bacteria from the surrounding environment to the roots and induce the production of bacterial lipochitooligosaccharide signal molecules referred to as Nod factors. The Nod factors elicit root hair curling and trigger the plant meristematic cells to divide and differentiate, leading to the formation of plant nodules. Root nodule invasion requires the action of additional signal molecules such as the exopolysaccharides produced by *S. meliloti*. Once inside the plant, the bacteria differentiate into morphologically distinct forms called bacteroids that actively fix atmospheric nitrogen (35). During this intimate association involving high bacterial cell density, cell-cell communication is likely to play an important role in regulating and coordinating the interaction between the symbiont and its host (19).

Quorum sensing, or population density-dependent regulation of gene expression, was first characterized in the symbiotic association between the bacterium *Photobacterium fischeri* (*Vibrio fischeri*) and its marine hosts (12, 13, 16). This phenomenon involves the production of pheromone-like signals called autoinducers, one class of which includes the *N*-acyl homoserine lactones (AHLs). At high cell densities, these membrane-permeant molecules accumulate within the cell and bind to and activate the LuxR regulator (25, 29). Once activated, LuxR binds upstream of the promoter of its target genes and enhances the activity of RNA polymerase. In *P. fischeri*, the target genes include the *lux* operon, responsible for bioluminescence, and *luxI*, the autoinducer synthase gene, whose activation results in the formation of a positive feedback loop (23, 40).

Cell density-dependent gene regulation is often used to control the expression of genes specific for symbiotic or pathogenic bacterium-host associations. For example, *Pseudomonas aeruginosa* utilizes the *lasR*/*lasI* and *rhlR*/*rhlI* systems to control the production of virulence factors, *Agrobacterium tumefaciens* depends on the *traR*/*traI* system for conjugal plasmid transfer, and *Rhizobium leguminosarum* has a multitiered quorum sensing system that regulates the nodulation of its plant host (18, 28, 30, 33, 50, 62).

The wild-type *S. meliloti* strain Rm1021 possesses at least two quorum sensing systems that have been initially characterized by Marketon and coworkers (19, 37). The Mel system controls the synthesis of short-chain AHLs, which preliminary evidence suggests play a role in nodulation and invasion (A. Patankar and J. González, unpublished data). The Sin system, composed of SinR (transcriptional regulator) and SinI (autoinducer synthase), is responsible for the synthesis of a series of long-chain AHLs that include C_{12}-HL, oxo-C_{14}-HL, $C_{16:1}$-HL, oxo-$C_{16:1}$-HL, and C_{18}-HL (38). Our laboratory has shown that at least one of these AHLs, $C_{16:1}$-HL, specifically activates the expression of the *exp* genes and the subsequent production of EPS II, one of the two *S. meliloti* exopolysaccharides involved in the plant nodule invasion process (36). A *sin*-deficient strain induces a smaller number of nitrogen-fixing nodules on alfalfa, and the nodulation process is also delayed in plants that are inoculated with this mutant strain (36). In the absence of EPS II and succinoglycan, the second symbiotically important exopolysaccharide, *S. meliloti* fails to invade the plant nodules and cannot establish a successful symbiosis (22, 46).

The expression of the *exp* genes not only relies on the acti-

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TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristics	Reference
Rm1021	Su47 str-21, *expR* mutant	31
Rm11511	Rm1021 *sinI*::KM	38
Rm11512	Rm1021 *sinR*::GM	38
Rm8530 (formerly *expR101*)	Rm1021 $expR^+$	42
Rm11527	Rm8530 *sinI*::KM	36
Rm9033	Rm8530 *expE2*::*lacZ*-GM	36
Rm11525	Rm9033 *sinI*::KM	36
Rm11533	Rm1021 *expE2*::*lacZ*-GM	36

vation by the *sin*-specified AHLs but also requires the presence of an additional regulator, ExpR (36). Pellock et al. (42) recently reported that the strain Rm1021, which does not produce EPS II under normal conditions, carries an insertion element within the *expR* gene. On the other hand, the Rm1021 derivative (Rm8530) that contains a functional copy of the *expR* gene is able to make the EPS II polymer (42). ExpR is a LuxR homolog whose function includes the activation of EPS II production in the presence of the *sin*-AHLs (36, 42). Activated LuxR-type regulators usually bind to a consensus sequence known as a *lux* box typically located upstream of the promoters of its target genes (11, 53). In the case of ExpR, no upstream *lux* box-like motif has been identified; therefore, the exact mechanism for the activation of the *exp* genes remains to be elucidated.

One of the questions that remained to be answered was whether the *sinR*/*sinI* locus and the ExpR regulator control other downstream targets in addition to the *exp* genes. Toward this end, we decided to explore the effect of the Sin/ExpR quorum sensing system on the whole genome expression profile of *S. meliloti*. We show here that both the *sinR*/*sinI* locus and the ExpR regulator not only controls the genes involved in EPS II production, but they also play a role in the expression of genes involved in an assortment of cellular processes such as motility, nitrogen fixation, and the transport of metal and small molecules. The regulators may perhaps work in concert with other unidentified regulators. These findings lead us to propose that the ExpR regulator, together with the Sin quorum sensing system, plays a wide role in the regulation of *S. meliloti* gene expression and the symbiotic process.

MATERIALS AND METHODS

Bacterial strains and medium conditions. Table 1 lists the bacterial strains used in this study. Starter cultures were grown in 5 ml of TYC broth (10 g of tryptone, 5 g of yeast extract, and 0.4 g of $CaCl_2$/liter) with streptomycin (500 μg/ml) for 2 days at 30°C. The strains were subcultured (1:100) in 25 ml of minimal mannitol glutamate (MGM) low-phosphate medium (50 mM morpholinepropanesulfonic acid, 19 mM sodium glutamate, 55 mM mannitol, 0.1 mM K_2HPO_4-KH_2PO_4 [stock consists of equal molar ratio of each], 1 mM $MgSO_4$, 0.25 mM $CaCl_2$, 0.004 mM biotin; pH 7) and grown at 30°C with constant shaking.

Contents and layout of Sm6k microarrays. In the present study, the Sm6k microarrays described by Rüberg and coworkers were used (4, 47). Each microarray contains 6,046 PCR fragments and 161 70-mer oligonucleotides as open reading frame (ORF)-specific probes and 3 alien DNA fragments (Spot Report Alien PCR product #1, Stratagene 252551; Spot Report Alien PCR product #2, Stratagene 252552; Spot Report Alien PCR product #3, Stratagene 252553; Stratagene, La Jolla, Calif.) that can serve as probes for spiking controls. Each probe was spotted in triplicate. DNA fragments were generated by two rounds of PCR amplification (47). In the first round, ORF-specific primers carrying H1 (GGTTCCACGTAAGCTTCC), B4 (GCGATTACCCTGTACACC), or M3 (GCCAGTACATCAATTGCC) 5′ extensions were used. These primary PCR products were reamplified by using these extensions as standard priming sites.

RNA purification. Bacterial cultures were grown to an optical density at 600 nm (OD_{600}) of 0.8 in MGM low-phosphate media (0.1 mM phosphate) supplemented with 500 μg of streptomycin/ml. Cells were harvested by centrifugation (10,000 × *g* for 1 min at 4°C), and cell pellets were immediately frozen in liquid nitrogen. Total RNA was purified by using an RNeasy minikit (Qiagen, Hilden, Germany). Cells were disrupted in the RLT buffer provided with the kit in Fast Protein tubes (Q BIOgene, Carlsbad, Calif.) by using a Ribolyser (Hybaid, Heidelberg, Germany) (30 sec, level 6.5) prior to spin column purification according to the RNeasy minikit RNA purification protocol. The RNA samples were treated with the Qiagen on-column RNase-free DNase kit and further purified.

The total RNA samples for the quantitative real-time PCR experiments were also obtained by using the RNeasy minikit. Cells were disrupted in the RLT buffer provided with the kit in Fast Protein tubes (Q BIOgene) by continuous vortexing for 5 min at 4°C before we proceeded with the RNA purification and on-column DNase digestion. Samples were DNase treated a second time with the TURBO RNase-free DNase from Ambion according to the manufacturer's instructions. An additional RNA clean-up step was performed, and the concentrations of the samples were determined for cDNA synthesis.

Labeling of hybridization probes and hybridization. For each comparison, hybridizations accounting for three biological replicates were conducted. Fluorescence-labeled cDNA was prepared according to the method of de Risi et al. (http://www.microarrays.org/protocols.html) from 12 μg of total RNA (10). Prehybridization and hybridization of microarrays were carried out as previously described (4, 47).

Data analysis. Image acquisition and data analysis were performed as described previously (4, 49). In brief, mean signal and mean local background intensities were obtained for each spot of the microarray images by using the ImaGene 5.0 software for spot detection, image segmentation, and signal quantification (Biodiscovery, Inc., Los Angeles, Calif.). The $\log_2$ value of the ratio of intensities was calculated for each spot by using the following formula: $M_i = \log_2(R_i/G_i)$. R_i and G_i were determined as follows: $R_i = I_{ch1_i} - Bg_{ch1_i}$and $G_i = I_{ch2_i} - BgI_{ch2_i}$, where I_{ch1_i} or I_{ch2_i} is the intensity of a spot in channel 1 or channel 2 and Bg_{ch1_i} or Bg_{ch2_i} is the background intensity of a spot in channel 1 or channel 2, respectively. A normalization method based on local regression that account for intensity and spatial dependence in dye biases was applied (65). Normalization and statistical analysis were carried out by using the *EMMA 1.1* microarray data analysis software developed at the Bioinformatics Resource Facility (Center of Biotechnology, Bielefeld University [www.genetik.uni-bielefeld.de/EMMA/]) (11). Genes were regarded as differentially expressed if $P \leq 0.05$ and $M \geq 1.00$ or $M \leq -1.00$ (at least a twofold difference).

To support groupings of coregulated genes, self-organizing maps (SOMs) (54) were applied by using the GeneCluster2.0 software package (http://www-genome.wi.mit.edu/cancer/software/genecluster2/gc2.html; Cancer Genomics Group, Whitehead/MIT Center for Genome Research). A 3X3 SOM was used for clustering.

Genes were classified according to the function predicted for their gene products according to the classification scheme suggested for clusters of orthologous groups of proteins (COGs) (55, 56).

Quantitative real-time PCR. The first-strand cDNA mixture for each strain was prepared with the RETROscript kit from Ambion by using 0.2 μg of total RNA per reaction, and 1 μl of the cDNA reaction was used as a template for the real-time PCR setup. The probe and oligonucleotide sequences included the following: *expE2* probe, 5′-[DFAM]CAACCCGTCCCGCTCGTCAGCAC[DBH1]-3′; *exsH* probe, 5′-[DFAM]TTGTCCGCCTCGTTGCCGAATGC[DBH1]-3′; *ndvA* probe, 5′-[DFAM]CCGCACCGAGCACCACGAGGATG[DBH1]-3′; *ndvB* probe, 5′-[DHEX]CGCCCACAACTCGCCGATCTTGAG[DBH1]-3′; *flaF* probe, 5′-[DFAM]CGCCGGACAACCAGCTCAACGAAG[DBH1]-3′; *cheY1* probe, 5′-[DFAM]CAGGCGGAGGATGGCGTCGAGG[DBH1]-3′; 16S probe, 5′-[Cy5]CAGCCATGCAGCACCTGTCTCCGA[BH2]-3′; *expE2* sense, 5′-GCCAAACACACGCTCGTCAT-3′; *expE2* antisense, 5′-GCCACTCTCCGCAAGAGAAA-3′; *exsH* sense, 5′-CGGCGAACTTCGAGAACCTC-3′; *exsH* antisense, 5′-TTCCCGACCCACCCTTTATGA-3′; *ndvA* sense, 5′-GGCGCTCATGCTTCTGATTC-3′; *ndvA* antisense, 5′-TCATCACGACCTTGCTGATCAT-3′; *ndvB* sense, 5′-TTTACGTTGCCCATACCCATAGT-3′; *ndvB* antisense, 5′-GATGAGGACGAAACGCAGGAT-3′; *flaF* sense, 5′-CTGGATCCGGTTCATCGAAGAT-3′; *flaF* antisense, 5′-GCCCTGGAAGTTGGAAGACTC-3′; *cheY1* sense, 5′-GCTTCTCGTCACCCTCAACAA-3′; *cheY1* antisense, 5′-ATTGGCCGTATCGAGCTTCTC-3′; 16S (Smc02675) sense, 5′-CTTAACCCAACATCTCACGACAC-3′; and 16S antisense, 5′-ACCTTACCAGCCCTTGACATC-3′. Each reaction mixture contains 0.3 μM sense oligonu-

TABLE 2. Microarray combinations for expression profile analysis

Expt	Comparison (strain vs strain)[a]						Observation
	Strain 1	*expR* status	*sin* status	Strain 2	*expR* status	*sin* status	
1	Rm8530	+ *expR*	− *sinI*	Rm8530	+ *expR*	+ *sinI*	Role of *sin*-AHLs in the presence of *expR*
2	Rm11511	− *expR*	− *sinI*	Rm1021	− *expR*	+ *sinI*	Role of *sin*-AHLs in the absence of *expR*
3	Rm11512	− *expR*	− *sinR*	Rm1021	− *expR*	+ *sinR*	Role of *sinR* in the absence of *expR*
4	Rm1021	− *expR*	+ *sinI*	Rm8530	+ *expR*	+ *sinI*	Role of *expR* in the presence of *sin*-AHLs
5	Rm11511	− *expR*	− *sinI*	Rm11527	+ *expR*	− *sinI*	Role of *expR* in the absence of *sin*-AHLs

[a] Status refers to the presence (+) or absence (−) of the indicated gene.

cleotide (0.1 μM for the 16S sense oligonucleotide), 0.3 μM antisense oligonucleotide (0.1 μM for the 16S antisense oligonucleotide), 0.2 μM TaqMan probe, 0.5 OmniMix HS PCR Beads (each PCR bead contains 1.5 U of *Taq* DNA polymerase, 10 mM Tris-HCl [pH 9.0], 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphate, and stabilizers, including bovine serum albumin) in a 25 μl-reaction volume. The experiment was performed with the Cepheid Smart Cycler version 2.0c programmed as follows: stage 1, 95°C for 120 s; and stage 2, 95°C for 15 s and 60°C for 30 s (two-temperature cycle repeated 40 times).

RESULTS

We systematically designed a series of microarray experiments to determine whether other genes, in addition to the *exp* operon (36), are regulated by the SinR/SinI quorum sensing system and/or the ExpR regulator. These microarray experiments involved the comparison of strains lacking one or more of the components of the Sin/ExpR system to explore their individual roles in the *S. meliloti* global gene expression. The strains under study were grown in low-phosphate conditions such as those previously found to maximize the production of EPS II, the only Sin quorum sensing controlled phenotype identified to date. The different experimental combinations are listed in Table 2.

In experiment 1, we sought to determine the role of the *sin*-AHLs in the presence of an active ExpR regulator. We compared strains retaining an intact *expR* gene (Rm8530) with either the ability to produce the *sin*-AHLs (*sinI*$^+$) or disrupted for this function (*sinI* mutant). Experiment 2 was designed to investigate the possibility that the *sinR*/*sinI* locus might control gene expression in *S. meliloti* through a separate regulatory network that did not involve the ExpR regulator. For this purpose, we conducted an expression profile analysis of a strain with a disrupted *expR* gene (Rm1021) versus a derivative also lacking the ExpR regulator and unable to make the *sin*-AHLs (Rm1021 *sinI* mutant). To determine the extent to which the SinR regulator controls additional genes, other than *sinI*, we compared the expression profiles of an *expR* mutant strain (Rm1021) versus a strain lacking both the ExpR and SinR regulators (Rm1021 *sinR* mutant) in experiment 3. Our earlier results clearly demonstrated that the ExpR regulator, in the presence of the *sin*-AHLs, plays a role in the expression of the symbiotically important *exp* genes (39). We designed experiment 4 to explore the possibility that the ExpR regulator could play a similar role in an assortment of other *S. meliloti* cellular processes. Toward this end, we carried out microarray analysis to compare an *expR* mutant (Rm1021) versus an *expR*$^+$ (Rm8530) strain. Finally, we also examined in experiment 5 the control of genes by the ExpR regulator in the absence of the Sin system by comparing the expression profiles of strains lacking a functional *sinI* gene but carrying either an intact or a disrupted *expR* gene. The data from these series of microarray analyses can be obtained in the supplemental material. These expression profiles were organized into groups by cluster analysis (Materials and Methods). The magnitudes of differential gene expression are presented in Table 3 as M_i values as described in Materials and Methods. The boldface italic values indicate genes that meet the criteria for significant M_i and *P* values, and the boldface roman numbers are genes that meet the cutoff for significant M_i but not for the *P* values. We also classified the genes according to their predicted functions (see Materials and Methods) and correlated them to the groups to which they belong based on the cluster analysis (Fig. 1). These groups (discussed below) provide a clear picture of the individual or combined role(s) that each component of the Sin/ExpR system plays in *S. meliloti* gene expression.

***S. meliloti* genes whose expression is dependent on SinI and ExpR (group A).** The genes that fall under this category seem to be differentially expressed under conditions that depend on the presence of both the *sin*-AHLs and the ExpR regulator (Table 3, group A, experiments 1 and 4). Many of the 26 genes in this group participate in the production of the exopolysaccharide (EPS) II. The *exp* operon was the only group of genes previously shown to be strongly activated by the Sin system (36). The microarray data correlates well with earlier observations that expression of the *exp* genes is strongly dependent on an intact ExpR regulator and the *sin*-AHLs (36). Our previous analysis with an *expE2-lacZ* fusion showed that the expression of the *expE2* gene was ~50-fold lower when the Sin system was inactivated (Table 4) (36). The expression of *expE2* was likewise dependent on the presence of an intact ExpR regulator (36, 42). This finding parallels the observation of the mucoid phenotype presented by the comparison of the different isogenic derivatives. The *expR*$^+$ strain is very mucoid when grown in MGM low-phosphate media (36). Disruption of either the *sinI* or the *expR* gene results in a nonmucoid phenotype (36). The lack of mucoidy caused by the absence of the *sin*-AHLs in a *sinI* mutant can be complemented in *trans* by the addition of AHLs extracted from a *sin*-proficient strain (Rm8530) (36). To further validate our microarray results, we examined the expression of the *exp* genes by a different method. Expression of the *expE2* gene in the *expR*$^+$, *expR*$^+$ *sin* mutant, and *expR* mutant *sin*$^+$ derivatives was also examined by real-time PCR analysis. In this assay, *expE2* expression exhibited at least a 14-fold increase in the *expR*$^+$ (Rm8530) strain compared to the *expR*$^+$ derivative, which was unable to make *sin*-AHLs, or a derivative with an inactive *expR* gene (Rm1021) (Table 4). The 16S cDNA was probed simultaneously in a multiplexing experiment as a control for the equal addition of template in each reaction mixture.

TABLE 3. Gene cluster analysis of the microarray data

Group and name (alias)	Group or gene product description	M_i value in expt[a]:				
		1	2	3	4	5
A	Genes differentially expressed dependent on SinI and ExpR					
SMc02726	Putative iron transport protein	***2.26***	0.04	0.05	***2.29***	0.08
SMc04059	Hypothetical protein	***2.14***	0.28	0.34	***1.00***	−0.80
SMc04114 (*pilA1*)	Putative pilin subunit protein	***1.50***	−0.09	0.21	***1.58***	−0.85
SMb20557	Conserved hypothetical protein	***−1.00***	−0.30	**1.54**	***−1.02***	−0.16
SMb21309 (*expE6*)	Putative membrane protein	***−1.07***	−0.23	0.32	***−1.28***	0.00
SMc04170	Putative two-component receiver domain protein	***−1.07***	0.28	0.53	***−1.47***	0.04
SMb21310 (*expE5*)	Putative membrane-anchored protein	***−1.15***	0.07	0.39	***−1.01***	0.02
SMb21544	Hypothetical exported protein	***−1.20***	**1.03**		***−1.08***	**−1.13**
SMb20951 (*exoI*)	Putative periplasmic protein	***−1.29***	0.05	−0.47	***−1.69***	−0.21
SMb20391	Putative cellulose synthase catalytic subunit protein	***−1.30***	0.00	−0.10	***−1.59***	0.12
SMb21319 (*expA1*)	Putative membrane-anchored protein	***−1.31***	0.19	0.26	***−1.93***	0.50
SMb21311 (*expE4*)	Putative glycosyltransferase, forming α-glycosyl linkages protein	***−1.36***	−0.21	0.26	***−2.06***	0.10
SMb21402	Hypothetical calcium binding protein	***−1.46***	−0.03	0.12	***−1.12***	0.69
SMb21318 (*expC*)	Putative glycosyltransferase, forming α-glycosyl linkages protein	***−1.50***	−0.08	0.27	***−2.22***	0.38
SMb20909	Hypothetical protein	***−1.58***	0.04	0.04	***−1.85***	−0.55
SMb20952	Hypothetical protein	***−1.68***	−0.32	0.94	***−2.15***	0.14
SMb21321 (*expA4*)	Putative membrane-anchored protein	***−2.03***	−0.04	0.20	***−3.26***	−0.26
SMb20932 (*exsH*)	Endo-1,3-1,4-β-glycanase, C-terminal secretion signal protein	***−2.07***	−0.05	0.34	***−2.46***	0.46
SMa2111	Hypothetical protein	***−2.18***	−0.04	−0.16	***−1.46***	0.58
SMb21312 (*expE3*)	Putative methyltransferase protein	***−2.44***	−0.30	0.40	***−3.26***	0.31
SMb21323 (*expA6*)	Putative protein, probably exported to periplasm	***−2.47***	0.02	0.12	***−3.22***	−0.41
SMb21317 (*expG*)	Transcriptional activator of exopolysaccharide II synthesis, MarR family protein	***−2.62***	0.07	0.48	***−2.62***	0.32
SMc04171	Putative hemolysin-type calcium-binding protein	***−2.73***	−0.06	0.08	***−2.75***	0.24
SMb21681	Hypothetical protein	***−3.06***	0.02	0.49	***−3.03***	0.21
SMb21543	Putative outer membrane secretion protein	***−3.15***	0.01	0.57	***−3.35***	0.52
SMb21314 (*expE1*)	Putative secreted calcium-binding protein	***−3.18***	−0.03	0.57	***−3.50***	0.54
B	Genes differentially expressed dependent on SinI and SinR and independent of ExpR					
SMc01403	Putative transcription regulator protein	0.16	***1.76***	***1.40***	**1.53**	0.42
SMc00779	Putative FAD-dependent oxidoreductase protein	0.47	***1.33***	***1.55***	−0.86	0.28
SMc00672 (*hisX*)	Histidine-binding periplasmic signal peptide protein	0.14	***1.04***	***1.25***	0.76	0.76
C	Genes differentially expressed dependent on SinR and independent of SinI					
SMa0089	Conserved hypothetical protein	−0.08	0.04	***4.21***	0.14	−0.15
SMc02111	Conserved hypothetical protein	−0.57	−0.25	***2.67***	−0.16	−0.17
SMa1727	Putative hydrolase	0.07	0.09	***2.36***	0.00	−0.39
SMa1262	Conserved hypothetical protein	0.08	−0.07	***1.99***	0.50	0.02
SMc00777	Conserved hypothetical protein	0.22		***1.88***		0.28
SMa0091	Hypothetical protein	0.00	0.03	***1.71***	0.04	0.35
SMb21097	Putative amino acid uptake ABC transporter periplasmic solute-binding protein precursor	0.24	0.00	***1.54***	−0.08	−0.10
SMc00670 (*hisV*)	Histidinc transport ATP-binding ABC transporter protein	0.43	0.43	***1.44***	**1.23**	0.90
SMc01843	Probable 5,10-methylenetetrahydrofolate reductase oxidoreductase protein	−0.16	0.35	***1.39***	0.91	0.34
SMb20836	Hypothetical heme-binding protein	0.18	0.40	***1.29***	0.75	−0.12
SMc02087 (*gltA*)	Citrate synthase protein	0.05	−0.03	***1.20***	0.10	0.39
SMa0645	Hypothetical protein	0.25	0.60	***1.19***	−0.05	0.35
SMc03998	Hypothetical transcription regulator protein	0.26	0.01	***1.17***	0.29	0.07
SMb20464	Hypothetical protein	−0.18	0.26	***1.16***	0.23	−0.17
SMa1036	Hypothetical protein	0.05	0.40	***1.14***	0.15	−0.38
SMa1729	Putative periplasmic binding protein	0.18	0.29	***1.10***	0.30	0.62
SMa1208 (*fixS1*)	FixS1 nitrogen fixation protein	−0.27	0.18	***1.07***	0.12	0.10
SMc00665	Hypothetical/unknown protein	−0.11	0.27	***1.07***	0.26	0.21
SMb21378	Hypothetical protein	0.00	0.58	***1.01***	0.23	0.42
SMb20032	Hypothetical protein	−0.02	−0.11	***−1.10***	0.05	−0.95
SMb20336	Conserved hypothetical protein	−0.09	−0.10	***−1.61***	−0.31	0.82
SMb20292	Hypothetical immunogenic protein	−0.34	−0.11	***−2.74***	−0.34	0.62
SMb20335	Conserved hypothetical protein	0.33	−0.19	***−3.48***	0.01	0.66
D1	Genes differentially expressed dependent on ExpR and independent of SinI					
SMa0445 (TRm1a)	TRmla transposase	0.13	0.12	0.18	***2.59***	***1.46***
SMa1030 (TRm1a)	TRmla transposase	0.27	0.20	0.15	***1.98***	***1.11***
SMb21440	Hypothetical protein	0.08	−0.13	0.04	***1.95***	***1.82***
SMb20918 (TRm1a)	Transposase of insertion sequence ISRm1 ORFA protein	0.09	−0.09	0.20	***1.71***	***1.07***
SMc02079	Putative outer membrane protein	0.06	0.15	0.31	***1.68***	***2.51***
SMc03898 (TRm1a)	Transposase for insertion sequence element ISRm1	0.29	−0.15	0.16	***1.65***	***1.06***
SMc01959 (TRm1a)	Transposase for insertion sequence element ISRm1	0.24	0.04	0.14	***1.55***	***1.07***
SMc03295 (TRm1a)	Transposase for insertion sequence element ISRm1	0.19	0.04	0.01	***1.04***	***1.19***
D2	Genes differentially expressed dependent on ExpR in the absence of SinI					
SMb21522 (*minE*)	Putative cell division inhibitor protein	−0.08	−0.29	0.07	0.69	***1.25***
SMb20837	Hypothetical protein	−0.01	−0.15	0.18	0.11	***1.13***
SMc03239 (*ppa*)	Probable inorganic pyrophosphatase protein	0.19	0.01	0.81	0.70	***1.11***
SMc00198	Hypothetical/unknown protein	−0.88	−0.60	−0.44	0.10	***1.09***

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TABLE 3—*Continued*

Group and name (alias)	Group or gene product description	M_i value in expt[a]:				
		1	2	3	4	5
SMc02298 (TRm1a)	Transposase for insertion sequence element ISRm1	0.05	0.19	−0.04	0.72	***1.09***
SMb21523 (*minD*)	Putative cell division inhibitor protein	0.20	−0.07	0.40	0.24	***1.08***
SMc03900 (*ndvA*)	β-1→2 glucan export ATP-binding protein	0.07	−0.26	0.18	−0.02	***1.03***
SMb20946 (*exoY*)	Galactosyltransferase protein	−0.14	−0.16	0.90	0.72	***1.01***
SMc00912	10-kDa chaperonin A protein	−0.41	−0.04	−0.16	−0.12	***1.00***
SMc01266 (*hemN*)	Conserved hypothetical protein	0.12	−0.79	−0.50	−0.72	***−1.02***
SMc03051 (*flbT*)	Putative flagellin synthesis repressor protein	0.95	0.13	0.39	−0.38	***−1.02***
SMc04319	Hypothetical protein	−0.02	0.70	0.31	−0.46	***−1.02***
SMc03052 (*flgD*)	Putative basal-body rod modification protein	0.79	−0.04	0.30	−0.15	***−1.04***
SMc03072	Conserved hypothetical protein	0.29	−0.10	0.38	−0.06	***−1.06***
SMc03031 (*flgA*)	Flagellar precursor transmembrane protein	0.58	−0.20	0.24	0.07	***−1.14***
SMb21072	Hypothetical membrane protein	−0.46	−0.62		**−2.05**	***−1.17***
SMb20651	Hypothetical protein	0.42	0.05		0.51	***−1.18***
SMc03034 (*flgH*)	Flagellar L-ring protein precursor basal body L-ring protein	0.71	0.01	0.25	−0.10	***−1.21***
SMc00507	Hypothetical/unknown protein	0.18	−0.07		−0.40	***−1.48***
SMa1387	Putative LysR-type transcriptional regulator	0.41	−0.06	0.10	0.32	***−1.52***
SMc03006 (*cheY1*)	Chemotaxis regulator protein	0.64	0.75	0.18	0.21	***−1.62***
SMc03024 (*flgF*)	Flagellar basal-body rod protein	0.09	0.08	0.41	0.21	***−1.65***
SMc03050 (*flaF*)	Putative flagellin synthesis regulator protein	0.97	−0.23	0.24	−0.71	***−1.74***
SMc03030 (*flgG*)	Flagellar basal-body rod protein	0.77	0.06	0.09	0.02	***−1.76***
D3	Genes activated or repressed by ExpR in the absence of SinI					
SMc03038 (*flaB*)	Flagellin B protein	***1.71***	0.14	0.54	−0.54	***−2.01***
SMc03037 (*flaA*)	Flagellin A protein	***1.61***	0.11	0.44	−0.41	***−2.04***
SMc03049 (*flgL*)	Putative flagellar hook-associated protein	***1.32***	−0.14	0.21	−0.74	***−1.28***
SMc03048 (*flgK*)	Putative flagellar hook-associated protein	***1.25***	−0.06	0.42	−0.55	***−1.28***
SMc03035 (*fliL*)	Flagellar transmembrane protein	***1.19***	−0.03	0.19	−0.44	***−1.63***
SMc03039 (*flaD*)	Probable flagellin D protein	***1.18***	0.07	0.48	−0.30	***−1.68***
SMc03047 (*flgE*)	Flagellar hook protein	***1.13***	−0.11	0.32	−0.06	***−1.14***
SMc03040 (*flaC*)	Flagellin protein	***1.08***	0.01	0.21	−0.18	***−1.32***
SMc03108	Hypothetical calcium-binding protein	***−1.57***	−0.03	0.10	−0.02	***1.59***
E1	Genes differentially expressed dependent on ExpR or SinR					
SMc03043 (*motC*)	Chemotaxis precursor (motility protein C) transmembrane	0.77	0.60	***1.08***	***1.00***	−0.11
E2	Genes differentially expressed dependent on ExpR or SinR in the absence of SinI					
SMa1541	Putative oxidoreductase	0.39	0.78	***1.12***	***1.10***	***1.04***
SMc03799	Conserved hypothetical protein	0.24	0.01	***−2.05***	0.14	***−1.22***
SMc04245 (*znuA*)	Probable high-affinity zinc uptake system ABC transporter protein	−0.16	0.04	***−2.17***	−0.15	***−1.27***
E3	Genes differentially expressed dependent on ExpR or SinR and dependent on SinI or an unknown factor					
SMb20604	Putative urea short-chain amide or branched-chain amino acid uptake ABC transporter permease protein, possibly fusion protein	0.91	***1.50***	***1.42***	0.71	***2.03***
SMc04127	Putative ATP-binding ABC transporter protein	0.53	***1.30***	***1.17***	0.53	***1.19***
F	Genes differentially expressed dependent on SinI and ExpR or an unknown regulatory gene and independent of SinR					
SMc00193	Hypothetical/unknown protein	***−2.60***	***−1.09***	−0.88	***−2.38***	−0.66
SMa2091	Hypothetical protein	0.06	***1.13***	0.66	***1.10***	0.42
G	Genes differentially expressed dependent on SinI in the absence of ExpR and independent of SinR					
SMc04018	Probable 5′-nucleotidase precursor (signal peptide) protein		***4.76***			
SMc03987	Putative transport transmembrane protein		***3.75***			
SMa2055	Hypothetical protein	−0.05	***1.69***			
SMa2019	Putative oxidoreductase	0.33	***1.45***	0.74	0.07	0.75
SMa1077 (*nex18*)	Nex18 symbiotically induced conserved protein	0.22	***1.15***	0.67	1.15	0.48
SMa0903	Hypothetical protein	−0.08	***1.13***		0.54	−0.63
SMc00168 (*sinI*)	Putative autoinducer synthase protein	0.60	***1.06***	−0.19	−0.81	−0.21
SMa0087	Hypothetical protein	0.24	***1.01***		0.79	**−2.60**
H	Genes differentially expressed dependent on SinI, SinR, or ExpR					
SMc00477	Conserved hypothetical protein	***1.00***	***1.30***	***2.06***	***1.45***	***1.29***
A or F	Genes differentially expressed dependent on ExpR and SinI or genes differentially expressed dependent on SinI and ExpR or an unknown regulatory gene and independent of SinR					
SMa1214 (*fixQ1*)	FixQ1 cbb_3-type cytochrome oxidase	0.47	**−1.20**		***4.12***	−0.53
SMc03253	Putative L-proline 3-hydroxylase protein	0.16	−0.11	−0.10	***3.46***	−0.43
SMc03254 (*fixT3*)	Putative antikinase protein	0.06	−0.34		***3.30***	0.54
SMa0762 (*fixK2*)	FixK2 transcription regulator	0.43	0.40	−0.27	***3.24***	−0.88
SMa1225 (*fixK1*)	FixK1 transcriptional activator	0.19	0.07	0.41	***3.13***	−0.51

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TABLE 3—*Continued*

Group and name (alias)	Group or gene product description	M_i value in expt[a]:				
		1	2	3	4	5
SMa0763	Hypothetical protein	−0.03	0.07	0.21	***2.61***	−0.11
SMa1223	Conserved hypothetical protein (ORF151)	0.06	0.37	0.54	***2.06***	−0.33
SMa1092	Hypothetical protein	0.41	0.01	0.43	***1.79***	−0.61
SMa1226 (*fixT1*)	FixT1 inhibitor of FixL autophosphorylation	0.27	−0.11	0.77	***1.71***	−0.05
SMc02877	Conserved hypothetical protein	0.26	0.99	0.77	***1.68***	0.52
SMa1266	HemN coproporphyrinogen III oxidase	0.15	0.33	0.21	***1.63***	0.27
SMa0667	Hypothetical protein	0.24	0.78	0.65	***1.55***	0.43
SMa1169	Hypothetical protein	0.13	0.57	0.81	***1.53***	0.38
SMa1231	Conserved hypothetical protein	0.14	−0.03	0.16	***1.53***	−0.08
SMa1995	Putative ABC transporter, permease protein	0.40	0.28	0.32	***1.52***	−0.19
SMa1335	Hypothetical protein	0.30	0.05	0.48	***1.47***	0.40
SMa1136	Hypothetical protein	−0.03	0.47	0.69	***1.46***	0.52
SMa0748	Putative MucR family transcriptional regulatory protein	−0.18	−0.16	−0.30	***1.45***	0.94
SMc01855	Hypothetical transmembrane protein	0.14	−0.08	0.05	***1.44***	0.47
SMc04081 (*sqdD*)	Glycosyl transferase (sulfolipid biosynthesis) protein	0.05	0.63	0.50	***1.31***	0.48
SMa0765 (*fixN2*)	FixN2 cytochrome *c* oxidase polypeptide I	0.07	0.19	0.07	***1.30***	−0.04
SMc01525 (*dppA2*)	Putative dipeptide binding periplasmic protein	0.79	0.21	0.26	***1.30***	−0.27
SMa1615	TRm1a transposase	0.15	0.12	0.22	***1.28***	0.49
SMc01947	Conserved hypothetical transmembrane protein	−0.30	−0.19	−0.02	***1.28***	0.15
SMa0760 (*fixT2*)	FixT2 transcription regulator	0.04	0.17	−0.18	***1.26***	−0.13
SMb21234 (TRm1a)	Probable transposase of insertion sequence ISRm1 orfA protein	−0.09	−0.08	−0.23	***1.25***	0.72
SMc01946 (*livK*)	Putative leucine-specific binding protein precursor	0.12	0.12	0.49	***1.23***	0.82
SMc03963 (*sqdC*)	Sulfolipid biosynthesis protein	0.07	0.60	0.51	***1.21***	0.62
SMc01545	Hypothetical protein	−0.14	0.73	0.48	***1.19***	−0.89
SMa1132	Hypothetical protein	−0.09	0.12	0.23	***1.14***	−0.35
SMc02725 (*trpE*)	Anthranylate synthase, glutamine amidotransferase protein	0.37	0.23	0.51	***1.14***	0.57
SMa1067	Putative transcriptional regulator	−0.02	0.13	0.54	***1.11***	0.11
SMc03832	Conserved hypothetical signal peptide protein	−0.07	−0.04	0.25	***1.11***	0.73
SMc04017 (*omp10*)	Probable outer membrane lipoprotein	−0.14	0.57	0.34	***1.10***	−0.05
SMa1170 (*cycB2*)	Putative CycB2 cytochrome c_{552}	−0.13	−0.02	0.03	***1.09***	−0.46
SMc00573 (*acpP*)	Acyl carrier protein	−0.29	0.11	−0.33	***1.08***	−0.44
SMa0541	Hypothetical protein	−0.29	0.41	0.53	***1.06***	−0.37
SMa2063	Hypothetical protein	−0.06	0.46	0.56	***1.06***	0.04
SMc01524	Putative dipeptidase protein	0.45	0.00	0.22	***1.05***	−0.33
SMa0900	Possible anti-restriction protein	−0.01	0.69	0.74	***1.03***	0.19
SMa1957	Hypothetical protein	0.11	0.43	0.43	***1.02***	0.28
SMc04007	Conserved hypothetical protein	−0.04	0.46	0.34	***1.02***	−0.16
SMa1896	Putative methionine sulfoxide reductase	0.25	0.96	0.94	***1.00***	0.80
SMc02940	Hypothetical protein	0.37	0.44	0.80	***1.00***	0.33
SMc00123	Conserved hypothetical protein	−0.39	−0.24	0.18	***−1.00***	0.01
SMb21118	Hypothetical protein	0.01	0.02	−0.02	***−1.03***	−0.61
SMc00251	Hypothetical protein	−0.12	−0.87	0.39	***−1.03***	−0.12
SMa1651	Putative ABC transporter, periplasmic solute-binding protein	0.15	0.10	−0.57	***−1.09***	−0.31
SMc01585 (*cspA3*)	Putative cold shock transcription regulator protein	−0.33	0.04	−0.01	***−1.09***	−0.34
SMa2379	Catalase/peroxidase	−0.90	−0.19	−0.27	***−1.10***	−0.03
SMc04292 (*cyaF3*)	Probable adenylate/guanylate cyclase protein	−0.19	0.01	0.53	***−1.12***	0.22
SMc04363	Hypothetical protein	0.27	−0.38	−0.05	***−1.18***	0.10
SMc04442	Putative acetyltransferase protein	−0.08	−0.16	0.31	***−1.28***	−0.09
SMb21324 (*expA7*)	Putative glucose-1-phosphate thymidyltransferase protein	−0.75	−0.04	0.25	***−1.31***	−0.11
SMb20811	Putative protein	−0.34	−0.11	0.35	***−1.32***	−0.47
SMb21315 (*expD2*)	Putative secretion protein, HlyD family membrane fusion protein, secretes ExpE1	−0.92	−0.12	0.27	***−1.33***	0.49
SMa2412 (*rhrA*)	RhrA transcriptional activator	−0.23	0.03	0.60	***−1.35***	0.01
SMb21552	Putative aminoglycoside 6′-*N*-acetyltransferase, similar to amikacin resistance protein	−0.34	−0.19	−0.13	***−1.36***	−0.08
SMb20133	Conserved hypothetical protein	−0.96	0.00	0.23	***−1.47***	0.24
SMb21320 (*expA23*)	Putative bifunctional glycosyltransferase, forming β-glycosyl and α-glycosyl linkages protein	−0.78	−0.01	0.34	***−1.48***	−0.06
SMa0357	Hypothetical protein	−0.42	−0.22	**−1.24**	***−1.53***	−0.86
SMb21313 (*expE2*)	Putative bifunctional glycosyltransferase, forming α-glycosyl and β-glycosyl linkages	−0.89	0.00	0.48	***−1.62***	0.16
SMa0125 (*groES3*)	GroES3 chaperonin	0.09	0.24	−0.39	***−2.84***	−0.47
SMb21229	Putative calcium-binding exported protein	***−1.08***	0.15	0.12	−0.16	0.57
SMc00979	Hypothetical transmembrane protein	***−1.25***	0.21	0.14	−0.86	0.34
SMa1587 (*eglC*)	EglC endo-1,3-1,4-β-glycanase	***−1.35***	−0.12	−0.06	−0.45	0.71

[a] Values in boldface italic type indicate genes that meet both M and *P* criteria; genes were grouped according to these values. Value in boldface roman type indicate genes that meet M but not *P* criteria. The data presented in this table are the M_i values of each gene. Positive M_i values indicate upregulation in strain 1, and negative M_i values indicate upregulation in strain 2. Cross hybridizations are listed the supplemental data. Experiment parameters for experiments 1 to 5 are as described in Table 2.

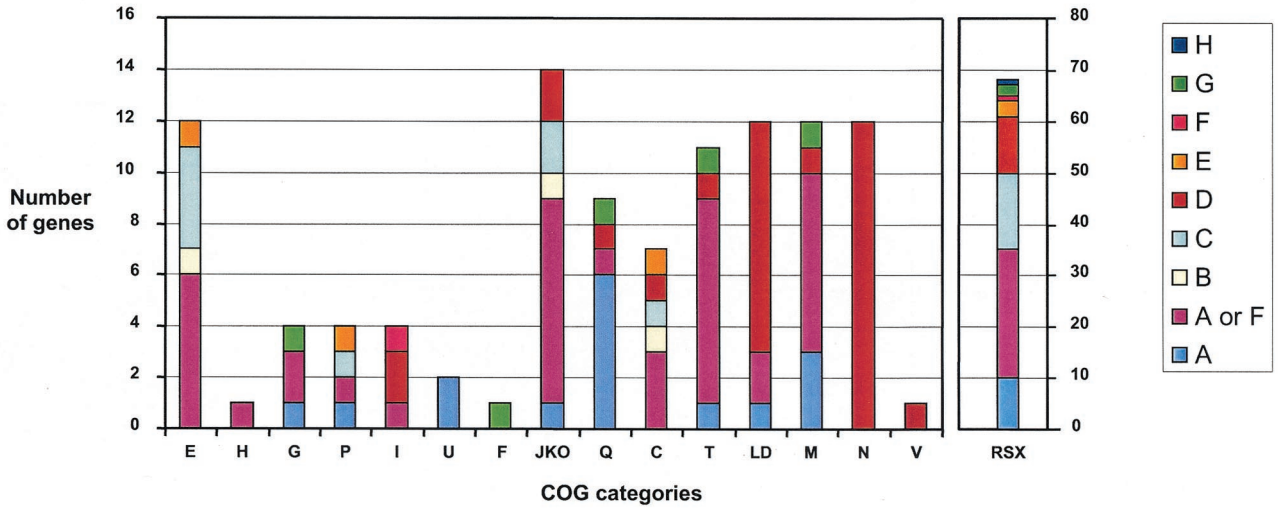

FIG. 1. All of the differentially expressed genes detected in the microarray experiments are grouped by cluster analysis based on the similarity of their regulation (groups A to H). The genes in groups A to H are further classified into different categories based on their function (*x* axis). The number of genes which have similar functions and modes of regulation is indicated in the *y* axis. COG categories: E, amino acid transport and metabolism; H, coenzyme transport and metabolism; G, carbohydrate transport and metabolism; P, inorganic ion transport and metabolism; U, intracellular trafficking; F, nucleotide transport and metabolism; J, translation; Q, secondary metabolite biosynthesis; C, energy production and conversion; T, signal transduction mechanisms; D, cell cycle control; M, cell wall; N, cell motility; V, defense mechanisms; S, function unknown; K, transcription; L, replication; O, posttranslational modification; R, general function prediction only; X, no function.

In addition to EPS II biosynthesis genes, other *S. meliloti* genes seem to be dependent on the *sin*-AHLs and the ExpR regulator. Among these are a couple of genes involved in the production of low-molecular-weight (LMW) succinoglycan. Two endoglycanase genes, *exsH* (SMb20932) and *eglC* (SMa1587) (see Table 3, group A or F, experiments 1 and 4), are expressed at a higher level in the presence of the *sin*-AHLs. Both of these genes were previously shown to be major contributors to the production of LMW succinoglycan, but their regulation remained unclear (66). The expression of *exsH* was analyzed by real-time PCR with *S. meliloti* strains that retain or lack one or two components of the Sin/ExpR system. The results match our microarray observations and show that *exsH* is upregulated by 10- to 12-fold in a strain containing intact *expR* and *sinI* genes (Table 5). Additional genes that are upregulated in the presence of a functional Sin/ExpR system are several genes encoding calcium-binding proteins (SMb21402 and SMc04171). Three genes in group A exhibit higher expression in strain derivatives that lack either the *sin*-AHLs (Rm8530 *sinI* mutant) or the ExpR regulator (Rm1021) compared to an *expR*$^+$ strain (Rm8530), including those involved in iron transport (SMc02726) and pilus formation (*pilA1*). This result suggests a possible inhibitory effect by the Sin/ExpR system on the expression of these genes.

Genes whose expression is dependent on SinI and SinR but is independent of ExpR (group B). This group contains genes that seem to be regulated exclusively by the Sin system and

TABLE 4. Analysis of *expE2* expression

Strain	Fold change as determined by:		
	DNA microarray analysis (M value)	Real-time PCR analysis[a] (Ct value)	β-Galactosidase assay[b] (Miller units)
Rm8530	–(NA)[e]	–(26.63)	−(1,186.2 ± 50.4)[b]
Rm8530 (*sinI*)	1.85 (−0.89)[c]	14.24 (33.75)	53.19 (22.3 ± 0.4)
Rm1021 (*expR*)	3.07 (1.62)[d]	14.50 (33.88)	98.68 (12.02 ± 0.2)

[a] 16S RNA was used as an internal control in a multiplex real-time PCR; the Ct values for 16S RNA for the three strains were 22.70, 22.62, and 22.38, respectively.
[b] These values were obtained with strains carrying a *lacZ* fusion to *expE2*.
[c] The M value for the *expE2* gene in the Rm8530 *sinI* mutant-versus-Rm8530 microarray experiment was −0.89, which is below our cutoff value.
[d] This value equals the $\log_2$ R/G, where R is the expression of *expE2* in Rm8530 and G is the expression of *expE2* in Rm1021. See Table 3.
[e] NA, not available.

TABLE 5. Expression analysis of representative quorum sensing regulated genes

Gene	Fold change (microarray)[a]	Fold change (real-time PCR)[b]	Relevant comparison
exsH	4	12 ↓[c]	*expR*$^+$ *sinI* mutant vs *expR*$^+$ *sinI*$^+$
exsH	5	11.2 ↓	*expR* mutant *sinI*$^+$ vs *expR*$^+$ *sinI*$^+$
ndvA	2	5.9 ↑	*expR* mutant *sinI* mutant vs *expR*$^+$ *sinI* mutant
ndvB	0.1	0.6	*expR* mutant *sinI* mutant vs *expR*$^+$ *sinI* mutant
flaF	3.5	7.1 ↓	*expR* mutant *sinI* mutant vs *expR*$^+$ *sinI* mutant
cheY1	3.2	7.6 ↓	*expR* mutant *sinI* mutant vs *expR*$^+$ *sinI* mutant

[a] The fold change is based on the M value from the microarray data. Refer to Table 3.
[b] The fold change is calculated from the Ct values obtained in the real-time PCR analyses. The experiments include 16S RNA multiplexed as an internal control.
[c] The arrow indicates the direction of expression in the strain listed first in the "relevant comparison" column.

autonomous from the ExpR regulator. The differences in their expression can be appraised from experiments 2 and 3 (Tables 2 and 3). All of the genes in this group show a higher level of expression in the absence of the *sin*-AHLs and/or the SinR regulator. The small number of genes in group B suggests that the SinR/SinI system is highly dependent on an active ExpR regulator for most of its activity.

Differentially expressed genes that are dependent on SinR but not SinI (group C). The genes in group C are differentially expressed in the presence or absence of the SinR regulator (experiment 3), but their expression seems to be independent of an active *sinI* gene or its encoded AHLs (Table 3, group C, experiment 2). Most of the genes in this group appear to be expressed more strongly in the absence of the SinR regulator. Among these are genes that play a role in nitrogen fixation (*fixS1*), citrate synthesis (*gltA*), transcriptional regulation (SMc03998), and other cellular processes. The regulation of the genes in group C does not seem to rely on a functional *sinI* (experiment 2) or an intact *expR* gene (experiment 4) since significant values are seen only in the *sinR*$^+$ versus *sinR* mutant combination (experiment 3).

Genes whose expression depends on the ExpR regulator but are either independent of SinI or differentially expressed in the absence of SinI (group D). The genes in this group are further categorized into subgroups based on the expression patterns in the microarray experiments. Group D1 represents genes whose expression depends on the presence of the ExpR regulator but are independent of the presence of an intact *sinI* gene or the *sin*-AHLs (experiments 4 and 5). The expression of group D1 genes does not differ significantly in experiment 2. As a result, we conclude that these genes rely on ExpR for expression but are unaffected by the presence or absence of the *sin*-AHLs. Most of the members of this group encode TRm1a transposases which, due to their high degree of identity (99 to 100%), cannot be differentiated.

The ExpR-dependent genes in group D2 are differentially expressed in the absence of a functional *sinI* gene. Their differential expression is observed only in experiment 5 (see Tables 2 and 3). Of the 24 genes in this group, 9 showed higher expression levels in the absence of the ExpR regulator. The products of these genes participate in activities such as inhibition of cell division (*minE* and *minD*), cyclic glucan production (*ndvA*), and succinoglycan biosynthesis (*exoY*). The expression of *ndvA* appears to be upregulated in the *expR sinI* mutant strain compared to the *expR*$^+$ *sinI* mutant strain (Table 3), and analysis by real-time PCR revealed about a 6-fold increase in expression (Table 5). The expression of *ndvB*, which does not appear in our microarray data, remained the same in all of the strains that we examined (Tables 3 and 5). The remaining 15 genes exhibited elevated levels of expression in the presence of ExpR and are involved in flagellum synthesis (*flbT*, *flaF*, and several *flg* genes) and chemotaxis (*cheY1*). Real-time PCR analysis was also performed for the *flaF* and *cheY1* genes, and the results show that both of these genes are upregulated in an *expR*$^+$ *sinI* mutant strain (Table 5).

The members of group D3 are activated or repressed by ExpR in the absence of an active *sinI* gene. As in group D2, they rely on the ExpR regulator but only show differential expression when the strain lacks the *sin*-AHLs (experiments 1 and 5). These genes (*flaABCD*, *flgEKL*, and *fliL*) exhibit increased expression in the presence of ExpR and in the absence of SinI (experiment 1) and a downregulation in a strain lacking both an active *expR* gene and the *sinI* gene (experiment 5).

Differentially expressed genes which rely on either ExpR or SinR and may depend on the *sin* AHLs (group E). The first subgroup (Table 3, group E1) in this category contains a single gene (*motC*) that showed higher expression in the absence of the SinR regulator (experiment 3) and the ExpR regulator (experiment 4), indicating a dependence on ExpR, SinR, or both regulators (Table 3, group E1, experiments 3 and 4).

Similarly, the genes in group E2 are assigned based on their dependence on ExpR (experiment 4) or SinR (experiment 3), but the differential expression occurs in the absence of SinI (experiment 5). Prominent in this group is a gene coding for a putative high-affinity zinc uptake ABC transporter protein (*znuA*) and a possible oxidoreductase-encoding gene (SMa1541) (Table 3, group E2, experiments 3, 4, and 5).

Two genes coding for various ABC-type transporters (SMb20604 and SMc04127) belong to group E3, which classifies them based upon their dependence on ExpR or SinR and possibly the *sin*-AHLs or an unknown factor (Table 3, group E3, experiments 2, 3, and 5).

Groups F and H. The genes assigned to these groups are mostly of unknown functions, and their regulation remains inconclusive at this time (Table 3, groups F and H). Due to their different expression patterns in experiments 1, 2, and 4, we conclude that the genes in group F (SMc00193 and SMa2091) are regulated by the *sin*-AHLs, the ExpR regulator or another gene regulator, but not by the SinR regulator. Group H contains a single gene (SMc00477), which is possibly dependent on ExpR, SinR, or SinI. Its expression varies in all five of the microarray experiments.

Differentially expressed genes that depend on SinI in the absence of ExpR but that are independent of SinR (group G). The genes listed in this category showed various expression levels when we compared a strain lacking the ExpR regulator with a strain that also lacks the *sinI* gene (Table 3, group G, experiment 2). These genes seem to rely on the *sin*-AHLs in an *expR* mutant background but remain independent of the SinR regulator. All of the genes exhibit an upregulated level of expression when both *sinI* and *expR* have been disrupted.

Differentially expressed genes that are dependent on ExpR or an unknown regulatory gene and SinI but are independent of SinR (group A or F). Most of the genes in this group have elevated expression in a strain lacking the ExpR regulator compared to an ExpR proficient strain (Table 3, group A or F, experiment 4). Since no differential expression of these genes is seen in experiment 5, we conclude that their expression also relies on the presence of the *sin*-AHLs. Many of the genes in this category play important roles in nitrogen fixation (*fixQ1*, *fixK2*, *fixK1*, *fixT1*, and *fixT2*), sulfolipid biosynthesis (*sqdC* and *sqdD*), and transcriptional regulation (SMa0748 and SMa1067). On the other hand, the genes involved in functions such as EPS II production (*exp* genes), regulation of iron transport (*rhrA*), and other physiological processes exhibit lower expression in a strain lacking an intact *expR* gene (Rm1021) in the same experiment.

DISCUSSION

Quorum sensing is used by many organisms, especially those that live in close association with eukaryotic hosts, to regulate cellular processes in a coordinated and efficient manner (17, 59). *S. meliloti* harbors at least three quorum sensing systems (19). The Tra system resides on the pRme41 plasmid of the Rm41 strain (38) and controls conjugal plasmid transfer (M. M. Marketon et al., unpublished data). The Mel system, present in both the Rm1021 and Rm41 strains, seems to play an important role in nodulation (A. Patankar and J. González, unpublished data). The Sin quorum sensing system was shown to be involved in EPS II production and the efficient establishment of symbiosis with its host *M. sativa* (36–38). We sought to determine whether additional genes are targeted and controlled by the Sin quorum sensing system and what role they may play in the symbiosis between alfalfa and *S. meliloti*. Expression profile analysis through DNA microarrays, conducted with strains that retain or lack the presence of a functional Sin system or the ExpR regulator, allow us to report the *S. meliloti* genes that are either directly or indirectly quorum sensing regulated. It is essential to point out that our microarray analysis was performed with cultures at an OD_{600} of 0.8. These experiments therefore provide a "snapshot" of what evidently is a dynamic process that varies as the population density changes in the growing bacterial culture. Differences in the expression of the quorum sensing controlled genes may occur as the culture goes through the various growth stages.

Initially, we compared the expression profiles of *expR*$^+$ *sinI* mutant versus *expR*$^+$ (Rm8530) strains to view the role that the *sin*-encoded AHLs play in gene expression (Table 3, group A). As expected, the *exp* genes are highly induced in the presence of the *sin*-AHLs, as previously shown by Marketon et al. (36). In that study, the expression of the *exp* operon was determined by analyzing the expression of selected *lacZ* fusions to various *exp* genes in strains that are either proficient or deficient in the synthesis of the *sin*-AHLs. Expression of the *exp* genes, particularly *expE2*, *expG*, and *expC*, depends not only on the production of the *sin*-AHLs but also on an active ExpR regulator (36, 42). The colony phenotype of the *expR*$^+$ (Rm8530), *expR*$^+$ *sinI* mutant, and *expR* mutant (Rm1021) strains on MGM low-phosphate plates confirms the role of the Sin system and ExpR regulator in the production of EPS II (36). The *expR*$^+$ strain exhibits a mucoid phenotype due to the presence of EPS II. However, *expR*$^+$ *sinI* mutant is unable to produce EPS II since activation of the *exp* genes is dependent on the presence of the *sin*-AHLs. Induction of the *exp* genes is dependent on the presence of an active ExpR; therefore, the *expR* mutant strain remains dry and unable to produce EPS II (36, 42). When we streak the *expR*$^+$ *sinI* mutant strain next to AHLs extracted from a *sin*-AHL producing strain, the mucoid phenotype is again evident. Furthermore, the addition of a specific AHL, $C_{16:1}$-HL, was able to activate the expression of the *expE2* gene (36). Subsequent work shows that oxo-$C_{14:1}$-HL and oxo-$C_{16:1}$-HL can also induce *expE2* expression in the presence of the ExpR regulator (E. Tredemeyer and J. González, unpublished data). The fact that we also see differential expression of the *exp* genes in the microarray analysis confirms these earlier results and justifies the use of *expE2* as an internal control to verify the accuracy and reproducibility of the microarray data. This is further supported by analysis of the expression of the *expE2* gene in the various *S. meliloti* derivatives by real-time PCR (Table 4). As predicted, the expression of *expE2* is higher in the strains containing a functional Sin system and ExpR regulator (Rm8530) than in the strains missing either an active Sin system or ExpR (Rm8530 *sinI* mutant or Rm1021, respectively). An important point to note in this comparison is the magnitude of the fold changes in *expE2* expression. The β-galactosidase assay shows a 50-fold change in expression, whereas the real-time PCR analysis reveals a 14-fold change when a Sin/ExpR-proficient strain is compared to a strain lacking either the SinI synthase or the ExpR regulator. On the other hand, the DNA microarray data shows a two- to threefold increase in *expE2* expression under these same conditions. Based on prior knowledge that expression of the *exp* genes affects biosynthesis of EPS II, we are confident that the microarray data are a faithful representation of the differences in *exp* gene expression and its dependence on the presence of the Sin/ExpR system. In fact, the differentially expressed genes detected in our microarray experiments may be an underrepresentation of the number of genes controlled by the Sin/ExpR system.

In addition to the activation of the *exp* operon and EPS II biosynthesis, the Sin system seems to regulate production of LMW succinoglycan, another symbiotically important exopolysaccharide produced by *S. meliloti* (3, 5, 8, 21, 44). A trimer of the succinoglycan octasaccharide molecule, when added in *trans*, allows exopolysaccharide-deficient strains of *S. meliloti* to invade plant nodules (3, 20, 41, 58). The present study is the first to suggest a role for quorum sensing in the production of LMW succinoglycan. The endoglycanases ExsH and EglC, both of which play a role in the synthesis of the LMW fraction of succinoglycan required for the successful invasion of the plant host (49, 66), seem to be controlled by quorum sensing. We have confirmed that indeed, the *exsH* gene is expressed at a higher level in the presence of the ExpR regulator and an active Sin system (see Table 5). In addition, our assays suggest that expression of some of the *exo* genes (*exoY* and *exoI*) is suppressed under conditions that lead to EPS II production. It has been previously demonstrated that the presence of ExpR and the *sin*-AHLs are crucial for the production of LMW EPS II (36, 42). The present data suggest that quorum sensing may also play a part in the synthesis of the invasion-proficient fraction of succinoglycan.

Furthermore, we report that the Sin system also regulates the expression of genes involved in motility and chemotaxis (1, 6, 51, 52, 67). Genes residing in at least four different gene clusters, including *fla*, *flg*, *fli*, and *pil*, are downregulated in the presence of the Sin system. To verify the microarray data, we also analyzed by real-time PCR the expression of *flaF* and *cheY1*, which are involved in the regulation of flagellin synthesis and chemotaxis, respectively. Both of these genes were indeed expressed at a higher level in the *expR*$^+$ *sinI* mutant strain, a finding that corresponds to the microarray data (Table 5). This observation is in contrast to what is seen in *Serratia liquefaciens*, in which motility is upregulated by quorum sensing (2, 32). These results suggest that the Sin quorum sensing system in *S. meliloti* represses production of the flagella once the bacteria have achieved a quorum. Preliminary results indicate that the *sin*-specific AHLs are able to suppress motility of

the *expR*$^+$ *sinI* mutant strain which exhibits high expression of the motility genes compared to the *expR*$^+$ strain (Rm8530) in the microarray analysis (H. Hoang and J. González, unpublished data). We speculate that control of the motility genes by the Sin system is also dependent on ExpR regulation based on the following observation. The increase in expression of the motility genes is observed only in microarray analyses which include the *expR*$^+$ strain in the presence or absence of *sinI* (Table 3, group D3, experiments 1 and 5). Moreover, there is no evidence of the increased expression of the flagellum genes in the profile of experiment 4 (Table 3, groups D2 and D3). These data suggest the need for both a functional ExpR regulator and the absence of the *sin*-AHLs for the induction of the motility genes.

In addition, we show that most of the genes controlled by the *sin*-AHLs are also dependent on the presence of an active ExpR regulator. The analysis in experiment 2, testing which genes are regulated specifically through *sinR*/*sinI* independent of *expR*, revealed few genes that are differentially expressed, most of which have no known function. It is possible that the *sin*-encoded AHLs bind ExpR, and the autoinducer-regulator complex then activates or represses its downstream targets. The ability to either activate or repress gene expression might depend on the binding of the autoinducer-regulator complex to its target site with respect to the target gene promoter. The existence of a consensus DNA-binding sequence for ExpR is a logical possibility, but one that remains to be determined. The *sin*-AHLs seem to regulate the expression of a small number of *S. meliloti* genes through the SinR regulator (Table 3, group B). Furthermore, SinR may regulate a small set of genes upon binding and activation by other AHLs not encoded for by *sinI*, since at least 10 genes show differential expression in experiment 3 but not in experiment 2 (Table 3, group C). Another possibility is that the *sin*-AHLs may bind and activate other LuxR-type regulators independent of SinR or ExpR. The *S. meliloti* genome contains at least six additional ORFs (SMc00658, SMc00877, SMc00878, SMc04032, Smc03015, and Smc03016) that code for potential LuxR homologs, and any or all of these may work in concert with the *sin*-AHLs to regulate a subset of genes.

Recently, Chen et al. presented a proteomic analysis of *S. meliloti* in response to particular AHLs at early log phase or early stationary phase (7). These researchers suggested that the production of several proteins is affected by the addition of AHLs, mainly C_{14}-HL and 3-oxo-$C_{16:1}$-HL. The *exp* genes previously shown to be quorum sensing regulated are not among those detected in their proteomic analysis. The reason for this may be that the wild-type *S. meliloti* Rm1021 strain was used for the analysis instead of a strain with an intact *expR* gene. Our results show that an intact ExpR regulator is necessary for the *sin*-encoded AHLs to exert their effect. In fact, we observe very few genes with significant differences in expression with our microarray analysis of Rm1021 *sinI* mutant versus Rm1021. Appreciable and accurate observations of quorum sensing effects, at least with regard to the *sin*-encoded AHLs, should be apparent with the *expR*$^+$ strains (36).

We also describe the genes whose expression is dependent on the presence or absence of an ExpR regulator (groups A, D, E, and F). This group includes genes that are involved in key functions such as exopolysaccharide production, nitrogen fixation, and metal transport in addition to genes with unidentified functions. The regulation of some of these genes depends on the presence of the Sin system, whereas in other cases it occurs in the absence of the *sin*-AHLs. For instance, expression of the various *fix* genes is clearly elevated in the absence of ExpR (Table 3, group A or F, experiment 4). Some of the regulated Fix proteins are in turn regulators of nitrogen fixation such as FixK1 and FixK2. A low-oxygen environment, similar to that found inside of the plant nodules, is conducive to upregulation of the *fix* genes through FixK1 and FixK2. Activation of the *nif* cascade relies on the activity of NifA, which also requires the regulators FixK1 and FixK2 for induction (14, 15, 43). It is unclear how the ExpR regulator fits into the multileveled regulation of nitrogen fixation. ExpR may act as a repressor to keep the expression of the *fix* genes in check until the conditions are appropriate for optimal nitrogen fixation (i.e., inside plant nodules). Deciphering this complex regulatory network would be a major contribution to our understanding of not only the process of nitrogen fixation but also the regulation of the *S. meliloti*-alfalfa symbiosis by quorum sensing. Interestingly, seven genes encoding putative Ca^{2+}-binding proteins, including ExpE1 and the endoglycanases ExsH and EglC were affected depending on the presence of *expR* (groups A, D3, and A or F). These proteins are known or likely to be secreted by type I secretion systems. Whether or not these putative extracellular proteins are important for symbiosis remains to be investigated.

Supported by a cluster analysis that facilitated grouping of the genes and based on the evidence obtained thus far, we have proposed a model for the regulation of gene expression by the Sin/ExpR quorum sensing system and other possible quorum sensing system(s) in *S. meliloti* (Fig. 2). One mode of regulation may occur through the concerted activities of the *sinI*-encoded AHLs and the ExpR regulator, which seems to control expression of the genes in group A and group E. These are the two largest groups. In these instances, the *sin*-AHLs probably bind and activate the ExpR regulator, which then controls the expression of its target genes in a typical quorum sensing relay. The *sin*-AHLs may also direct gene expression through the SinR regulator for the genes in group B and a few genes in group E (group E3). We also predict that the regulation of several genes, such as those in group D, may depend on the ExpR regulator, either in the absence of the *sin*-AHLs or independently of the *sinI*-encoded AHLs. An additional regulatory pathway could involve the control by the SinR regulator of the genes in group C. In this case, non-*sin*-AHLs or an additional factor might induce the activating or inhibitory effects of the SinR regulator. Yet another means of control could exist for the genes in group F, wherein regulation might occur via the ExpR regulator or another regulator upon activation by the *sin*-AHLs. Another possibility is that the genes in group F could be controlled by the ExpR regulator or another regulator upon activation by an unknown factor or AHLs made by a separate autoinducer synthase. We have proposed that *S. meliloti* Rm1021 possesses an additional quorum sensing system termed the Mel system (38). Preliminary evidence suggests that an HdtS homolog (SMc00714) may be responsible for the synthesis of short-chain AHLs in this strain. Finally, the mode of regulation for the genes in group G seems to depend on the activity of the *sin*-AHLs and an unidentified regulatory factor

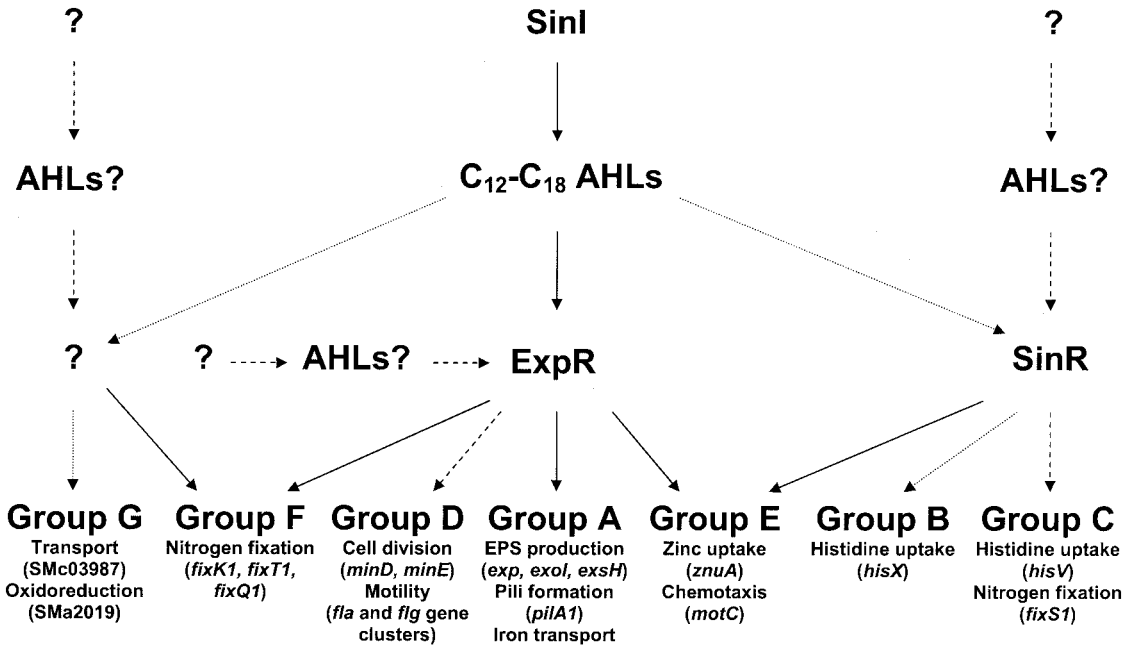

FIG. 2. Model for the regulation of *S. meliloti* gene expression by the SinR/SinI/ExpR quorum sensing system and other possible quorum sensing system(s). Continuous arrows denote relationships that can be affected by more than one way of regulation. Dotted and dashed arrows indicate unbranched relationships.

other than the ExpR and SinR regulators. As mentioned above, the *S. meliloti* genome encodes at least six additional putative LuxR-type regulators. It would be interesting to determine whether one or more of these factors might play a role in regulating the genes in group G and/or group F. Among the differentially expressed genes, eight regulatory genes of known function and six putative regulatory genes of unknown function were identified. Some of the observed regulatory effects of the quorum sensing systems may therefore be indirect. This model presents a very intricate and widespread network for gene regulation by the *S. meliloti* quorum sensing system(s).

Of the nitrogen-fixing rhizobia, quorum sensing is best characterized in *R. leguminosarum* bv. *viciae* (for a review, see reference 63). Several quorum-sensing systems (*rai*, *rhi*, *cin*, and *tra*) have been identified and are intertwined in a complex regulatory network (9, 33, 45, 61, 64). Early work identified *rhiI* (45) and showed that it was responsible for the synthesis of several short-chain AHLs, including C_6-HL, C_8-HL, and another compound comigrating with C_7-HL (45). In addition to short-chain AHLs, a long-chain AHL (27, 60) was identified as 3-OH-$C_{14:1}$-HL (24, 48). The production of 3-OH-$C_{14:1}$-HL is a result of the *cinRI* locus, located on the chromosome (33). The *cinI* AHL synthase is positively autoregulated by CinR and 3-OH-$C_{14:1}$-HL. Regulation of *cinR* is unclear at this time, since a mutation in *cinR* or *cinI* or even the lack of the *rhi* and *tra* systems does not seem to affect *cinR* expression (33). However, *cinR* expression is population density dependent (33). This finding is somewhat similar to what we observe in the Sin system, where only a small set of genes seems to be regulated by *sinI*/*sinR*. The important difference with the *R. leguminosarum* Cin quorum sensing system is that autoregulation of *sinI* by *sinR* is not observed. We have previously shown a decrease in the production of the *S. meliloti sin* AHLs in a *sinR* mutant strain (37). Surprisingly, the SinR-dependent expression of the *sinI* gene in our microarray experiments falls below our cutoff values. This is, however, consistent with unpublished data from our laboratory that shows a very low level of control over the *sinI* gene by SinR. It is possible that, in contrast to the Cin system in *R. leguminosarum*, which sits at the top of the quorum sensing network, the Sin system in *S. meliloti* is not at the apex of the quorum sensing hierarchy.

It is interesting to speculate on how the ExpR regulator may control gene expression. One possibility is that ExpR could be differentially activated by the different *sin* or the *mel* AHLs made by *S. meliloti*. The Sin system makes at least five different AHLs, and the Mel system is responsible for another three AHLs. Another possibility is that ExpR could have the capacity to act as a regulator even in the non-AHL bound form. This could explain the fact that ExpR seems to activate (or repress) transcription in the absence of the *sin* AHLs. Another intriguing possibility is that ExpR could bind to different promoters based on the activating AHL.

In light of our findings, we propose that the ExpR regulator has a comprehensive regulatory role in the *S. meliloti*-alfalfa symbiosis, both within and outside of the plant environment. It would be informative to determine the regulatory mechanisms, beyond that of the Sin system, which control the activity of the ExpR regulator. Possibilities include environmental cues such as phosphate and nitrogen concentrations, the oxygen tension of the surrounding milieu, or other *S. meliloti* quorum sensing systems such as the Mel system. Another possibility is that the plant host itself may play a role in the activation of the *S. meliloti* quorum sensing system(s). The observation that alfalfa produces autoinducer mimics (57) suggests that the host may interfere in the bacterial signaling system. Our genomic anal-

ysis of the Sin/ExpR quorum sensing system opens the door for the future analysis of the complex signaling systems in the *S. meliloti*-alfalfa symbiotic process.

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