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Competitive and Cooperative Effects in Quorum-Sensing-Regulated Galactoglucan Biosynthesis in *Sinorhizobium meliloti*[▽]

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The symbiotic nitrogen-fixing bacterium *Sinorhizobium meliloti* possesses the Sin quorum-sensing system based on *N*-acyl homoserine lactones (AHLs) as signal molecules. The Sin system consists of SinI, the AHL synthase, and SinR, the LuxR-type regulator. This system regulates the expression of a multitude of *S. meliloti* genes through ExpR, another LuxR-type regulator. Analysis of the activity of the *sinI* promoter showed that the expression of *sinI* is dependent on *sinR* and enhanced by a combination of *expR* and Sin AHLs. The characterization of the ExpR binding site upstream of *sinI* and the identification of binding sites upstream of the galactoglucan biosynthesis genes *wgaA* (*expA1*) and *wgeA* (*expE1*) allowed the definition of a consensus sequence for these binding sites. Based on this consensus, two additional ExpR binding sites in the promoter regions of *exoI* and *exsH*, two genes related to the production of succinoglycan, were found. The specific binding of ExpR to the *wgaA* and *wgeA* promoters was enhanced in the presence of oxo-C_{14}-HL. Positive regulation of the galactoglucan biosynthesis genes by ExpR was shown to be dependent on WggR (ExpG) and influenced by MucR, both of which are previously characterized regulators of these genes. Based on these results, a reworked model of the Sin-ExpR quorum-sensing regulation scheme of galactoglucan production in *S. meliloti* is suggested.

The soil bacterium *Sinorhizobium meliloti* converts atmospheric dinitrogen to ammonia in symbiotic association with the host plant *Medicago sativa* (alfalfa). During the establishment of successful symbiosis, the extracellular polysaccharides produced by *S. meliloti* are crucial for this process. Mutants that are unable to produce at least one of the three symbiotically active polysaccharides, including succinoglycan, galactoglucan, or K antigen, are defective in nodule invasion and primarily induce the formation of symbiotically ineffective root nodules that are devoid of bacteria and bacteroids (18, 28, 41). The mechanisms by which each of these polysaccharides functions revolve around mediating infection thread initiation and extension on alfalfa (11, 37).

The galactoglucan biosynthesis genes are located in a 32-kb gene cluster on pSymB that is composed of 21 genes organized into five putative operons. The nomenclature of these genes, previously named the *exp* cluster (6), was recently revised (GenBank accession no. AL591985). The five putative operons are as follows: *wga* (*expA*) (nine genes), *wgcA* (*expC*) (one gene), *wggR* (*expG*) (one gene), *wgd* (*expD*) (two genes), and *wge* (*expE*) (eight genes) (6). The regulation of the galactoglucan biosynthesis genes is controlled by WggR, a transcriptional regulator that activates the expression of *wgaA* (*expA1*), *wggR* and/or *wgdA* (*expG* and *expD1*, respectively), and *wgeA* (*expE1*) by binding to sites consisting of a conserved palindromic region and two associated sequence motifs in the promoter regions of these genes (2, 4). Another major regulator of galactoglucan production in *S. meliloti* is MucR, a protein containing a C_2H_2 zinc finger DNA binding motif (26). In addition to activating the production of succinoglycan through binding sites in the promoter regions of the succinoglycan genes *exoH* and *exoY* (8), MucR inhibits the production of galactoglucan through additional sites in the promoter regions of the galactoglucan biosynthesis genes *wgaA*, *wgeA*, *wgdA*, and *wggR* (1, 43). Yet another protein, PhoB, is involved in regulating the galactoglucan biosynthesis genes under phosphate-limiting growth conditions, probably through direct interactions with the so-called PHO boxes in the promoter regions of these genes (13, 50). Recent work on the regulation of galactoglucan production revealed a very complex system involving two promoters (distal and proximal) for *wggR* and for each of the *wga*, *wge*, and *wgd* operons, and a model involving WggR, MucR, and PhoB has been proposed (1, 43).

Wild-type laboratory *S. meliloti* strain Rm1021, whose genome has been sequenced (16), produces a non-symbiotically-active galactoglucan under low-phosphate conditions (21, 34, 51) or upon the disruption of the *mucR* gene (26). However, the production of symbiotically active galactoglucan requires a functional ExpR, which is not present in Rm1021 (38). Characterization of the *expR* locus, located on the 3.4-Mb main chromosome (18), revealed an insertion sequence (ISRm2011-1) within the coding region of the gene (38). A spontaneous mutation involving the precise, reading frame-restoring excision of the insertion sequence from the coding region of *expR* resulted in a functional ExpR and mucoid colony morphology, which is indicative of high levels of galactoglucan production, including symbiotically active galactoglucan (38). ExpR is a member of the LuxR family of proteins, many of which are receptors for *N*-acyl homoserine lactones (AHLs) and are transcription regulators involved in the control of gene expression in response to changes in population density, a process known as quorum sensing (14, 38). Quorum-

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TABLE 1. Strains and plasmids

Strain or plasmid	Description	Reference(s) or source
Strains		
S. meliloti		
Rm2011	Wild type; Nx^r Sm^r	10
Rm101	Rm2011 *mucR*Ω; Spc^r cassette from pHP45Ω inserted into the P*macI* site within *mucR*	6
SmSRΔG	Rm2011 Δ*wggR*; deletion of the *wggR* gene comprising 490 nucleotides of the 3′ terminus of the *wggR* coding region and 17 nucleotides downstream of *wggR*	43
SmBBΔG101	Rm2011 Δ*wggR mucR*Ω; double mutant	1
2011mTn*5*STM.2.01.B05	Rm2011 *sinR*::mini-Tn*5*	39
Rm1021	Wild type; Nx^r Sm^r	27
Rm8530	Rm1021 $expR^+$	18
Rm11511	Rm1021 *sinI*::Km	33
Rm11527	Rm8530 *sinI*::Km	32
Rm1021 *sinR*	Rm1021 *sinR*::mini-Tn*5*	This work
Rm8530 *sinR*	Rm8530 *sinR*::mini-Tn*5*	This work
E. coli		
DH5α	F^- *endA1 supE44 thi-1* λ-*recA1 gyrA*96 *relA1 deoR*Δ(*lacZYA-argF*)*U169*	22
S17-1	*E. coli* 294 Thi RP4-2-Tc::Mu-Km::Tn*7* integrated into the chromosome	44
M15pREP4	Nx^s Str^s Rif^s Thi^- Lac^- Ara^+ Gal^+ Mtl^- F^- $RecA^+$ Uvr^+ Lon^+	Qiagen, Hilden, Germany
Plasmids		
pSRPP18	Promoter probe vector, pUC derivative; promoterless *lacZ* gene; integrates between the *exoP* terminator and the *thiD* gene	1
pCR-TOPO	TOPO cloning kit	Invitrogen, Karlsruhe, Germany
pJN105	*araC*-PBAD cassette cloned in pBBR1MCS5; Gm^r	36
pJN*expR*	pJN105 containing *expR*	3
pHU231	Tc^r; pRK290 with a 388-bp HaeII insert containing pUC18 polylinker	25, 42
pLK64	pPHU231 containing *sinI*-EGFP translational fusion; Tc^r	This work

sensing systems have been discovered in *S. meliloti* (31, 33). One of these, the Sin system, consists of the autoinducer synthase SinI and its LuxR-type regulator, SinR. SinI is responsible for the production of a series of long-chain AHLs, including C_{12}-HL, oxo-C_{14}-HL, oxo-$C_{16:1}$-HL, $C_{16:1}$-HL, and C_{18}-HL. A disruption of *sinI* was found to abolish galactoglucan production as well as the expression of several genes in the galactoglucan biosynthesis operons. This phenotype was complemented by the addition of AHL extracts from the wild-type strain, but not from a *sinI* mutant, and by the addition of synthetic $C_{16:1}$-HL (32). The absence of symbiotically active galactoglucan in a *sinI* mutant was confirmed in plant nodulation assays, emphasizing the role of quorum sensing in symbiosis (32). In addition to regulating the galactoglucan biosynthesis genes, the Sin-ExpR combination also regulates 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 (17, 23).

Given that ExpR is significant in the quorum-sensing-based regulation of so many *S. meliloti* genes, the question of its exact mechanism of regulation is rather interesting. We therefore sought to unravel the pathway by which quorum sensing and ExpR regulate the genes responsible for the production of galactoglucan. LuxR-type regulators, of which ExpR is a member, usually bind to a DNA consensus sequence known as a *lux* box, which is typically located upstream of the promoters of its target genes. These regulators bind to the *lux* box upon activation by binding to an AHL (12, 46). A putative *lux* box 70 bp upstream of *sinI* has been identified, but ExpR does not bind to it. Rather, as we previously reported, ExpR binds to a site approximately 100 bp upstream of *sinI* and results in approximately four-times-higher *sinI* mRNA levels (3), indicating positive regulation of *sinI* by ExpR, presumably upon AHL activation. However, this cannot be the only function of ExpR, because the upregulation of *sinI* occurs both in $expR^+$ and *expR* strains (3), but only the $expR^+$ strain produces detectable levels of galactoglucan.

In this study, we characterized the effect of ExpR on the Sin quorum-sensing system and identified additional ExpR DNA binding sites in the promoter regions of the galactoglucan and succinoglycan biosynthesis gene clusters. Furthermore, the effect of ExpR-stimulated galactoglucan biosynthesis gene transcription was studied in relation to those of the transcriptional regulators WggR and MucR. Cooperative and competitive interactions between these regulators are discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains used in this study are listed in Table 1. *S. meliloti* strains were incubated at 30°C in Luria-Bertani (LB) medium supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ (LBmc) or tryptone-yeast medium (7). *Escherichia coli* strains were incubated at 37°C in LB medium. Antibiotics were added at the following concentrations: 120 μg ml^{-1} neomycin (Nm), 40 μg ml^{-1} gentamicin (Gm), 10 μg ml^{-1} nalidixic acid (Nx), 600 μg ml^{-1} streptomycin (Sm), and 10 μg ml^{-1} tetracycline (Tc) for *S. meliloti* and 50 μg ml^{-1} kanamycin (Kan), 100 μg ml^{-1} ampicillin (Ap), and 10 μg ml^{-1} Gm for *E. coli*.

Plasmids. The construction of plasmids pJNexpR (3) and pSRPP18 (1), used in this work, was described previously. For the fusion of the *sinI* promoter with the gene coding for enhanced green fluorescent protein (EGFP), the *sinI* promoter region along with the region containing the first 9 codons of SinI were

amplified using primers sinI_forward_HindIII (5′-CCTAAAGCTTCAACGAT TCTCGGCATATCC) and sinI_reverse_XbaI (5′-TCCTTCTAGAACCGTTTC CGTTCACTATCCT). The EGFP coding sequence was amplified using primers EGFP_forward_XbaI (5′-AAGATCTAGAGTGAGCAAGGGCGAGGAGCT) and GFP_reverse_EcoRI (5′-GTACGAATTCTTACTTGTACAGCTCGTCCA TG). The PCR-amplified DNA fragments were cloned into EcoRI-HindIII sites of pPHU231, yielding plasmid pLK64.

Expression and purification of His_6-ExpR. The expression and purification of recombinant His_6-ExpR were performed essentially as described previously (3) except that the purified protein was mixed with glycerol (1:1 volume) and stored at −20°C, which resulted in prolonged stability.

Binding site cloning and DNA labeling. The DNA probes from the promoter regions of *sinI*, *wgaA*, *wgeA*, and *wggR* and/or *wgdA* used in the electrophoretic mobility shift assays (EMSAs) were prepared as described previously (3). Specific primers labeled with 5′ Cy3 were used in a PCR with genomic DNA as the template to produce the Cy3-labeled fragments. The *sinI* promoter region was derived from a 216-bp region that included 31 bp of the 3′ end of *sinR*, the 156-bp intergenic region between *sinR* and *sinI*, and 29 bp of the 5′ end of *sinI* using primers 5′-TGTTCGACATGCTCTGATCC and 5′-CGACCGTTTCCGTTCA CTAT. The *wgaA* promoter was derived from a 301-bp region that included 115 bp of the 3′ end of *wgcA*, the 183-bp intergenic region between *wgcA* and *wgaA*, and 3 bp of the 5′ end of *wgaA* using primers 5′-CAGAACGGTCGAACAG AGGT and 5′-CATCAACTCTTGCACGCAGC. The *wgeA* promoter was derived from a 301-bp region that included 41 bp of the 3′ end of *wgdB* (*expD2*), the 274-bp intergenic region between *wgdB* and *wgeA*, and 11 bp of the 5′ end of *wgeA* by using primers 5′-CAGTCTCCGACAGTTTCAAC and 5′-CATCAAC TCTTGCACGCAGC. The *wgdA* and *wggR* promoters were derived from a 334-bp region that included 131 bp of the 5′ end of *wgdA*, 7 bp of the 5′ end of *wggR*, and the 196-bp intergenic region between *wggR* and *wgdA* by using primers 5′-ATAAAGAAGCGTCACGACGA and 5′-TCTCCATTGGGAACGTACTT. The *exsH* promoter was derived from a 238-bp region that included 230 bp upstream of *exsH* plus 8 bp of the 5′ end of *exsH* by using primers 5′-CGCGG TACCAAGTCGTGACATCGTCAATC and 5′-CGCGGATCCACCACGGCG TTCAATACGGTT. The *exoI* promoter was derived from a 273-bp region that included 270 bp upstream of *exoI* plus 3 bp of the 5′ end of *exoI* by using primers 5′-CGCGGTACCACGGCAACAACATGGATGTTC and 5′-CGCGGATCCC ATTCCCCATCCCCGTTCAG. For the cloning of the ExpR binding sites, oligonucleotides (indicated by the gray boxes in Fig. 3) were annealed and cloned into vector pCR using the TOPO cloning kit (Invitrogen) according to the manufacturer's instructions. DNA fragments from the pCR-oligonucleotide constructs were amplified and Cy3 labeled using pUC18 universal sequencing primers 5′-AGCGGATAACAATTTCACACAGGA and 5′-GTTTTCCCAGTCAC GAC.

Rapid amplification of 5′ cDNA ends. The rapid amplification of 5′ cDNA ends was performed essentially according to the kit manufacturer's instructions (Roche). Cells were grown in LBmc medium to late logarithmic phase, centrifuged, and frozen in liquid nitrogen. RNA was extracted and purified using the RNeasy purification kit (Qiagen). RNA was then reverse transcribed at 55°C for 1 h using primer 5′-ATCGGTGACCGTGACGATATGG. A homopolymeric A tail was added to the 3′ end of the cDNA, and the cDNA was then amplified by PCR using a poly(T) primer and primer 5′-ATGGTGACCTGGTTCGATGC. The resulting PCR product was cloned using the TOPO cloning kit (Invitrogen) according to the manufacturer's instructions. Multiple positive clones were sequenced, and the transcription start was mapped to the promoter region of *sinI*.

PCR-based mutation of the ExpR binding site upstream of *sinI*. For the mutation of each nucleotide within the ExpR binding site upstream of *sinI*, a series of complementary primers based on the binding site were designed. Within each set of complementary primers, a single nucleotide was replaced. From each set of complementary primers, the forward primer was used together with the universal reverse primer and the reverse primer was used with the universal forward primer using the UpsinI fragment cloned into pUC18 as the template (3). The resulting PCR products were gel purified, combined to allow annealing, and used as a template for a second PCR together with the universal primers to produce a Cy3-labeled fragment containing a mutated nucleotide.

EGFP fluorescence assay. *S. meliloti* strains were first cultivated in liquid LBmc medium. Cell suspensions (20 μl) with an optical density at 600 nm (OD_{600}) of 0.5 were spotted onto LBmc plates supplemented with streptomycin and tetracycline, with or without added oxo-C_{14}-HL, and grown at 30°C for 20 to 22 h. The cells were collected and resuspended in 0.9% NaCl to an OD_{600} of 1.0. The cell density (OD_{600}) and EGFP fluorescence (excitation at 485 nm and emission at 538 nm with a 97% scanning rate) were measured using a Tecan Infinite M200 reader (Tecan Trading AG, Switzerland) using 200 μl of the cell suspension in 96-well microtiter plates.

Analysis of promoter expression using the promoter probe vector. *S. meliloti* strains carrying the promoter probe constructs were grown at 30°C either to late log phase in LBmc liquid medium or overnight on LBmc agar containing 120 μg. ml^{-1} Nm. Cells harvested from LBmc agar were resuspended in 0.9% NaCl. The cell density (OD_{600}) and relative β-galactosidase activity were measured using a Tecan Infinite M200 reader (Tecan Trading AG, Switzerland) using 100 μl of the cell suspension in 96-well microtiter plates. The β-galactosidase activity assay was performed, and relative units (Miller units) were calculated as described previously (35).

EMSA. The EMSA protocol was described previously (3). The Cy3-labeled DNA fragments were mixed with purified His_6-ExpR in a reaction buffer containing approximately 0.1 mg ml^{-1} of sonicated herring testes DNA and 1.0 mg ml^{-1} bovine serum albumin (Sigma) in a final volume of 20 μl of DNA binding buffer (20 mM Tris-HCl [pH 8.0], 5 mM KCl). In the 20-μl reaction mixture, the Cy3-labeled DNA was included at 0.05 pmol; His_6-ExpR was included at 0.1 pmol for the *sinI*-derived fragment and 3.0 pmol for fragments derived from the galactoglucan biosynthesis gene cluster as well as *exsH*- and *exoI*-derived fragments. Oxo-C_{14}-HL was included at 20 pmol. The reaction mixtures were incubated at 24°C for 20 min. Loading buffer (5 μl; 78% glycerol) was added, and the reaction mixtures were loaded onto a 2% agarose gel. Following electrophoresis at 5 V/cm for 2 h, gel images were acquired using a Typhoon 8600 variable-mode imager (Amersham Bioscience, Freiburg, Germany).

RESULTS

Characterizing the UpsinI ExpR binding site. As established in our previous report (3), plasmid pJN105, carrying the His_6-*expR* gene, complemented the nonfunctional *expR* locus in Rm1021, demonstrating that the His_6-ExpR fusion protein functions in vivo. In that study, purified His_6-ExpR was shown to bind to a site within the promoter region of *sinI* based on DNA footprinting. However, the specific nucleotides that are important for the binding of ExpR were unknown. In this study, a total of 35 bp was systematically modified within the *sinI* promoter using a "gene SOEing-type" PCR approach (24). The effect on the relative strength of binding of His_6-ExpR to each DNA fragment that included a modified nucleotide was observed using gel shift assays in the presence of oxo-C_{14}-HL, one of the identified Sin AHLs demonstrated to activate binding between His_6-ExpR and the *sinI* promoter region (3) and previously shown to cause a maximal induction of *sinI* expression in *S. meliloti* (29). Figure 1 shows the resulting shift after mixing His_6-ExpR, the modified Cy3-labeled *sinI* promoter fragment, and oxo-C_{14}-HL. Of the 35 nucleotides that were modified, 12 were found to be important for His_6-ExpR binding. Of these 12 nucleotides, 4 nucleotides, indicated by an increase in size in Fig. 1, were found to be critical for binding, although residual binding remained observable in each case. Also, as indicated in Fig. 1, the effects of four of the mutations on the expression of *sinI* were individually measured using the promoter probe vector containing a fusion of the *sinI* promoter region to *lacZ* in order to compare the in vitro and in vivo results. The selection of mutations was based on the EMSA result, where the *sinI* promoter-carrying mutations at both critical and insignificant locations were included. The first of the mutations (from left to right) corresponds to an insignificant reduction of binding in the EMSA and a reduction in expression to 70% compared to that of the wild type. The second mutation corresponds to a significant reduction in binding in the EMSA and to a reduction in expression to 60%. The third mutation appears to have resulted in an improved binding in the EMSA, although the level of expression was not significantly altered. The remaining mutation corresponds to a significant reduction in binding but not in expression. Under

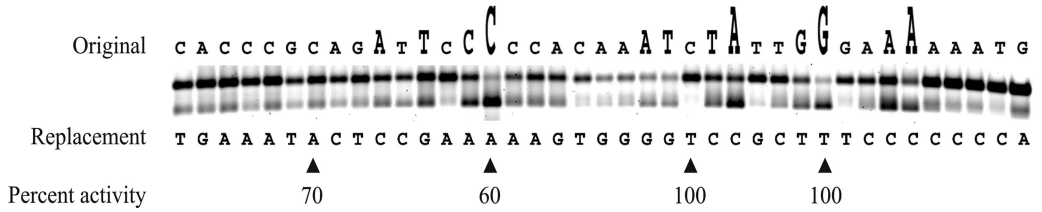

FIG. 1. Mutation-based characterization of the ExpR binding site upstream of *sinI*. Purified His_6-ExpR was mixed with 10 μM oxo-C_{14}-HL and Cy3-labeled UpsinI fragments, each carrying a single point mutation, and the effect of the mutation was determined using gel shift assays. Each shift is a representation of the effect caused by a single modifying nucleotide on ExpR-DNA binding strength. A weak shift indicates a significant disruptive effect caused by the modification, and a strong shift indicates a moderate or negligible effect. The letter above each shift indicates the original nucleotide, and the letter below each shift indicates the modifying nucleotide. The nucleotides very important for ExpR binding are indicated by a large increase in the size of the letter above the shift; nucleotides of lesser importance are indicated by an intermediate increase in size. The resulting shift using the native Cy3-labeled UpsinI fragment (not shown) showed no observable difference from the first five shifts on the left side of the figure. The gel shift experiment was repeated three times. Four UpsinI fragments, each with a single point mutation (indicated by the arrows below), were cloned into the promoter probe vector, in addition to the native UpsinI fragment. Percent activity indicates the β-galactosidase activity that was measured from the mutated fragment in comparison to that from the native fragment.

identical conditions, using both the promoter probe vector (results not shown) and plasmid pLK64, which contains a fusion of the *sinI* promoter region to the EGFP gene (see Fig. 4), the absence of *expR* resulted in a reduction of *sinI* expression to approximately 30% of the levels of a strain carrying a functional *expR*. Thus, it appears that although a high level of *sinI* expression is dependent on the presence of ExpR, a reduction in binding strength between ExpR and the *sinI* promoter, as observed in vitro, does not necessarily correspond to a reduction in the activation of *sinI* expression by ExpR in vivo.

ExpR binds to several promoter regions in the galactoglucan and succinoglycan biosynthesis gene clusters. DNA fragments directly upstream of the galactoglucan biosynthesis genes *wgaA*, *wgdA*, *wgcA*, *wgeA*, and *wggR* (Fig. 2A), representing the promoter regions of the five putative galactoglucan biosynthesis operons, were included in gel shift assays with purified His_6-ExpR. Of these regions, only those from *wgaA* and *wgeA* shared homology with the ExpR binding site upstream of *sinI* (Fig. 2B) and bound to His_6-ExpR in an oxo-C_{14}-HL-enhanced manner (Fig. 2C). Covering the homologous regions within the promoter regions of *wgaA* and *wgeA*, 31 and 34 nucleotides (indicated by the dark gray boxes in Fig. 3), respectively, were cloned into vector pCR. Universal sequencing primers based on the vector were used to amplify and Cy3 label DNA fragments containing the cloned regions and the surrounding vector sequence. In addition, a 33-nucleotide sequence (indicated by a dark gray box in Fig. 3) from the promoter region upstream of *sinI*, covering the ExpR binding site, was also cloned in the same manner and included as a positive control. A Cy3-labeled PCR product from the empty vector was included as a negative control. Figure 2C shows the resulting mobility after mixing His_6-ExpR, the Cy3-labeled fragment, and oxo-C_{14}-HL and confirms that the ExpR binding sites are indeed located within the cloned regions consisting of <35 bp (Fig. 2B) derived from the promoter regions of *sinI*, *wgaA*, and *wgeA*. The identification of a general ExpR binding site consensus allowed the location of other potential ExpR binding sites in the promoter regions of two more genes, *exoI* and *exsH*, both shown to exhibit ExpR/Sin quorum-sensing-dependent expression (19). Unlike the promoter regions of *sinI*, *wgaA*, and *wgeA*, where the His_6-ExpR-induced shifts are maximal in the presence of oxo-C_{14}-HL, the promoter regions of both *exoI* and *exsH* showed a shift in the presence of His_6-ExpR independent of the presence of oxo-C_{14}-HL. Labeled DNA derived from several other gene promoters, including SMc00690, SMc02032, SMc04171, SMa1067, and SMc01116, were included on the basis of homology to the ExpR binding site consensus but did not show a shift under the same conditions (results not shown).

The transcription start for *sinI* was determined by rapid amplification of 5′ cDNA ends and is located 28 bp upstream of the translation start, 37 bp downstream from the middle of the *lux* box, and approximately 80 bp downstream of the ExpR binding site (Fig. 3). The ExpR binding site upstream of *wgaA* is centered approximately 35 bp downstream of the WggR binding site (2), either overlapping or immediately downstream of the distal transcription start, 47 bp upstream of the proximal transcription start (1), and 138 bp upstream of the ATG (Fig. 3). Interestingly, MucR was shown to bind between the WggR and ExpR binding sites or possibly overlap the ExpR binding site (1). The ExpR binding site upstream of *wgeA* is centered approximately 44 bp downstream of the WggR binding site (2), overlapping the distal transcription start, 132 bp upstream of the proximal transcription start (1), and 188 bp upstream of the ATG (Fig. 3). The MucR binding site was once again shown to be located between the WggR and ExpR binding sites (1).

Expression of *sinI* is dependent on *sinR* and is activated by *expR* in the presence of Sin AHLs. In order to characterize the regulation of *sinI* expression, we measured fluorescence in *S. meliloti* strains Rm1021, Rm8530 (Rm1021 $expR^+$), Rm11511 (Rm1021 *sinI*), Rm11527 (Rm8530 *sinI*), Rm1021 *sinR*, and Rm8530 *sinR*, carrying plasmid pLK64, in which the *sinI* promoter region is fused to the EGFP gene. During growth on LBmc agar, the expression of *sinI*-EGFP was activated in the presence of ExpR, since an approximately three-times-higher level of *sinI* expression was measured in Rm8530 ($expR^+$ $sinI^+$ $sinR^+$) than in Rm1021 ($expR$ $sinI^+$ $sinR^+$) (Fig. 4). The activation by ExpR is dependent on the Sin AHLs, since *sinI* expression was reduced in strain Rm11527 ($expR^+$ *sinI*) but restored by the addition of 10 nM oxo-C_{14}-HL to the medium. Activation by ExpR is also completely dependent on SinR, since only background levels of fluorescence could be detected in both Rm8530 *sinR* and Rm1021 *sinR* strains ($expR^+$ $sinI^+$

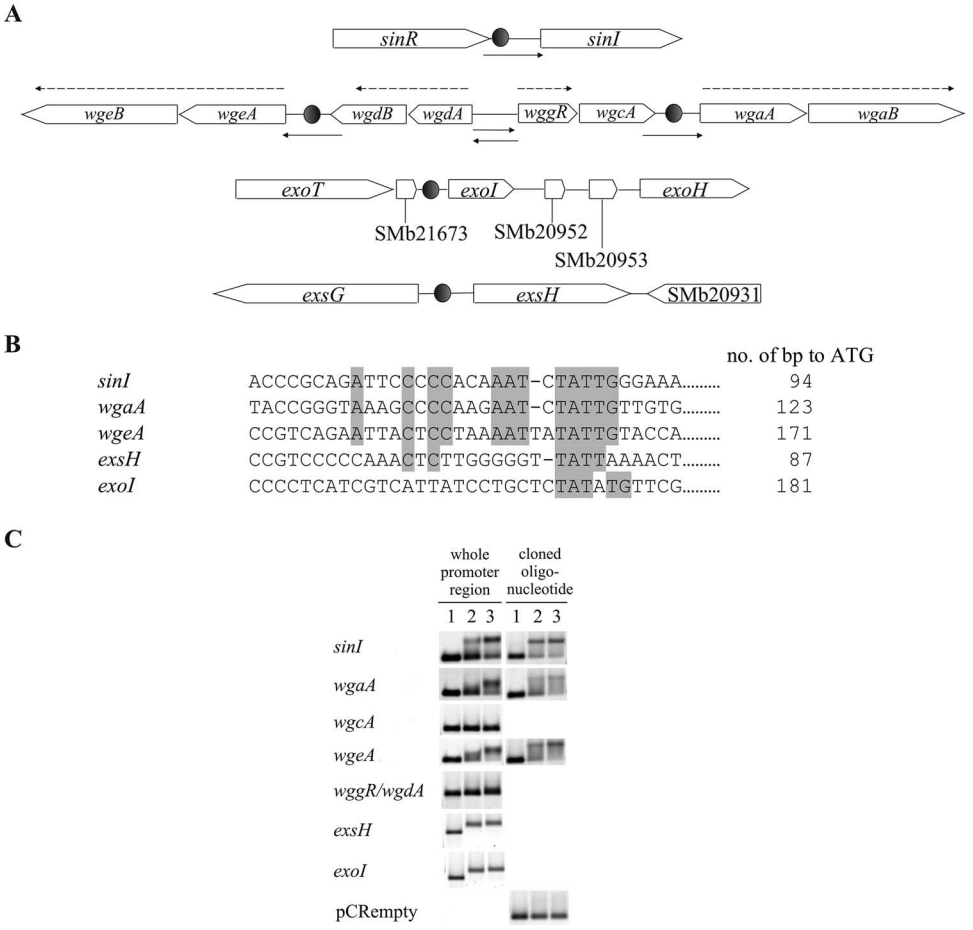

FIG. 2. (A) The galactoglucan biosynthesis gene region from *wgaA* to *wgeB* (*expE2*) (10,330 bp) from the galactoglucan biosynthesis gene cluster of *S. meliloti* (16). Promoter regions used for gel shift assays and determination of expression levels are indicated by solid arrows. ExpR binding sites are indicated by filled circles. Transcriptional units are marked by broken arrows. (B) ExpR binding-site consensus. The identified ExpR binding sites from the promoter regions of *sinI*, *wgaA*, and *wgeA* were aligned in the 5′-to-3′ orientation to show homology. Sequences from the promoter regions of *exsH* and *exoI* exhibiting weak homology to the consensus are included. (C) AHL-enhanced DNA binding of purified His_6-ExpR to the galactoglucan biosynthesis gene promoter regions. (Left) Cy3-labeled promoter regions of *wgaA*, *wgeA*, and *wgdA* and/or *wggR* were used in the gel shift assay. (Right) Oligonucleotides (indicated by the dark gray boxes in Fig. 3) covering the ExpR binding sites within the *sinI*, *wgaA*, and *wgeA* promoters cloned into vector pCR. DNA fragments from the pCR-oligonucleotide constructs were amplified and Cy3 labeled and are included in the gel shift assays, along with a Cy3-labeled fragment from the empty vector as a negative control. For each panel, lane 1 is the Cy3-labeled DNA only, lane 2 is Cy3-labeled DNA mixed with His_6-ExpR, and lane 3 is a mixture of Cy3-labeled DNA, His_6-ExpR, and oxo-C_{14}-HL.

sinI promoter region
GCCCGGGACA GGACCGGCCA CCCGCAGATT CCCCCACAAA TCTATTGGGA AAAAATGAGG
AAATAAACTG TCACTATAGA CAGTTACATG TGTCATCCGA GCCTGACAGC ATCGCTACAT
CGCGTAATCA CGCATGGAGC GAAAAAATG

wgaA promoter region
ATATTGCTTC AATTTTTGAA GTATAAATAC CGCGTAAAGC CCCAAGAATC TATTGTTGTG
CACTGCGGAA TCCACGGATA TGGTTTGCTC CATGAGGTTC TGGACCGCGA ATATTCCCGG
ACCATCATTT TTTCATCTTC TGGATAAATC GATTTATTCT GGGGTCGCTG CGTGCAAGAG
TTGATG

wgeA promoter region
CCTGTCTAGC TCTTGATATC TCTTCTCGTC GCAAATTACT TTAAAATTTG AAGCGCTTTG
CCTGCATTTC CGTCAGAATT ACTCCTAAAA TTATATTGTA CCATATTTGG CACAGCATGG
AGATATGTTT CGGGCACCCT CTTTCTATCA AAATATCGCC GTTTTATTTT ATGCATCTGT
GTTGCGTTCG TAATTATTGC AGTGCACACT CCGGCACGCA ATCCTCGGCT CCGTGCCGAT
GACGCATGCA TCAAACCACG AAGAGGAGAA GCAGATG

FIG. 3. Promoter regions containing ExpR binding sites. Empty boxes indicate WggR binding sites (2), dark gray boxes indicate ExpR binding sites (oligonucleotides cloned into vector pCR), and the light gray box in the *sinI* promoter region indicates the *lux* box. Transcriptional start sites for *wgaA*, *wgeA* (1), and *sinI* are indicated by arrows with a "+1." Translational starts are underlined.

sinR and *expR sinI*$^+$ *sinR*, respectively). The Sin AHLs may be required for the full activity of SinR, although this is unclear, since the positive effect of Sin AHLs on *sinI* expression is only weak in the absence of ExpR (in strain Rm1021 [*expR sinI*$^+$ *sinR*$^+$]), and *sinI* expression was not activated by the addition of oxo-C_{14}-HL to strain Rm11511 (*expR sinI sinR*$^+$). However, SinR was able to promote *sinI* expression to moderate levels even in the absence of ExpR, SinI, or synthetic oxo-C_{14}-HL, since fluorescence levels were higher in Rm11511 (*expR sinI sinR*$^+$) and Rm11527 (*expR*$^+$ *sinI sinR*$^+$) than in Rm1021 *sinR* (*expR sinI*$^+$ *sinR*) and Rm8530 *sinR* (*expR*$^+$ *sinI*$^+$ *sinR*).

ExpR-activated expression of galactoglucan biosynthesis genes is dependent on WggR. The promoter fragments of galactoglucan biosynthesis operons, including *wga*, *wgd*, *wge*, and *wggR*, were cloned into promoter probe vector pSRPP18, a mobilizable suicide vector carrying a promoterless *lacZ* gene (1). Following the integration of the plasmid into the genome

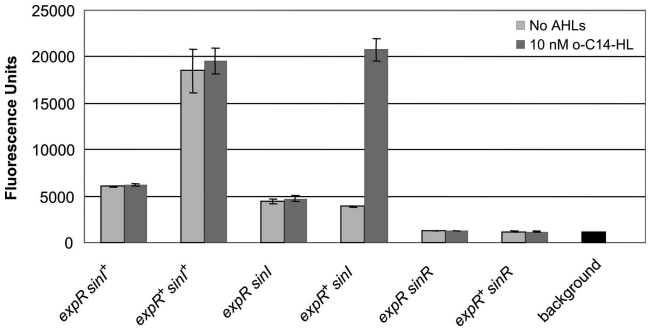

FIG. 4. Expression of *sinI* is dependent on *sinR* and is induced by *expR* and Sin AHLs. s*inI* expression was measured as EGFP fluorescence in strains carrying plasmid pLK64 in the absence (dark gray bars) and presence (light bars) of synthetic oxo-C_{14}-HL (abbreviated as o-C_{14}-HL). Background fluorescence was determined in Rm1021 devoid of plasmid pLK64 and is shown at the far right of the graph. Strains are included as follows: lane 1, Rm1021 (*sinI*$^+$ *expR*); lane 2, Rm8530 (*sinI*$^+$ *expR*$^+$); lane 3, Rm11511 (*sinI expR*); lane 4, Rm11527 (*sinI expR*$^+$); lane 5, Rm1021 *sinR* (*sinR sinI*$^+$ *expR*); lane 6, Rm8530 *sinR* (*sinR sinI*$^+$ *expR*$^+$).

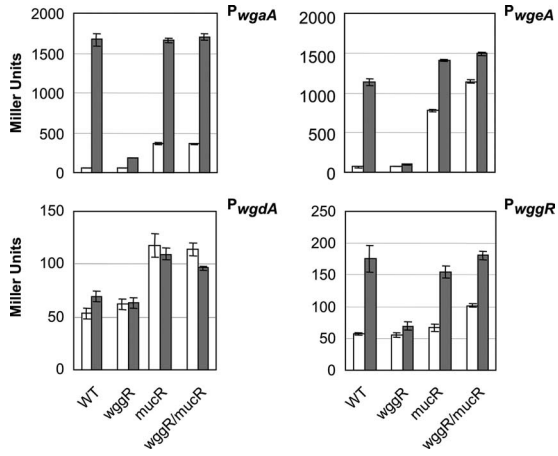

FIG. 5. Effect of ExpR on expression of promoters of galactoglucan biosynthesis genes. The promoter regions of *wgaA*, *wgdA*, *wgeA*, and *wggR* were fused to *lacZ* via the promoter probe vector (1), and the resulting LacZ activity was measured in the absence (pJN105) (open boxes) and presence (pJNexpR) (gray boxes) of ExpR and various *wggR* and *mucR* backgrounds after growth in LBmc liquid medium to late log phase. LacZ activity was also measured in strains containing the empty promoter probe vector (without an inserted promoter) and was approximately 50 ± 5 Miller units (not shown).

by a single homologous recombination event downstream of *exoP*, the promoter strength, indicated by LacZ activity, was measured in four genetic backgrounds: *wggR*$^+$ *mucR*$^+$ *expR* (Rm2011), *wggR mucR*$^+$ *expR* (SmSRΔG), *wggR*$^+$ *mucR expR* (Rm101), and *wggR mucR expR* (SmBBΔG101) (Fig. 5). The effects of a functional *expR* locus on the promoter fragments were measured in each of the four genetic backgrounds using either pJNexpR, which was constructed as previously described (3), or empty vector pJN105 as a negative control. During growth on LBmc agar, the presence of a functional *expR* resulted in a mucoid phenotype that is easily distinguishable from the "dry" phenotype of strains without *expR*, as was previously reported (32). Consistent with this observation, a functional *expR* resulted in a dramatic increase in the activation of the *wgaA* promoter (28 times) and the *wgeA* promoter (17 times) but much lower activation of the *wggR* promoter (2.7 times). The presence of *expR* did not result in a significant increase in activity of the *wgdA* promoter. The putative promoter for *wgcA* was also measured but did not show any activity in any of our experiments (results not shown). In a *wggR* background, this ExpR-dependent activation is markedly reduced for the *wgaA*, *wgeA*, and *wggR* promoters, suggesting that ExpR is dependent on the presence of WggR for most of the activation of these promoters but that a low level of activation by ExpR is also present in the absence of WggR.

Compared to the wild type in the absence of *expR*, the effect of a disruption of *mucR* was an increase in the levels of expression of *wgaA* (six times), *wgdA* (2.2 times), and *wgeA* (11 times), consistent with MucR as a repressor for the galactoglucan biosynthesis genes, as was reported previously (1, 26, 43). No significant change in *wggR* expression in the absence of *mucR* was observed under our conditions. In previous reports, however, the increase in expression of the galactoglucan biosynthesis genes due to the disruption of *mucR* was higher. This discrepancy is probably due to the use of different growth media. In strains carrying homogenote fusions of *lacZ* to these genes, growth in minimal medium supplemented with 2 mM phosphate showed a 10-times increase in the level of expression of *wggR* and a 40-times increase in the level of expression of *wgdA* due to the disruption of *mucR* (43). A disruption of *mucR* in strains carrying the identical promoter probe constructs used in this study resulted in a four-times increase in the levels of expression of both *wgdA* and *wggR* during growth in minimal medium supplemented with 2 mM phosphate (1).

In a *mucR* background, the effect of the presence of *expR* was again a very high level of expression of *wgaA* (28 times), similar to that in a *mucR*$^+$ *expR*$^+$ background, while that of *wgeA* was increased (21 times) by approximately 20% compared to that in the *mucR*$^+$ *expR*$^+$ background. In this case, it appears that MucR slightly negatively regulates the *wgeA* promoter in the presence of *expR* but not the *wgaA* promoter under the same conditions. The expression of *wgdA* was unchanged by the presence of *expR* in a *mucR* background, while that of *wggR* was slightly increased (2.7 times).

The effect of disrupting both *wggR* and *mucR* (*wggR mucR*) was an increased level of expression of both *wgeA* and *wggR* (~50%) compared to that in a *wggR*$^+$ *mucR* background but not for *wgaA* or *wgdA*. This result suggests that in the absence of MucR, WggR appears to function as a negative regulator, as was reported previously (1). With the addition of *expR* to a *wggR mucR* background, we observed the same high levels of expression of *wgaA* as in the *expR*$^+$ *wggR*$^+$ *mucR*$^+$ and *expR*$^+$ *wggR*$^+$ *mucR* backgrounds, an even higher level of expression of *wgeA* (30% higher than levels of expression in a *wggR mucR* background), no significant change in the levels of expression of *wgdA*, and a slight increase (1.7 times) in the level of expression of *wggR*, implying that in the absence of MucR, WggR is not required for the positive effect of ExpR.

DISCUSSION

Previous studies have shown that the Sin quorum-sensing system in *S. meliloti* consists of SinI, the AHL synthase, and

SinR, a LuxR-type regulator (33). The genes for both proteins are located at the *sinR-sinI* locus, in which *sinI* is located downstream of *sinR*, separated by a 157-bp intergenic region. This intergenic region contains a binding site for ExpR, another LuxR-type regulator, and a so-called *lux* box, a 16-bp sequence identified on the basis of its homology with the binding sites of LuxR-type regulators from *Vibrio fischeri* (9) and *Agrobacterium tumefaciens* (3, 52). *lux* boxes are typically located upstream of the promoters of its target genes and serve as recognition sites for the LuxR-type regulators upon activation by an AHL (12, 46). In this investigation, we confirm that the expression of *sinI* is positively regulated by *expR* in the presence of Sin AHLs and *sinR*, as was previously reported, and that ExpR does not bind to the *lux* box but rather binds to a 24-bp binding site located 65 bp upstream of the *lux* box and 100 bp upstream of *sinI* (3). A DNA binding site for SinR has not yet been identified, but it is possible that the *lux* box serves as the binding site for SinR in regulating *sinI* expression. We have identified the *sinI* transcription start and show that the −35 region of the *sinI* promoter is covered by the *lux* box, consistent with the idea that the *lux* box is important for the initiation of *sinI* transcription. However, the exact mode of interaction between the *lux* box, SinR, and ExpR in the activation of *sinI* expression remains to be elucidated.

We also show that the activation of *sinI* is absolutely dependent on *sinR*, since the expression of *sinI* was not detectable in the *sinR* mutant strains independent of the presence or absence of *expR* or Sin AHLs. Interestingly, *sinR* promoted *sinI* expression to a moderate constitutive level even in the absence of *expR* or *sinI*. This finding is intriguing because it indicates that either SinR can be activated by another *S. meliloti* quorum-sensing system or SinR does not require AHLs for this moderate induction of *sinI* transcription. Perhaps this aspect of the Sin system serves to guarantee a basic Sin AHL-independent expression of *sinI* at low cell densities that is required for a prompt response to quorum-sensing induction. We also note that *expR* and Sin AHLs, together with *sinR*, mediate a high level of expression of *sinI*. Thus, while the Sin system is not dependent on *expR*, it is probable that the AHL-activated ExpR serves to ensure the appropriate Sin quorum-sensing response to increasing cell density and that in the absence of ExpR, the Sin system is restricted to a weaker and slower quorum-sensing response.

The Sin system has been shown to be involved in the regulation of galactoglucan production. The disruption of the autoinducer synthase gene *sinI* abolished galactoglucan production, resulting in a nonmucoid phenotype on phosphate-rich medium (32). Furthermore, the levels of expression of several of the galactoglucan biosynthesis genes, including *wgcA*, *wgeB* (*expE2*), and *wgdA*, were dramatically reduced during log-phase growth in low-phosphate medium upon the disruption of *sinI* but were restored by the addition of AHLs from extracts from either the wild-type strain or one of the previously identified Sin-specified AHLs, synthetic palmitoleyl HL ($C_{16:1}$-HL) (32, 33). In addition to the Sin system, ExpR is also involved in the regulation of galactoglucan production. Several studies have shown that the Sin effect on galactoglucan production requires a functional ExpR (32, 38). However, the details of the regulation of galactoglucan production by the Sin-ExpR system have remained an enigma. Although both genome and proteome approaches have shown the Sin-ExpR system to be involved in the regulation of a multitude of other genes throughout the genome (17, 23), the question of whether ExpR is acting directly or indirectly on its target genes has been unanswered and was one of the specific questions addressed in this investigation. As mentioned above, the single ExpR binding site upstream of *sinI* was described previously (3). However, while the effect of an active ExpR results in an increase in the level of expression of *sinI*, such an effect alone is insufficient to explain the dramatic increase in the level of galactoglucan production and the genome-wide changes in gene expression. We postulated that either ExpR binds directly to the promoters of its target genes or some other regulatory intermediate controlled by the Sin-ExpR system is binding to sites within the promoters. The discovery of additional AHL-dependent ExpR binding sites in the promoters of the galactoglucan biosynthesis operons in this investigation confirms our assumptions and allows a better understanding as to precisely how the Sin-ExpR system regulates galactoglucan production. Furthermore, a characterization of the ExpR binding site upstream of *sinI*, together with a comparison of this site to binding sites in the *wgaA* and *wgeA* promoters, allows a homology-based search for other ExpR binding sites that show Sin-ExpR-dependent regulation, as revealed by previous studies involving transcription profiles (23). Accordingly, the detection of ExpR binding sites in the promoter regions of two additional genes, *exoI* and *exsH*, demonstrates the success of using this approach. *exoI* codes for a predicted periplasmic protein of unknown function that is not required for succinoglycan production (5, 20), and *exsH* codes for an endo-1,3-1,4-β-glycanase which depolymerizes high-molecular-weight succinoglycan (49) to produce symbiotically active low-molecular-weight succinoglycan (48). Both were previously shown to be upregulated in response to Sin-ExpR regulation (19, 23).

The binding of His_6-ExpR to the promoter regions of *sinI*, *wgaA*, *wgeA*, *exoI*, and *exsH* is specific, since under the same conditions, His_6-ExpR did not bind to the *wgdA*, *wggR*, or *wgcA* promoter regions, nor did it bind to several other promoters that contained some homology to the ExpR consensus binding sequence. The low level of transcription activity measured from the *wgdA*, *wggR*, and putative *wgcA* promoters appeared to support this conclusion. Interestingly, a comparison of the ExpR binding sites revealed a consensus sequence unlike the *lux* box observed in *V. fischeri* (45, 46) or the *tra* box in *A. tumefaciens* (15, 30, 52). The ExpR binding site upstream of *sinI* contains a low level of similarity with the sites in the *wgaA* and *wgeA* promoters and an even lower level of similarity with those in the *exoI* and *exsH* promoters, suggesting that ExpR operates quite differently from the other LuxR-type regulators. This observation is also consistent with the solubility and stability of ExpR in the absence of AHLs, in contrast to other LuxR-type regulators such as TraR and LuxR (40, 47, 52).

We have also noted that ExpR is capable of various levels of binding to its target sites in the absence of AHLs in the gel shift experiments, particularly for the *exsH* and *exoI* promoter regions. Although the possibility that this is due to suboptimal conditions for the gel shift assays cannot be ruled out, another possibility is that this phenomenon occurs in vivo and plays a role in the regulation of the expression of these promoters. It appears that the interaction between the AHL and ExpR not

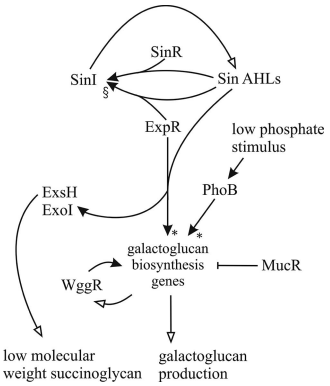

FIG. 6. Model of regulation of galactoglucan production in *S. meliloti*. Empty arrows indicate synthesis. Filled arrows indicate regulation, with "↑" and "⊤" indicating positive and negative regulation, respectively. Dependency in regulation is indicated beside the filled arrows: "§" indicates a dependency on SinR, where the positive regulation of *sinI* by ExpR and Sin AHLs is dependent on the presence of SinR, and "∗" indicates a dependency on WggR, where the positive regulation of the galactoglucan biosynthesis genes by ExpR and Sin AHLs and by PhoB is dependent on the presence of WggR (1).

only enhances the strength of binding of ExpR to the DNA site, as was observed previously (3), but also provides the necessary configuration of an ExpR-DNA complex to allow the RNA polymerase to bind, since the positive regulation of these promoters by ExpR occurs only in the presence of AHLs. However, ExpR binding to a subset of its target sites in the absence of AHLs may negatively regulate the transcription of the downstream gene, serving as another level of control of transcription at low cell densities. In support of this concept, succinoglycan production was recently shown to be reduced in a *sinI expR*$^+$ strain but not in any of the *sinI*$^+$ *expR*$^+$, *sinI*$^+$ *expR*, or *sinI expR* strains, consistent with a positive regulation by Sin-ExpR and a negative regulatory effect by ExpR in the absence of the Sin AHLs (19).

Knowledge of the regulation of galactoglucan production in *S. meliloti* was recently updated (1). The promoters of the *wga*, *wgd*, and *wge* operons were characterized with respect to transcription starts and the effect of three regulators, PhoB, WggR, and MucR, on the transcription of these promoters during growth in media containing both low and high phosphate concentrations (1). A model illustrating the complex regulation of galactoglucan production by these transcription regulators together with their DNA binding sites within the promoter regions and the distal and proximal transcription starts was presented. The Sin-ExpR quorum-sensing effect presented in this study can be added as yet another level of regulation of galactoglucan production, as shown in Fig. 6. Interestingly, the location of the ExpR binding sites within the *wgaA* and *wgeA* promoters almost immediately downstream of the proposed PhoB and WggR binding sites and possibly overlapping the MucR binding sites suggests some sort of cooperation and/or competition among the four regulators. Activation by ExpR is almost completely dependent on the presence of WggR when MucR is present, but in the absence of MucR, WggR is no longer required for the activation by ExpR. This WggR-dependent effect is also seen with PhoB-dependent induction under conditions of phosphate starvation (1) and suggests that WggR functions as a general mediator of galactoglucan biosynthesis gene expression for at least two regulators under different conditions. The close proximity of the PhoB, WggR, MucR, and ExpR binding sites suggests that there exists a complex regulation of the galactoglucan biosynthesis genes involving protein-protein interactions and, possibly, some competition for the DNA target sites. Furthermore, the ExpR binding sites overlap the distal transcription starts of *wgaA* and *wgeA*, suggesting that ExpR regulates both *wgaA* and *wgeA* by inhibiting the distal promoters and activating the proximal promoters, although this has not been confirmed.

No binding between the *wggR* promoter region and ExpR was observed in the gel shift assays despite the presence of a low level of activation of expression from the *wggR* promoter due to the presence of *expR*. One possibility is that our gel shift assay conditions are not optimal enough to detect a weak interaction between the *wggR* promoter and ExpR, although we consider this to be unlikely, since, as mentioned above, no homology to the known ExpR binding sites upstream of *wggR* was detected. Perhaps more likely is that the low level of activation of *wggR* expression by ExpR is indirect, i.e., through another transcription regulator that is yet to be discovered. Yet another possibility is that the binding strength of ExpR is dependent on variations in the structure of the AHL molecule, as was demonstrated previously for the interaction between ExpR and its binding site upstream of *sinI* (3). That study found that ExpR was activated by a range of AHLs and that, depending on the structure of the acyl chain, the strength of binding between ExpR and DNA varied. The most critical attribute was the number of carbons in the acyl chain, where more than 12 carbons resulted in a dramatic increase in ExpR activation. In this study, we have used one of the functional Sin AHLs, oxo-C_{14}-HL, which was previously shown to cause a maximal induction of *sinI* expression in *S. meliloti* (29). It remains to be seen how the other AHLs affect the strength of binding between ExpR and its target promoter regions and the resulting transcription activity from these promoters.

As noted above, galactoglucan can be produced either upon a "low-phosphate" stimulation of the galactoglucan biosynthesis genes or through the Sin-ExpR quorum-sensing system. However, only quorum-sensing stimulation results in low-molecular-weight galactoglucan that is important for symbiosis (34, 38, 43, 51). Under phosphate-limiting conditions, the *wga*, *wgd*, and *wge* operons were all stimulated at comparable levels through positive regulation by WggR and PhoB (1). Under quorum-sensing regulation, however, only those of *wgaA* and *wgeA* were dramatically upregulated in the presence of both ExpR and WggR. One possibility is that this strong upregulation of one or more of the products from the *wga* and/or *wge* operon is responsible for the production of low-molecular-weight galactoglucan. Alternatively, ExpR may directly regulate the transcription of a gene encoding a hydrolase that results in the generation of low-molecular-weight galactoglucan. The detection of an ExpR binding site upstream of *exsH*, a succinoglycan glucanase, leading to the generation of low-molecular-weight succinoglycan (19), lends support to this hypothesis. As yet another alternative, ExpR or one or more of the products from the open reading frames within the *S. meliloti* genome that are predicted to encode LuxR homologs (SMc00658, SMc00877, SMc00878, and SMc04032) are re-

sponsible for the production of symbiotically active galactoglucan by the regulation of other genes via DNA binding sites that remain to be identified. Further investigations into the role of quorum sensing in the regulation of various cell processes in *S. meliloti*, including the production of exopolysaccharides, will allow a greater understanding of the complex formation of symbiosis.

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