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A LuxR Homolog Controls Production of Symbiotically Active Extracellular Polysaccharide II by *Sinorhizobium meliloti*

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Production of complex extracellular polysaccharides (EPSs) by the nitrogen-fixing soil bacterium *Sinorhizobium meliloti* is required for efficient invasion of root nodules on the host plant alfalfa. Any one of three *S. meliloti* polysaccharides, succinoglycan, EPS II, or K antigen, can mediate infection thread initiation and extension (root nodule invasion) on alfalfa. Of these three polysaccharides, the only symbiotically active polysaccharide produced by *S. meliloti* wild-type strain Rm1021 is succinoglycan. The *expR101* mutation is required to turn on production of symbiotically active forms of EPS II in strain Rm1021. In this study, we have determined the nature of the *expR101* mutation in *S. meliloti*. The *expR101* mutation, a spontaneous dominant mutation, results from precise, reading frame-restoring excision of an insertion sequence from the coding region of *expR*, a gene whose predicted protein product is highly homologous to the *Rhizobium leguminosarum* bv. viciae RhiR protein and a number of other homologs of *Vibrio fischeri* LuxR that function as receptors for *N*-acylhomoserine lactones (AHLs) in quorum-sensing regulation of gene expression. *S. meliloti* ExpR activates transcription of genes involved in EPS II production in a density-dependent fashion, and it does so at much lower cell densities than many quorum-sensing systems. High-pressure liquid chromatographic fractionation of *S. meliloti* culture filtrate extracts revealed at least three peaks with AHL activity, one of which activated ExpR-dependent expression of the *expE* operon.

The soil bacterium *Sinorhizobium meliloti* fixes atmospheric dinitrogen to ammonia in symbiotic association with the host plant *Medicago sativa* (alfalfa). A successful symbiosis is the result of a complex series of interactions between the host and the symbiont (10, 18, 48, 55, 78). A broad range of plant compounds can function to influence the production of Nod factors by *S. meliloti*. Nod factors stimulate root hair curling and root nodule formation. *S. meliloti* cells colonize curled root hairs and invade developing root nodules via extended invaginations of the root hair cell membrane called infection threads. The *S. meliloti* cells are individually surrounded by host cell membrane and released into the host nodule cells, where they differentiate into bacteroids, the nitrogen-fixing form of the bacteria.

Extracellular polysaccharides (EPSs) produced by *S. meliloti* are crucial for establishing a successful nitrogen-fixing symbiosis with alfalfa. *S. meliloti* mutants that are unable to produce symbiotically active polysaccharides are defective in nodule invasion and primarily induce the formation of symbiotically ineffective root nodules that are devoid of bacteria and bacteroids (34, 45, 62). Any one of three *S. meliloti* polysaccharides, succinoglycan, EPS II, or K antigen, can mediate alfalfa root nodule invasion. By using green fluorescent protein-expressing *S. meliloti* strains, we have recently demonstrated that each of these three polysaccharides functions to mediate infection thread initiation and extension on alfalfa (16, 56). However, under laboratory conditions, there are quantitative and qualitative differences in the manners in which succinoglycan, EPS II, and K-antigen function (56). This suggests that certain polysaccharides are able to function more efficiently under different conditions, thus providing strains that produce multiple polysaccharides with a selective symbiotic advantage under variable conditions.

Succinoglycan, EPS II, and K antigen are structurally diverse polysaccharides. The succinoglycan repeating unit is composed of one galactose and seven glucose residues with pyruvyl, acetyl, and succinyl modifications (2, 61). EPS II has a galactoglucan repeating unit modified with acetyl and pyruvyl moieties (34, 39). The K-antigen repeating unit is a disaccharide containing glucuronic acid and 5,7-diamino-3,5,7,9-tetradeoxynonulosonic acid (63). Despite the structural diversity of these three polysaccharides, genetic and biochemical evidence strongly suggests that particular low-molecular-weight forms of each polysaccharide are the symbiotically active species (i.e., the forms able to promote nodule invasion) (6, 35, 64, 77, 81).

Under nonstarvation conditions, wild-type laboratory *S. meliloti* strain Rm1021, whose genome has recently been sequenced (4, 11, 27, 32), produces measurable quantities of succinoglycan but does not produce detectable EPS II and does not make symbiotically active K antigen. EPS II production in Rm1021 can be induced by the *expR101* mutation (34), by a null allele of the *mucR* gene (42, 88), or by growth under very low phosphate conditions (89). However, only the *expR101* mutation, a spontaneous mutation in Rm1021 that results in mucoid colony morphology, stimulates production of

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symbiotically active EPS II. Both the *mucR*::Tn*5* mutation and growth under very low phosphate conditions result in production of high-molecular-weight EPS II, a form that is symbiotically inactive (36, 52), whereas *expR101*-induced EPS II ranges from a high molecular weight to a low molecular weight and includes EPS II molecules with 15 to 20 disaccharide repeating units, the fraction active in promoting nodule invasion (36).

To date, molecular analysis of the genetics of EPS II production has revealed a cluster of genes (the *exp* gene cluster) required for EPS II production (34). The *exp* gene cluster has been sequenced (7), revealing 21 *exp* genes that are organized into five operons, including the *expA* (9 genes), *expC* (1 gene), *expD* (2 genes), *expE* (8 genes), and *expG* (1 gene) transcriptional units. However, among the predicted *exp* gene products are several proteins whose roles in EPS II production are not easily explained. Furthermore, there are more predicted glycosyltransferases (five) present than might have been expected to synthesize a polysaccharide with only two sugars in the repeating unit. Thus, it is unclear exactly what roles these multiple *exp* gene products have in the synthesis of EPS II. In addition, it is not clear how the molecular weight distribution of EPS II is controlled and which gene products are responsible for adding the pyruvyl and acetyl modifications to the EPS II galactoglucan backbone, as genes predicted to encode these functions appear to be absent. A regulatory gene, *mucR*, has also been subjected to molecular analysis. MucR is 80% identical to the Ros protein from *Agrobacterium tumefaciens* and is a negative regulator of EPS II production and a positive regulator of succinoglycan production in *S. meliloti* (42).

In a previous study, the *expR101* locus was mapped to the ~3.4-Mb *S. meliloti* main chromosome (34). Cotransduction analyses showed that the *expR* locus was ~66% transductionally linked to one allele of the *ndvB* gene and ~7% transductionally linked to the *trp-33* locus (33). However, prior to this work, the nature of the *expR101* mutation had not been elucidated. In order to gain insight into how the production of symbiotically active EPS II by Rm1021 is controlled, we have cloned and characterized the *expR101* mutation.

We have found that the production of symbiotically active EPS II in *S. meliloti* strain Rm1021 is dependent on the presence of a functional copy of the *expR* gene. The predicted *expR* gene product is a member of the LuxR family of proteins, many of which are receptors for *N*-acylhomoserine lactones (AHLs) and are transcriptional regulators involved in the control of gene expression in response to changes in population density, a process known as quorum sensing (30, 84). ExpR controls the transcription of the *exp* genes (and hence production of symbiotically active EPS II) in a density-dependent manner. Compared to many other LuxR family systems, ExpR-dependent activation of *exp* transcription is induced at relatively low cell densities.

TABLE 1. *S. meliloti* strains used in this study

Strain	Genotype	Reference
Rm1021	SU47 *str-21 expR102*::IS*Rm*2011-1	51
Rm7210	Rm1021 *exoY210*::Tn*5*	45
Rm8530	Rm1021 $expR^+$ (formerly *expR101*)	34
Rm10006	Rm1021 $expR^+$ ΩTn*5-233* #3–15	34
Rm9000	Rm1021 $expR^+$ *exoY210*::Tn*5*	35
Rm10002	Rm1021 *expA3*::Tn*5*	34
Rm9025	Rm1021 *expR103*::*lacZ*-Gm	This study
Rm9026	Rm8530 *expR103*::*lacZ*-Gm *exoY210*::Tn*5*	This study
Rm9028	Rm8530 *expR103*::*lacZ*-Gm	This study
RmAR1014	Rm2011 *expA1*::*lacZ*-Gm	7
RmAR1016	Rm2011 *expC*::*lacZ*-Gm	7
RmAR1018	Rm2011 *expG*::*lacZ*-Gm	7
RmAR1020	Rm2011 *expD1*::*lacZ*-Gm	7
RmAR1022	Rm2011 *expE2*::*lacZ*-Gm	7
Rm9030-2	Rm1021 $expR^+$ *expA1*::*lacZ*-Gm	This study
Rm9031	Rm1021 $expR^+$ *expC*::*lacZ*-Gm	This study
Rm9034	Rm1021 $expR^+$ *expG*::*lacZ*-Gm	This study
Rm9032	Rm1021 $expR^+$ *expD1*::*lacZ*-Gm	This study
Rm9033	Rm1021 $expR^+$ *expE2*::*lacZ*-Gm	This study

MATERIALS AND METHODS

Bacterial strains and culture media. The *S. meliloti* strains used in this study are listed in Table 1. Strains were grown at either 30°C (*S. meliloti*) or 37°C (*Escherichia coli*) in LBMC medium (Luria-Bertani [LB] [70] liquid or agar supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$) or in TYC medium (TY [9] liquid or agar supplemented with 12 mM $CaCl_2$). In the conditioned-medium experiments, LBMC medium was buffered with 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) to a pH of 6.75 (LBMCP). Antibiotics were used at the following concentrations: streptomycin, 500 μg/ml; neomycin, 200 μg/ml; gentamicin, 50 μg/ml for *S. meliloti* and 5 μg/ml for *E. coli*; spectinomycin, 100 μg/ml; tetracycline, 10 μg/ml; kanamycin, 25 μg/ml.

For AHL extraction, *S. meliloti* strain Rm1021 was grown in a defined NM salts medium (65) supplemented with 0.028 M glucose and Gotz vitamins and adjusted to a pH between 6.6 and 6.8. To prevent precipitation, $MgSO_4 \cdot 7H_2O$, $CaCl_2$, and $FeSO_4$-citric acid stocks were autoclaved separately. Extracts were prepared from the defined-medium-grown culture to facilitate future chromatographic studies and AHL identification.

The AHL reporter strains used were *E. coli* HB101/pSB401 (*luxRI*′::*luxBCDAE*) (85), *E. coli* HB101/pSB1075 (*lasRI*′::*luxBCDAE*) (85), *E. coli* HB101/pSB536 (*ahyRI*′::*luxBCDAE*) (75), *Chromobacterium violaceum* CV026 (50), and *Pseudomonas aureofaciens* 30-84I (86). These strains were maintained as frozen glycerol stocks and subcultured into the appropriate media as necessary. For the AHL bioassays, luminescent *E. coli* reporter strains were first grown overnight in LB broth with appropriate antibiotics. These cultures were then diluted 100-fold in fresh LB, cultured for 2 h, again diluted 100-fold in fresh LB, and then allowed to grow to an optical density at 600 nm (OD_{600}) of 0.2. The culture was then spun down, and the pellet was resuspended in 10 volumes of fresh LB and vortexed for the bioassays as described below.

Alfalfa nodulation assays. Alfalfa nodulation assays were performed as previously described (45). Each plant was inoculated with 1 ml of a cell suspension with an OD_{600} of 0.05. Plants were scored 4 weeks after inoculation for foliage condition, plant height, and the presence of pink, nitrogen-fixing nodules.

Molecular cloning of the *expR101* mutation. The molecular cloning of the *expR101* mutation was accomplished by preparing genomic DNA (3) from *S. meliloti* strain Rm10006 (Rm1021 *expR101* ΩTn*5-233* #3-15) (34), partially restricting the genomic DNA with *Eco*RI (New England Biolabs, Beverly, Mass.), and ligating 20- to 35-kb genomic DNA fragments to *Eco*RI-restricted pLAFR1 (29) by using T4 DNA ligase (New England Biolabs). The resultant recombinant cosmid DNA was delivered into *E. coli* strain HB101 (Gibco BRL, Gaithersburg, Md.) with the Gigapack III-XL kit (Stratagene, La Jolla, Calif.). Tetracycline-resistant HB101 cells (those carrying recombinant cosmids) were screened for the ability to confer resistance to spectinomycin and kanamycin, the antibiotic resistance markers located on Tn*5-233* (19) that function in *E. coli*. The insertion target for ΩTn*5-233* #3-15 was found to be the 9-bp sequence including nucleotides 3997 to 4105 in the *ndvB* open reading frame (ORF) (nucleotides 4229 to 4237 in the total published sequence [40]). This *ndvB* allele confers the expected symbiotic defect (22) but not a hypoosmotic adaptation defect (21) (data not shown).

Diagnostic PCR analysis of the *expR* region and DNA sequencing. Both strands of the *expR* region were sequenced with two templates, pBSKSII+ (Stratagene) subclones of the *expR* region and PCR products generated from genomic DNA templates with oligonucleotide primers (Gibco BRL) specific to the *expR* region. DNA sequencing was performed by the Molecular Biology Core Facility at Dartmouth College with reaction products generated by following the recommended reaction and purification protocol.

The two primers used to amplify the *expR* region both for DNA sequencing

and for the diagnostic PCR were Rm*ndvA*5′out (5′-GCGAGGAGATCCTGCCCGAG-3′) and Rm*pyc*5′out (5′-AGAGTGGCGTGAACATTCGG-3′). We used 2.5 U of *Pfu* enzyme (Stratagene) and the manufacturer's recommended buffer conditions. The template consisted of 1 μl of a cell suspension consisting of a small scoop of cells from a 10% dimethyl sulfoxide frozen permanent strain stock suspended in 100 μl of water. Primers were used at a concentration of 1 μM, and deoxynucleoside triphosphates (Pharmacia Biotech, Piscataway, N.J.) were used at a concentration of 200 μM. The PCR program used was as follows: (i) 95°C for 5 min, (ii) 94°C for 30 s, (iii) 65°C for 30 s, (iv) 72°C for 5 min, (v) go to step ii 29 times, (vi) hold at 4°C.

Construction of an *expR*-null allele and a *lacZ* transcriptional fusion. The genomic *expR*-null allele and transcriptional fusion was generated as follows. A 2.2-kb PCR product with an *Xba*I restriction site engineered at the *expR* 5′ end was generated with the *expR101* locus from Rm8530 genomic DNA as the template and the primer and deoxynucleoside triphosphate concentrations listed above. The manufacturer's recommended buffer and amplification conditions for Platinum *Taq* Hifi (Gibco BRL) were used. The PCR product generated was purified with a PCR purification kit (Qiagen, Valencia, Calif.). Following restriction with *Xba*I and *Hin*dIII, the PCR product was ligated to *Xba*I/*Hin*dIII-restricted pK19*mob*GII (41) with T4 DNA ligase (New England Biolabs), generating pK19*expR*. The knockout construct was generated by inserting a 4.0-kb *lacZ*-Gm *Sph*I cassette from pAB2001 (8) into the unique *Sph*I site in the *expR* ORF on pK19*expR* and screening for an insertion with the *lacZ*-Gm cassette in the proper orientation to generate a transcriptional fusion. The resultant knockout plasmid, pK19*expR*::*lac*Z-Gm, was mobilized into Rm8530, and homologous recombinant candidates were identified by gentamicin resistance, kanamycin sensitivity, and white color on plates containing 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid. The *expR*::*lacZ*-Gm allele was transduced into Rm8530 (generating Rm9028) or Rm1021 (generating Rm9025) before use in subsequent assays. This analysis verified that the expected colony morphology phenotype cotransduced with the antibiotic resistance marker. The proper insertion of the *expR*::*lacZ*-Gm allele in the *S. meliloti* genome was confirmed by both PCR analysis and Southern blot analysis.

Effects of conditioned medium and culture filtrate extracts. *S. meliloti* cultures grown in unbuffered LBMC medium have a basic terminal pH of ~8. Because AHL molecules are unstable at a basic pH (71), conditioned medium was buffered with 50 mM PIPES (pH 6.75). Conditioned medium was generated by growing cultures of Rm1021 to an OD_{600} of ~3.0 in LBMCP medium and then filter sterilizing the culture supernatant. Before use in quorum-sensing experiments, conditioned LBMCP medium was supplemented with 0.05 volume of a filter-sterilized solution containing 100 g of Bacto Tryptone per liter and 50 g of yeast extract per liter. Addition of this concentrate to fresh LBMCP medium did not influence the transcriptional activity of our fusions. Cells from mid-log-phase cultures (OD_{600} between 0.6 and 0.8) were diluted to an OD_{600} of 0.002 and allowed to grow for 8 h in either fresh LBMCP medium, conditioned LBMCP medium, or LBMCP medium supplemented with dilutions of culture filtrate extract. After the growth period, cultures had reached an OD_{600} of between 0.010 and 0.020, at which point β-galactosidase activity was measured. β-Galactosidase activity assays were performed as previously described (53), with cells from 2 ml of each culture.

To prepare crude AHL extracts, cell-free culture supernatant from a 1-liter NM glucose-nitrate culture of *S. meliloti* Rm1021 (OD_{600} of 1.1 to 1.3) was extracted twice with 500 ml of ethyl acetate acidified with 1 ml of acetic acid per liter. The extracts were pooled, dried over anhydrous sodium sulfate, and then filtered and rotary evaporated to dryness. The dry residue was dissolved in acetonitrile.

AHL detection and fractionation. For reverse-phase high-pressure liquid chromatography (HPLC) of *S. meliloti* AHLs, the residue from 6 liters of bacterial culture was dissolved in 1 ml of 50% acetonitrile-water, injected onto a 50% acetonitrile-water-equilibrated semipreparatory C_{18} column (Whatman Partisil 10 ODS-3), and then eluted for 80 min with a step gradient (see Fig. 5) at a flow rate of 2 ml/min. Absorbance was monitored at 210 nm. Sequential 1-min fractions were collected.

Aliquots of each fraction were bioassayed with the AHL reporters listed above. Serial dilutions of the HPLC fractions were dried in 96-well microtiter plates (Life Sciences Inc., Denver, Colo.) in a laminar-flow hood and then inoculated with 80 μl of the AHL reporter suspension in LB. After 3 h of incubation, the luminescence of the *E. coli* reporters was measured with a Wallac Victor-2 microtiter plate reader (Perkin-Elmer Inc., Gaithersburg, Md.). Pigment production by *C. violaceum* CV026 and *P. aureofaciens* was assayed as previously described (50, 76, 86). For the bioassays with *S. meliloti exp*::*lacZ* fusions, aliquots of the fractions were dried in Eppendorf tubes and then mixed with the cultures as described above.

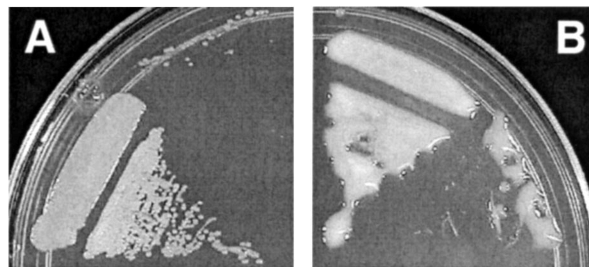

FIG. 1. The *expR101* mutation in *S. meliloti* strain Rm1021 results in a mucoid colony morphology. Colonies formed by wild-type strain Rm1021 (A), which does not produce EPS II, are dry, whereas colonies formed by Rm9000 (Rm1021 $expR^+$ [formerly *expR101*] *exoY210*::Tn*5*) (B), which produces EPS II, are mucoid. The colonies pictured were grown on LBMC agar.

Synthetic 3-oxo-C_{12}-L-HSL (3-oxo-C_{12}-L-homoserine lactone), C_4-L-HSL, C_6-L-HSL, C_8-L-HSL, and C_{14}-L-HSL were purchased from Aurora Biosciences (Coralville, Iowa).

RESULTS

Molecular cloning of the *expR101* mutation. The *expR101* mutant was originally noticed during a screen of *S. meliloti* Rm1021 Tn*5*-derived mutants because of its mucoid colony morphology (Fig. 1). The mucoid colony phenotype proved to be unlinked to the Tn*5* insertion, indicating that the *expR101* mutation had arisen spontaneously (34). Attempts to complement or suppress the mucoid colony morphology of the *expR101*-containing strain with cosmids from an Rm1021 pLAFR1 genomic library (29) were unsuccessful (J. Glazebrook and G. C. Walker, unpublished results; B. J. Pellock and G. C. Walker, unpublished results), suggesting that the *expR101* mutation was dominant.

To clone the *expR101* mutation, we utilized a direct cloning approach that exploited a transposon insertion (termed ΩTn*5-233* #3-15 [34] and located in the *ndvB* ORF [see Materials and Methods for details]) that was ~95% linked (5 to 10 kb) to the *expR101* mucoid colony phenotype in generalized transductions with φM12 (26). We used genomic DNA from Rm10006 (Rm1021 *expR101* ΩTn*5-233* #3-15) to construct a cosmid library (see Materials and Methods for details) and isolated p1-37, a cosmid that carried Tn*5-233* #3-15 and more than 10 kb of chromosomal DNA flanking the transposon on each side (data not shown).

To determine whether the *expR101* mutation was present on p1-37, we constructed an Rm1021/p1-37 transconjugant and examined its colony morphology. On both LBMC agar and TYC agar plates (see Materials and Methods) Rm1021/p1-37 had a mucoid colony morphology (similar to that shown in Fig. 1B) whereas Rm1021 carrying the vector control had a dry colony morphology (similar to that shown in Fig. 1A). p1-37 also induced a mucoid colony morphology when present in Rm7210 (Rm1021 *exoY210*::Tn*5*), a strain incapable of producing succinoglycan. However, p1-37 failed to induce a mucoid colony morphology in Rm10002 (Rm1021 *expA3*::Tn*5*), a strain incapable of producing EPS II. Taken together, our results strongly suggested that we had cloned the *expR101* mutation on p1-37, that the *expR101* mutation is dominant over the *expR* allele in wild-type strain Rm1021, and that the mu-

coid colony phenotype induced by p1-37 is a result of EPS II production, not succinoglycan production.

The predicted *S. meliloti expR* gene product is a LuxR homolog. By using the ability to induce a mucoid colony morphology in Rm7210 as an assay for the presence of the *expR101* mutation, we subcloned p1-37 into pSW213, a broad-host-range IncP vector (15), and examined the colony morphologies of Rm7210 subclone transconjugants on both LBMC and TYC plates. We isolated two overlapping subclones, pK5 and pH 6, that induced a mucoid colony phenotype in Rm7210. The ca. 3.2-kb *Kpn*I-*Hin*dIII fragment common to pK5 and pH 6 (subcloned to generate pKH3.2) was also able to induce a mucoid colony phenotype in Rm7210.

We determined the DNA sequence of this 3,274-bp *Kpn*I-*Hin*dIII fragment and compared the DNA sequences and predicted protein sequences to the GenBank database with a BLAST search (1). Sequence analysis revealed the 5′ ends of the *ndvA* (74) ORF and the 5′ end of an ORF predicted to encode pyruvate carboxylase (*pyc*) flanking one complete ORF, which we have designated *expR*. The *expR* protein product was predicted to be 246 amino acids long and showed significant homology to the *Rhizobium leguminosarum* bv. viciae RhiR protein (17), the *E. coli* SdiA protein (82), and a number of other *Vibrio fischeri* LuxR (24, 25) homologs. The predicted ExpR sequence contained all seven of the amino acid residues conserved in most LuxR homologs (31).

To test whether the *expR* ORF on the 3,274-bp *Kpn*I-*Hin*dIII fragment was responsible for the ability of this fragment to induce a mucoid colony phenotype in Rm7210, we deleted the 3′ terminus of the *expR* ORF by removing a 1.4-kb *Sph*I fragment internal to the 3,274-bp *Kpn*I-*Hin*dIII fragment, creating pKH3.2Δ*Sph*I. This deletion eliminated the ability of pKH3.2 to induce a mucoid colony phenotype.

The *expR* ORF in *S. meliloti* Rm1021 is disrupted by an insertion sequence (IS) element. When we PCR amplified the *expR* region (located on the *S. meliloti* main chromosome) from the wild-type and *expR101* strains for sequence analysis, we were surprised to find that, although the PCR product from the *expR101* mutant was of the expected size (0.9 kb), the PCR product from wild-type Rm1021 with the same primer pair was 2.2 kb (Fig. 2A). Sequence analysis of this 2.2-kb PCR product indicated that the *expR* ORF was disrupted in Rm1021 by a copy of IS*Rm*2011-1 (Fig. 2B), a previously described 1,319-bp IS element (43, 72). This result was corroborated by the published sequence of the *S. meliloti* main chromosome (11). Previous Southern blot analyses suggested that eight copies of IS*Rm*2011-1 exist in the Rm1021 genome (72), although it is now clear from the composite genome sequence that, including the copy that disrupts the *expR* ORF, Rm1021 has nine copies of this IS element (http://sequence.toulouse.inra.fr/meliloti.html). We observed eight IS*Rm*2011-1-hybridizing bands in Rm1021 with both *Eco*RI-restricted genomic DNA and *Eco*RV-restricted genomic DNA (Fig. 2C and D). However, in Rm8530 (Rm1021 *expR101*), we detected only seven copies of IS*Rm*2011-1 (Fig. 2C and D), suggesting that the *expR*-disrupting copy of IS*Rm*2011-1 in Rm8530 had been eliminated from the genome.

IS*Rm*2011-1 is a member of the IS*Rm*1 family of IS elements. Previous work indicated that members of this IS element family create 5-bp target site duplications upon insertion (69, 83). The IS*Rm*2011-1 insertion in *expR* appears to have created a 5-bp target site duplication in the *expR* coding sequence. Precise excision of the IS element and the duplicated 5-bp target site recreated a functional *expR* ORF. Thus, it is highly likely that the *expR101* mutation is a return to the state of the *expR* locus prior to the insertion of the IS element. Since the *expR101* mutation appears to have recreated a functional *expR* allele, we have renamed the *expR101* allele *expR*$^+$. The *expR* allele present in strain Rm1021 will be designated *expR102*::IS*Rm*2011-1.

Disruption of the *expR*$^+$ allele in the *expR101* mutant eliminates EPS II production. To demonstrate unequivocally that it is the presence of an intact *expR* ORF in the *expR101* mutant that is responsible for EPS II production, we disrupted the *expR*$^+$ allele in strain Rm8530 (Rm1021 *expR*$^+$ [formerly *expR101*]) (see Materials and Methods). In contrast to strain Rm8530, which has a mucoid colony phenotype, strain Rm9028 (Rm8530 *expR103*::*lacZ*-Gm) had a dry colony phenotype (data not shown), confirming that *expR*$^+$ is required for the mucoid colony morphology of strain Rm8530. As expected, introduction of the *expR103*::*lacZ*-Gm allele into Rm1021 (which already has a dry colony morphology) did not impact its colony phenotype (data not shown).

To confirm that a strain carrying the *expR103*::*lacZ*-Gm allele was incapable of producing any EPS II that could function in symbiosis, we generated Rm9026 (*expR103*::*lacZ*-Gm *exoY210*::Tn*5*), a derivative of strain Rm9028 that is unable to produce succinoglycan. Rm9026 induced only white, ineffective nodules when inoculated onto alfalfa, whereas Rm9000 (Rm1021 *expR*$^+$ *exoY210*::Tn*5*), which is able to produce symbiotically active EPS II, had the ability to induce pink, symbiotically effective nodules on alfalfa (data not shown). This indicated that disruption of the *expR*$^+$ allele prevented production of symbiotically active EPS II. In addition, we could not detect any low-molecular-weight EPS II (35) in Rm9026 culture supernatants by Dionex high-performance anion-exchange chromatography coupled with highly sensitive pulsed amperometric detection (data not shown).

Extracellular signals control ExpR-dependent transcription of *expC* and *expE*. Because the predicted protein sequence of ExpR has significant homology to both the AHL- and DNA-binding domains of canonical AHL receptors, we were interested in knowing whether ExpR functions to activate target gene transcription in a density-dependent fashion. To determine whether ExpR activates transcription of *exp* genes in response to an extracellular factor(s) produced by *S. meliloti*, we measured the activity of *lacZ* transcriptional fusions to *expA1*, *expC*, *expG*, *expD1*, and *expE2* in both fresh medium and medium conditioned by growth of Rm1021 (see Materials and Methods). We performed these measurements in both the Rm1021 *expR102*::IS*Rm*2011-1 and *expR*$^+$ strain backgrounds. In the absence of a functional *expR* ORF, the levels of transcription of the five *exp*::*lacZ* fusions were relatively low (usually ~10 Miller units) and were very similar in fresh and conditioned media (Fig. 3A). In the *expR*$^+$ background in fresh medium, expression of each of the *exp*::*lacZ* fusions was equal to (for *expA1* and *expD1*) or two- to fourfold higher (for *expG*, *expC*, and *expE2*) than that observed in the Rm1021 background (Fig. 3B). Overall, however, the activity of these fusions in fresh medium (at less than 50 Miller units) was modest. In

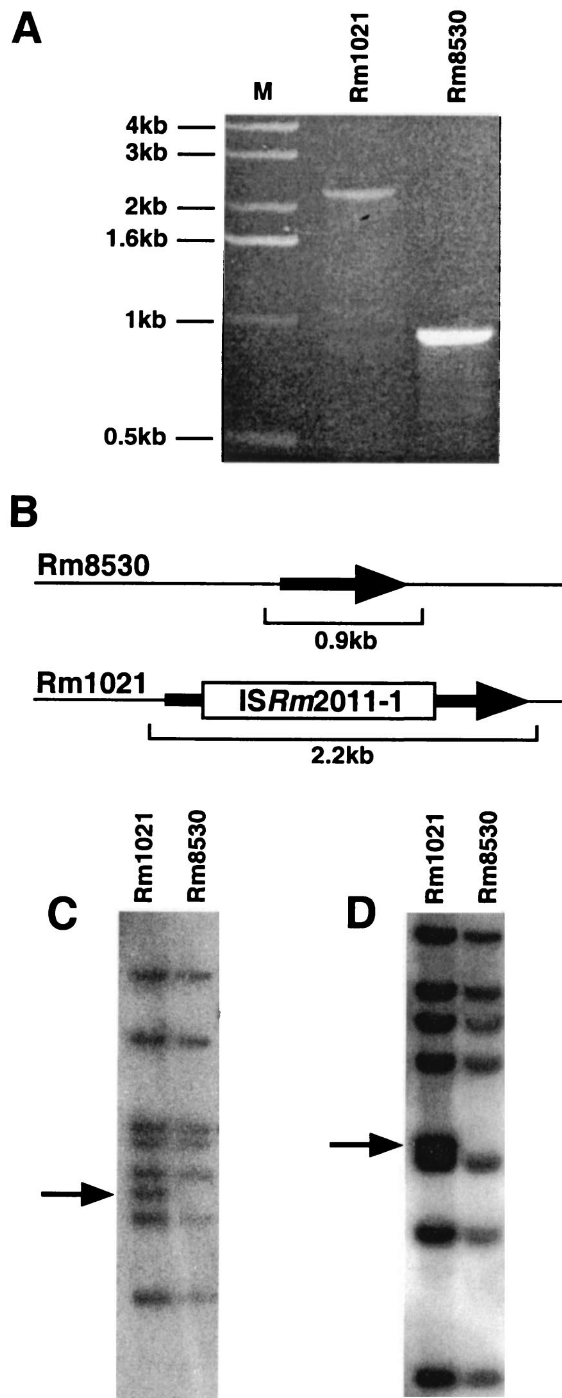

FIG. 2. The *expR* ORF in *S. meliloti* strain Rm1021 is disrupted by a 1,319-bp IS element. (A) A 0.8% agarose gel showing the *expR* region PCR products from Rm1021 (2.2 kb) and from Rm8530 (Rm1021 *expR*$^+$ [formerly *expR101*]) (0.9 kb). The marker (M) lane contains the Gibco BRL 1-kb ladder. (B) Schematic representation of the *expR* ORFs from Rm1021 and Rm8530. The scale bars represent the diagnostic PCR products produced from each strain. (C and D) Autoradiographs of Southern blots of *S. meliloti* DNA probed with a sequence specific for IS*Rm*2011-1. (C) IS*Rm*2011-1 fingerprint of *Eco*RI-restricted genomic DNA from Rm1021 and Rm8530 (Rm1021 *expR*$^+$ [formerly *expR101*]). (D) IS*Rm*2011-1 fingerprint of *Eco*RV-restricted genomic DNA from Rm1021 and Rm8530 (Rm1021 *expR*$^+$ [formerly *expR101*]). The arrows indicate the IS*Rm*2011-1-hybridizing bands present in Rm1021 that are missing in Rm8530.

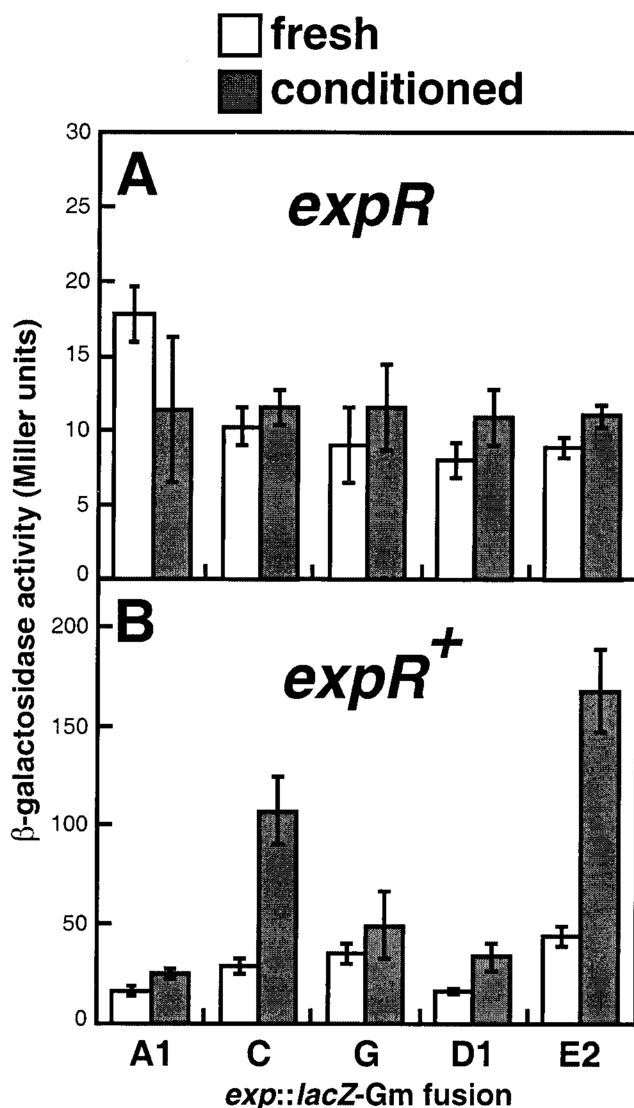

FIG. 3. Results of conditioned-medium experiments. The transcription of *lacZ* transcriptional fusions to five *exp* genes (*expA1*, *expC*, *expG*, *expD1*, and *expE2*) was measured in both fresh and conditioned media (see Materials and Methods). The activity of each fusion was determined in the *expR* mutant (A) and *expR*$^+$ (B) backgrounds. Assays for each strain tested were performed on at least three independent cultures and repeated with independent preparations of conditioned medium. Error margins represent 1 standard deviation. The β-galactosidase activities of Rm1021 and Rm8530 (Rm1021 *expR*$^+$) were nearly identical in both fresh and conditioned media (~5 Miller units).

contrast, in conditioned medium in the *expR*$^+$ background, we observed substantial activity by both the *expC* and *expE2* fusions (Fig. 3B). The *expA1* and *expG* transcriptional fusions were not induced by conditioned medium, and the *expD1* fusion showed only a modest increase in activity in conditioned medium (Fig. 3B). Taken together, these results strongly suggested that some density-dependent signal produced by Rm1021 activated the transcription of *exp* genes in an ExpR-dependent fashion.

Intriguingly, ExpR is tuned to activate *exp* gene transcription

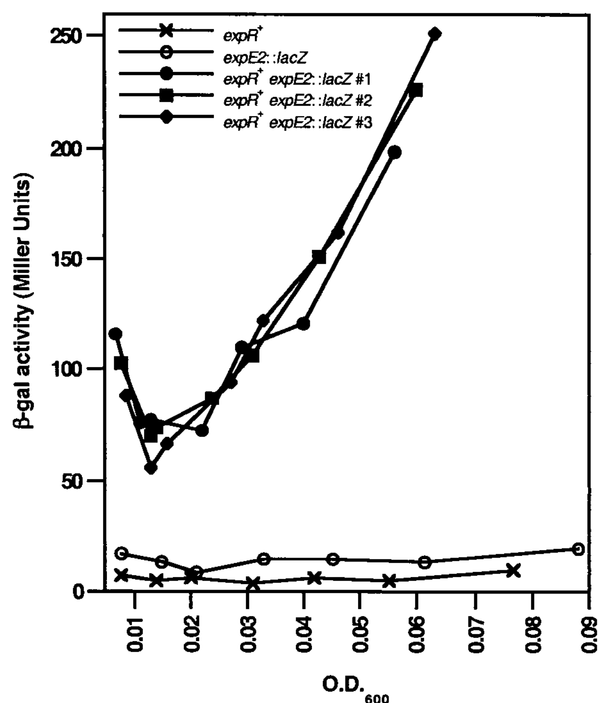

FIG. 4. ExpR mediates density-dependent transcriptional activation of *exp* genes at low cell densities. Mid-log-phase cultures of Rm8530 (×), RmAR1022 (○), and three independent cultures of Rm9033 (●, ■, and ♦) were diluted to an OD_{600} of 0.002 in fresh LBMCP and allowed to grow for 14 h. Samples were analyzed for β-galactosidase (β-gal) activity at 6.5, 8, 9.5, 11, 12, 13, and 14 h. Following dilution, β-galactosidase activity in Rm9033 cultures (*expR*$^+$ *expE2*::*lacZ*) falls until an OD_{600} of 0.015 to 0.02 is reached and then begins increasing again at an OD_{600} of 0.02 to 0.03. The results shown are typical of those seen in multiple iterations of similar experiments. Similar results were obtained with the *expC*::*lacZ* fusion.

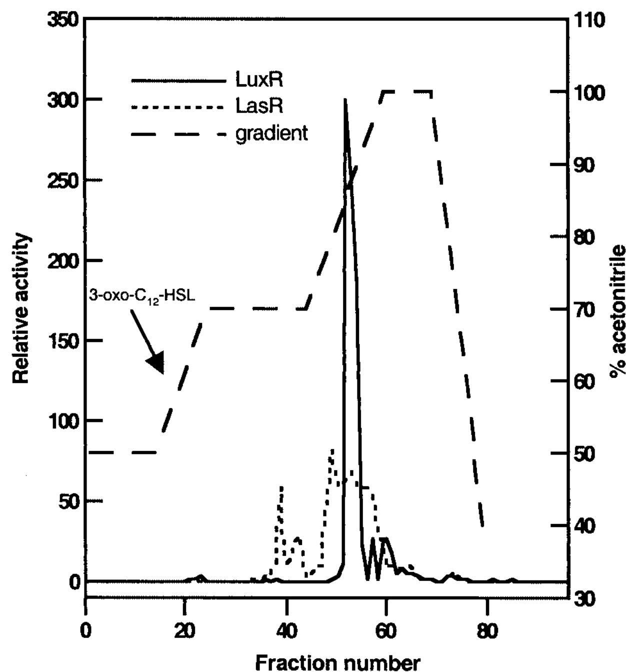

FIG. 5. Reverse-phase HPLC fractionation of AHLs present in the ethyl acetate culture filtrate extracts of *S. meliloti* strain Rm1021. One-minute fractions from a C_{18} HPLC column were collected, and serial dilutions were assayed with the AHL reporters as described in Materials and Methods. Luminescence was measured with a Wallac Victor-2 microtiter plate reader. The acetonitrile concentration in the elution gradient was 50 to 100%. The retention time of a synthetic 3-oxo-C_{12}-HSL standard is indicated. Similar AHL activity profiles were obtained with culture filtrate extracts of an *expR*$^+$ strain (Rm8530).

at low cell densities (Fig. 4). To observe density-dependent transcription of the *exp* genes by ExpR required that we extensively dilute mid-log-phase cultures and allow the cells to grow for several generations prior to the transcription assay (see Materials and Methods). This allowed *exp* transcription to return to baseline levels. Following cellular growth in fresh medium, we consistently observed that *expC* and *expE* transcription began to be activated at an OD_{600} between 0.02 and 0.03 (which corresponds to approximately 10^7 CFU/ml for *S. meliloti* [data not shown]) and was strongly activated before the cells reached an OD_{600} of 0.07 (Fig. 4). Consistent with this observation, use of medium conditioned by growth of *S. meliloti* to a relatively low cell density (OD_{600} of ~0.1) produced results similar to those shown in Fig. 3 (data not shown). This was an interesting result because many LuxR homologs are tuned to respond to cell densities much higher than those that activate ExpR (see Discussion).

Transcription of *expE* and *expC* was also activated in an ExpR-dependent manner by ethyl acetate extracts of *S. meliloti* strain Rm1021 culture supernatants. Addition of this extract activated ExpR-dependent *expC* and *expE* transcription 2- to 2.5-fold (data not shown). Our ethyl acetate extracts also activated the LuxR- and LasR-based AHL reporter strains (data not shown), which are responsive to AHLs with long, 3-oxo-alkanoyl side chains (85). We further separated compounds present in these extracts by reverse-phase HPLC (Fig. 5). *S. meliloti* compounds that activated both the LuxR and LasR reporters eluted at high acetonitrile concentrations, and HPLC fractions 52 to 55 also activated ExpR-dependent *expE* expression (data not shown). Commercially available AHLs, including C_4-L-HSL, C_6-L-HSL, and C_8-L-HSL (Aurora Biosciences), did not activate the ExpR-dependent fusions, although we consistently observed a very modest (1.2- to 1.4-fold) stimulation of *expE* transcription by C_{14}-L-HSL.

Under our culture conditions, the presence of a functional copy of ExpR did not seem to change the AHL profile (Fig. 5) we have observed for *S. meliloti*; AHL activity profiles of *expR*$^+$ cultures were generally similar to those from Rm1021 (data not shown). No additional AHL activities were detected in the *expR*$^+$ culture filtrates with *expR*$^+$ *expE*::*lacZ* or with AHL biosensors. Neither the culture filtrate extracts from *S. meliloti* strain Rm1021 or *S. meliloti* strain Rm8530 (Rm1021 *expR*$^+$) nor any of the HPLC fractions activated CviR-, AhyR-, and PhzR-based reporters (*C. violaceum* CV026, *E. coli* pSB536, and *P. aureofaciens* 30-84I, respectively), all of which respond most strongly to AHLs with alkanoyl (C_4 to C_8) side chains (50, 75, 86). Taken together, our results suggest that the putative AHL molecule responsible for ExpR-dependent stimulation of *exp* transcription is not an AHL with a short (C_4 to C_8) al-

TABLE 2. Status of the *expR* locus in *S. meliloti* strains

Strain	Reference	Size (kb) of *expR* PCR product	Status of *expR* ORF
Rm1021	51	2.2	*expR102*::IS*Rm*2011-1
Rm8530	34	0.9	Intact (*expR*$^+$)
SU47	79	2.2	*expR102*::IS*Rm*2011-1[a]
Rm2011	12	2.2	*expR102*::IS*Rm*2011-1[a]
Rm5000	26	2.2	*expR102*::IS*Rm*2011-1[b]
RCR2011	68	2.2	*expR102*::IS*Rm*2011-1[b]
Rm41	59	0.9	Intact[c]
YE-2S1	87	0.9	Intact[d]
102F34	20	0.9	Disrupted by 11-bp deletion

[a] Genotype confirmed via DNA sequencing.
[b] This genotype is inferred from the relatedness of these strains to SU47 and the size of the PCR product.
[c] Rm41 *expR* has a sequence distinct from those of Rm1021 and YE-2S1.
[d] YE-2S1 *expR* has a sequence distinct from those of Rm1021 and Rm41.

kanoyl side chain and that the ExpR receptor is very specific in the type of molecule that it recognizes.

Analysis of the *expR* ORF in other *S. meliloti* strains. During the course of our analyses of the *expR101* mutation, we became curious about the status of the *expR* ORF in a number of other *S. meliloti* strains that have been used in genetic laboratory studies and performed diagnostic PCRs on the *expR* regions of a number of *S. meliloti* strains. The results of these analyses are summarized in Table 2. The PCR product from strain SU47 (the parent strain of Rm1021, Rm2011, Rm5000, and RCR2011) and those from Rm2011, Rm5000, and RCR2011 were 2.2 kb, implying that these strains, like Rm1021, had an IS*Rm*2011-1-disrupted *expR* ORF. This was confirmed by sequence analysis for SU47 and Rm2011. Strain YE-2Sl, an independently isolated strain that constitutively produces both succinoglycan and EPS II, had an intact *expR* ORF. Strain Rm41, which is more mucoid than Rm1021 but much less mucoid than Rm8530 and YE-2Sl, also had an intact *expR* ORF. Another independently isolated *S. meliloti* strain that has been genetically analyzed, strain 102F34, gave a 0.9-kb *expR* PCR product, suggesting that this strain does not have IS*Rm*2011-1 inserted in the *expR* ORF. However, when we sequenced the *expR* region from strain 102F34, we discovered that, although no IS element is present, the 102F34 *expR* ORF has an 11-bp deletion in its coding sequence, a deletion predicted to result in premature translational termination of the predicted *expR* gene product in strain 102F34. This is consistent with the dry colony morphology of strain 102F34, and our sequence data agree completely with the previously published *ndvA* region sequence data (74).

DISCUSSION

The only known circumstance under which *S. meliloti* strain Rm1021 synthesizes EPS II in a symbiotically active form is when it carries a mutation originally designated *expR101*. In this report, we have shown that widely used laboratory strain Rm1021 (whose genome has recently been sequenced [32]) and several related strains have a copy of IS*Rm*2011-1 (43, 72) within the coding region of *expR*, a gene that encodes a LuxR homolog. The *expR101* mutation evidently resulted from precise excision of this IS, which restored the reading frame of the *expR* gene, with the excised copy of IS*Rm*2011-1 having been lost from this strain. The presence of a functional *expR* ORF on a plasmid or in the genome is sufficient to promote the production of symbiotically active EPS II, and disruption of the *expR101* allele eliminates EPS II production. We have therefore renamed the *expR101* allele *expR*$^+$. Rm1021 and related strains carry the *expR102*::IS*Rm*2011-1 allele.

The *expR* gene product, which is a LuxR homolog, significantly activates transcription of the *expC* and *expE* operons in conditioned medium and in response to specific HPLC fractions that also activate two AHL reporter systems. This result is consistent with our observation that, of the five *exp*::*lacZ* fusions tested, *expC* and *expE* are by far the most strongly expressed in an *expR*$^+$ background (B. J. Pellock, J. Lloret, and G. C. Walker, unpublished results). To the best of our knowledge, this is the first description of quorum-sensing regulation in *S. meliloti*. AHL-dependent quorum-sensing systems that impact nodulation efficiency have been described in *R. leguminosarum* (17, 37, 47, 66) and *R. etli* (67). However, it is not clear exactly how density-dependent gene expression mediated by these *R. etli* and *R. leguminosarum* systems is involved in the nodulation process. Previous studies have also shown that a number of distinct plant-associated and free-living bacteria use quorum-sensing systems to regulate EPS synthesis and colony mucoidy (for example, see references 28, 58, 80, and 90), and in some cases, nonmucoid derivatives are impaired in the ability to infect or colonize their plant hosts (80, 90).

A striking feature of ExpR-mediated density-dependent production of EPS II is that *exp* transcription is stimulated at much lower cell densities than in many of the other described LuxR-type systems (Fig. 4). For example, substantial activation of the *V. fischeri* LuxR system occurs when the OD_{600} of a culture is between 0.1 and 0.3, depending on the strain tested (23, 54). Additionally, the *Pseudomonas aeruginosa* LasR and RhlR systems induce target gene expression at much higher densities than *S. meliloti* ExpR (57). *R. leguminosarum*, which has a multitiered regulatory network involving multiple AHL molecules and several LuxI and LuxR homologs (17, 37, 47, 66), also contains a quorum-sensing system (CinR) that is tuned to respond to low cell densities. In fact, we initially overlooked the density-dependent regulation of the *exp* genes by ExpR, and it was this *R. leguminosarum* work that prompted us to explore the possibility that *expR* is tuned to activate *exp* gene transcription at low cell densities. However, ExpR is more closely related to many other LuxR family members than it is to CinR (data not shown), suggesting that *S. meliloti* ExpR and *R. leguminosarum* CinR are distinct systems tuned to respond to low cell densities.

The results presented here have changed our understanding of how *S. meliloti* controls the production of symbiotically active EPS II. Prior to this work, it was thought that *expR*$^+$ strains constitutively produce EPS II. However, we now know that this is not the case. We had previously overlooked the density-dependent production of EPS II because the cell densities used in typical laboratory experiments are much greater than those at which ExpR begins activating transcription of the *exp* genes. Since the densities of *S. meliloti* found in soil are typically less than 10^7 cells per gram of soil (73), it may be that expression of the *exp* genes in an *expR*$^+$ strain in the field will be at low levels until the cells are in an environment where the

local density is above the threshold for activation of *exp* gene expression. This could act to conserve cell resources until synthesis of symbiotic polysaccharides is required, for example, inside colonized curled root hairs and infection threads. However, further studies are needed to determine the quantity and molecular weight distribution of EPS II produced at various cell densities and to determine the precise roles of the *exp* gene products in EPS II synthesis.

The *expC*- and *expE*-activating substances present in the conditioned medium and the ethyl acetate culture filtrate extracts have not been chemically identified. However, because our ethyl acetate extracts and HPLC fractions activate both ExpR-dependent transcription of some *exp* genes and long-chain AHL reporter systems, it seems likely that ExpR also responds to an AHL. The side chains of the *expE*-active Rm1021 AHL(s) are likely to be very nonpolar. Gray et al. have described purification of a LasR-activating compound from *S. meliloti* Rm1021 culture supernatants that eluted from a C_{18} column later than *R. leguminosarum* 3OH,C_{14}:1-HSL (37). This suggests that the putative AHL(s) produced by strain Rm1021 is structurally distinct from *R. leguminosarum* 3OH,C_{14}:1-HSL and has carbon chains at least as long as C_{14}. Independent chromatographic analyses also indicate that the AHLs produced by *S. meliloti* strain Rm1021 are very hydrophobic (13, 58). Further experimentation is required to determine the structures of the Rm1021 AHL molecules and to identify which of these Rm1021 AHLs activate ExpR-mediated transcription of the *exp* genes.

Interestingly, the *S. meliloti* genome (http://sequence.toulouse.inra.fr/meliloti.html) contains a number of genes that may have roles in quorum sensing. In addition to *expR* (the intact gene is SMc03899 plus SMc03896), five ORFs (SMc00170, SMc00877, SMc00878, SMc00658, and SMc04032) in the *S. meliloti* genome are predicted to encode possible LuxR family members. Two candidate AHL synthase genes are also present: one gene (SMc00168) predicted to encode a LuxI homolog and one gene (SMc00714) predicted to encode a homolog of the *Pseudomonas fluorescens* HdtS protein (44). It is not clear whether all of these predicted genes function in density-dependent gene expression, but recent work indicates that SMc00170 and SMc00168 may be part of an *S. meliloti* quorum-sensing network similar to that found in *R. leguminosarum* (49).

In a number of bacteria, IS elements modulate polysaccharide-dependent phase variation but it is not clear whether this occurs in *S. meliloti*. Some examples of this include *Neisseria meningitidis* switching from a capsule-producing form to a capsule-deficient form (38), IS-mediated reversible production of EPS in *Pseudoalteromonas atlantica* (5), and phase variation of *Xanthomonas oryzae* pv. oryzae with regard to xanthan gum production (60). It is possible that, in the field, *S. meliloti* uses the IS*Rm*2011-1 insertion in *expR* as a genetically plastic switch to control EPS II production. However, under laboratory conditions, both the mucoid colony phenotype of *expR*$^+$ strains and the nonmucoid colony phenotype of *expR*::IS*Rm*2011-1 strains are highly stable (Pellock and Walker, unpublished). Additionally, it has been reported that *S. meliloti* IS element genomic fingerprint patterns are highly stable over long periods of time under laboratory conditions (72). Consistent with this observation, our IS*Rm*2011-1 fingerprinting analysis of SU47 and its daughters (data not shown) revealed a hybridization pattern identical to that reported previously (72). Since the *expR* ORF is disrupted by IS*Rm*2011-1 in all of the daughters of SU47 that we tested, insertion of the IS element almost certainly occurred before these daughters of SU47 were isolated. However, it is not known whether the *expR* ORF was already disrupted in the original SU47 field isolate or whether the IS insertion occurred later under laboratory conditions and a SU47 isolate with a less mucoid colony phenotype was chosen for further study.

IS*Rm*2011-1 is a member of the IS*1* family of IS elements, which are thought to transpose by a replicative mechanism (14). Since the copy of IS*Rm*2011-1 that disrupts *expR* has been eliminated from the genome (Fig. 2C and D), it seems likely that this precise excision resulted from a microhomologous recombination event between the 5-bp target site and its duplicate. Precise excisions of IS elements occur with a frequency of 10^{-6} to 10^{-10} per element per generation (46), which is consistent with the high stability of the IS*Rm*2011-1 insertion in *expR*. The fact that the *expR*$^+$ allele is also very stable suggests that the *expR* gene is not a hot spot for IS*Rm*2011-1 insertion, as appears to be the case for insertions of IS*Rm*1 in the *nif* gene region (69).

The results of this study raise a number of intriguing questions about the regulation of the production of EPS II and other symbiotically active *S. meliloti* polysaccharides. The identity of the cell density signal(s) that stimulates ExpR-dependent gene expression remains to be determined. It will also be of interest to determine whether the production of succinoglycan and/or symbiotically active K antigen is controlled in a density-dependent fashion, whether any non-*exp* genes are regulated by ExpR, and how the expression of *expR* itself is regulated. In addition, further biochemical and genetic studies are needed to determine the exact mechanism of ExpR action. The question of how the molecular weight distribution of EPS II is controlled also remains open.

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