Genetic and Biochemical Analyses of Succinoglycan, EPS II, and K antigen, Polysaccharides Important in the *Sinorhizobium meliloti* - Alfalfa Symbiosis

by

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ABSTRACT

Polysaccharides produced by *Sinorhizobium meliloti* play crucial roles in the establishing a successful, nitrogen-fixing symbiosis between *S. meliloti* and alfalfa. *S. meliloti* strains that fail to produce a symbiotically active form of at least one of three polysaccharides, succinoglycan, EPS II, or K antigen, fail to invade alfalfa root nodules and are therefore severely compromised in symbiosis. Low-molecular weight forms of each of the three polysaccharides appear to be the forms active in nodule invasion. We have been interested in determining exactly where in the symbiosis these polysaccharides are required and measuring the efficiencies of nodule invasion mediated by each polysaccharide. We have also been interested in determining how *S. meliloti* controls the production of low-molecular-weight forms of EPS II and determining which structural features of succinoglycan are important for its nodule invasion-promoting activity.

We report here that, similar to succinoglycan, EPS II and K antigen function to mediate infection thread initiation and extension on alfalfa. However, succinoglycan, EPS II, and K antigen can each mediate alfalfa root nodule invasion by *S. meliloti*, they do so with significantly different efficiencies. There are also qualitative differences in the morphologies of the infection threads mediated by the different polysaccharides.

We have also cloned and characterized the *expR101* mutation from *S. meliloti* strain Rm1021, a mutation that turns on production of symbiotically active EPS II. The *expR101* mutation resulted when an insertion sequence element that disrupts the Rm1021 *expR* open reading frame excised, restoring a functional *expR* ORF.

In structure-function experiments in which we rescued the nodule invasion defect of a symbiotic polysaccharide-deficient strain, we have found that a low-molecular-weight fraction of succinoglycan, specifically a trimer of the succinoglycan repeating unit, is the fraction active in promoting infection thread growth and thus nodule invasion on alfalfa. We have also determined that the succinyl and acetyl modifications of succinoglycan are dispensable for this activity.

Thesis Supervisor: Graham C. Walker
Title: Professor of Biology

Table of Contents

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Chapter 1

Introduction

Understanding the Roles of Symbiotic Polysaccharides in Rhizobia-Legume Symbioses*

* Manuscript in preparation for submission to J. Bacteriol. as a mini-review

INTRODUCTION

Nitrogen-fixing symbioses between rhizobia and their host legumes are the result of complex signaling conversations between the bacteria and the host plant [for general reviews see (24, 36, 83, 94, 114)]. Flavonoid compounds released by legumes attract rhizobia and activate rhizobial production of lipochitooligosaccharide Nod factors. Nod factors stimulate root hair curling and induce the growth of root nodules. Rhizobia enclosed in curled root hairs can colonize the curled root hairs and invade developing root nodules through extended invaginations of the root hair cell membrane called infection threads. Infection threads allow rhizobia to proceed from the inside of colonized, curled root hairs down through root hair cells to the cells of the developing root nodule. The rhizobia in the infection thread are released into root nodule cells, becoming enclosed in host membrane compartments in a process that resembles phagocytosis. The rhizobia then differentiate into bacteroids, the nitrogen-fixing form of the rhizobia.

Over the past two decades, it has become clear that many rhizobia produce polysaccharides that are important for the successful colonization of host root nodules. Specifically, forms of these polysaccharides appear to play critical roles in the efficient induction and growth of infection threads. Rhizobial polysaccharides are important in many rhizobia-legume symbioses, including those between *Sinorhizobium meliloti* and *Medicago sativa* (alfalfa) (55, 79, 113), between *Rhizobium leguminosarum* bv. *viciae* and *R. leguminosarum* bv. *trifolii* and many of their hosts (19, 20, 37, 38, 67, 75, 134, 136), and between *Rhizobium* sp. NGR234 and *Leucaena leucocephala* (31). Since the most intensively

studied examples of symbiotically important rhizobial polysaccharides are those produced by *S. meliloti*, they are the principal foci of this review.

POLYSACCHARIDES INVOLVED IN ALFALFA ROOT NODULE INVASION BY *SINORHIZOBIUM MELILOTI*

S. meliloti strains that are defective in the production of certain symbiotically important polysaccharides are blocked at the root nodule invasion step of symbiosis and thus primarily induce the formation of root nodules devoid of bacteria and bacteroids. *S. meliloti* strain Rm1021 (85) is capable of producing symbiotically active forms of two exopolysaccharides, termed succinoglycan (79) and EPS II (also referred to as galactoglucan) (55). In addition to these two exopolysaccharides, *S. meliloti* strain Rm41, can also produce a symbiotically active form of K antigen, a strain-specific, cell-associated polysaccharide (113). Though succinoglycan, EPS II, and K antigen are structurally diverse (Fig. 1A, B, C), the presence of any one of these polysaccharides in a symbiotically active form is sufficient to mediate alfalfa root nodule invasion by *S. meliloti*.

Most of the studies involving succinoglycan and EPS II have been performed in *S. meliloti* strain Rm1021 or its closely related sister strain Rm2011 (27). Rm1021 does not usually produce detectable EPS II and produces only symbiotically inactive forms of its K antigen (113). Thus, Rm1021 is dependent on succinoglycan for establishing a successful symbiosis with alfalfa. EPS II production by Rm1021 can be induced by growth in very low phosphate conditions (152) or by a mutation in either the *mucR* gene (70, 151) or the *expR* gene (55), which encode regulators of EPS II production. However, the only known circumstance under which strain Rm1021 produces symbiotically active EPS II is when it carries a special allele of *expR* originally termed *expR101*. Though Rm1021 does not synthesize symbiotically active forms of its K antigen, strain Rm41 (101), an independently isolated *S. meliloti* strain, is capable of producing a K antigen fraction [structurally distinct from the K antigen of Rm1021

(110)] that can mediate alfalfa root nodule invasion (102, 113). Most studies of the symbiotic role of K antigen have been performed using a derivative of Rm41 carrying a mutation that prevents the synthesis of succinoglycan and EPS II.

In retrospect, it is interesting to note that, had a strain that produces more than one symbiotically active polysaccharide been chosen for the original analysis of the importance of *S. meliloti* polysaccharides in symbiosis, the crucial role played by rhizobial polysaccharides in the alfalfa root nodule invasion process might not have been obvious.

Genetics of Symbiotically Important Polysaccharide Production

Succinoglycan. Analysis of the genetics and biochemistry of succinoglycan production has resulted in one of the best-characterized models of how bacteria synthesize complex polysaccharides. DNA sequence analysis of the genes implicated in succinoglycan biosynthesis provided a framework for assigning putative functions to the gene products and guided the construction of strains for subsequent biochemical analyses.

The identification of genes involved in succinoglycan production was facilitated by the serendipitous observation that the laundry whitener calcofluor fluoresces brightly under long wave ultraviolet (UV) light when it binds to succinoglycan. Genetic screens for mutants of *S. meliloti* Rm1021 that failed to fluoresce under UV light on plates containing calcofluor led to the identification of the *exo* gene cluster (Fig. 2A) (79, 82), which is located on pSymB, one of two symbiotic replicons that, along with a larger main chromosome, comprise the *S. meliloti* genome. The *exo* gene cluster (12-14, 57, 58, 105) consists of 19 genes involved in the biosynthesis of the succinoglycan backbone (*exoY*, *exoF*, *exoA*, *exoL*, *exoM*, *exoO*, *exoU*, *exoW*, and *exo?*), modification of the succinoglycan repeating unit (*exoH*, *exoV*, and *exoZ*),

regulation of succinoglycan production (*exoX*), succinoglycan polymerization and export (*exoP*, *exoQ*, and *exoT*), synthesis of precursors [galE (*exoB)* and *exoN*], succinoglycan molecular weight distribution (*exoK*). There is also one gene of unknown function (*exoI*). The *exo* cluster has since been extended to include the *exsA* gene (possibly involved in succinoglycan export), the *exsB* gene (a regulator of succinoglycan production) and the *exsH* gene (involved in succinoglycan molecular weight control) (15, 144).

The succinoglycan octasaccharide subunits are synthesized on membrane-bound (C-55 undecaprenyl phosphate) lipid carriers present in the cytoplasmic membrane (130, 131). Thorough chromatographic characterization of the radiolabeled succinoglycan biosynthetic intermediates produced by various *exo* mutants allowed the construction of a detailed model of succinoglycan biosynthesis (Fig. 3) (109). Galactose is the first sugar added, followed by the seven glucose residues. The acetyl, succinyl, and pyruvyl modifications are added during the synthesis of the sugar backbone. It is thought that the assembly of the repeating units occurs on the cytoplasmic face of the inner membrane, but it is not known how the completed succinoglycan subunits are exported. It has been postulated that polymerization of the repeating units occurs in the periplasm and that export of the polymer is coincident with polymerization (109).

EPS II. A cluster of *S. meliloti* genes (the *exp* genes) required for EPS II production has also been identified on pSymB (Fig. 2B) (55). The *exp* gene cluster contains 21 genes, each of which is required for EPS II production. Although the *exp* gene cluster has been sequenced (16), it is currently unclear exactly how the *exp* gene products participate in EPS II production. In contrast to our understanding of *exo* gene involvement in succinoglycan synthesis, there are a number of *exp* gene products whose roles in EPS II production are not easily explained. For

example, more predicted glycosyltransferases (five) are present in the *exp* gene cluster than might be expected to synthesize a polysaccharide with only a disaccharide repeating unit. Additionally, there are four genes predicted to encode a set of proteins that synthesize dTDP-L-rhamnose (a sugar not present in EPS II), as well as genes that encode components of a protein export system (90), a secreted calcium binding protein (90), and a predicted methyltransferase. Furthermore, it is not clear 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 from the *exp* cluster.

K antigen: A number of distinct loci involved in K-antigen production by Rm41 have been identified. The *rkp-1* locus, formerly known as the *fix-23* region (74, 102), contains ten open reading frames (*rkpA* to *–J*) required for K antigen production and the adsorption of the Rm41-specific bacteriophage ϕ16-3 (72, 100). The ORFs of the *rkp-1* locus are predicted to encode fatty acid synthases and proteins that modify and transfer lipids to capsular polysaccharides . It has been proposed that the *rkpABCDEFGHIJ* gene products are involved in synthesizing a lipid carrier required for K antigen biosynthesis. Further screens for non-*rkp-1* Tn*5* insertion mutants that were resistant to ϕ16-3 led to the discovery of the *rkp-2* and *rkp-3* loci as well as two distinct mutants designated class III and class IV (26, 71). The *rkp-2* locus contains the *rkpK* gene, which appears to code for a UDP-glucose dehydrogenase (71). The *rkp-3* locus, which is located near the previously described *rkpZ* gene (see below) has recently been characterized . The *rkp-3* region contains genes predicted to encode the machinery that assembles the K antigen repeating unit. The class III and class IV K antigen mutants are distinct from the *rkp-1*, *rkp-2*, and *rkp-3* mutants, but the mutations are unlinked to the Tn*5* insertions carried in these strains and may be complex, non-recessive mutations (26).

Regulation of the Production of Symbiotically Important *S. meliloti* Polysaccharides

Succinoglycan. A number of environmental conditions influence succinoglycan production by. *S. meliloti*: succinoglycan production is stimulated by low phosphate levels, low nitrogen levels, low sulfur concentrations, and high phosphate levels (79, 86). High sodium chloride or nitrogen levels reduce the amount of succinoglycan produced (23, 42). Additionally, ten regulatory genes that function at a variety of steps in succinoglycan production have been identified (Table 1). In this review we will focus on the best characterized, succinoglycan-specific regulators.

Two transcriptional regulators of succinoglycan production are *exoR*, a negative regulator, and *exoS*, a positive regulator (42). Strains carrying a null allele of *exoR* produce copious quantities of succinoglycan (11-fold more than wild type). The ExoR gene product lacks homology to known transcriptional regulators (104). However, ExoR appears to be involved in succinoglycan synthesis in response to nitrogen levels, as an *exoR* null strain does not reduce succinoglycan production in response to nitrogen as a wild type strain does. Succinoglycan production is also regulated by the ExoS-ChvI two-component regulatory system (a member of the EnvZ-OmpR family of two-component regulatory systems) (32), which has close homologs in *Brucella abortus* (127) and *Agrobacterium tumefaciens* (29). The *exoS* gene encodes the sensor and *chvI* encodes the response regulator, though what ExoS senses remains obscure. The sole available Tn*5* insertion allele of *exoS*, *exoS96*::Tn*5*, results in the production of an amino-truncated ExoS protein that constitutively stimulates succinoglycan production.

Succinoglycan production is also controlled post-transcriptionally. A negative, post-translational regulator of succinoglycan production is produced by the *exoX* gene (105, 150). Null alleles of *exoX* result in increased succinoglycan production. The ratio of ExoX to ExoY (which catalyzes the transfer of galactose to the lipid carrier, the first step of succinoglycan biosynthesis) is important in regulating succinoglycan production (see Table 1). ExoX appears to effect this regulation post-translationally, perhaps through a direct interaction with ExoY. The *exsB* gene, which is linked to the *exo* gene cluster, is a post-transcriptional, negative regulator of succinoglycan production; a strain carrying a null allele of *exsB* produces 3-fold more succinoglycan than a wild type strain (15). MucR, originally identified as a negative regulator of EPS II production, positively regulates succinoglycan production at a post-transcriptional level (70).

EPS II. The regulation of EPS II production in *S. meliloti* is also complicated. The complexity of EPS II regulation and of the structure of the *exp* gene cluster itself may well be a consequence of the fact that EPS II appears to be used by *S. meliloti* for at least two distinct purposes: 1) protection from environmental stress and 2) symbiosis.

S. meliloti strain Rm1021 does not usually produce EPS II. However, when grown under very low phosphate conditions, Rm1021 produces very HMW EPS II, a fraction that is not symbiotically active (86, 152). This response to phosphate limitation requires the *phoB* gene and also involves *expG*, a positive regulator of EPS II production located in the *exp* gene cluster (120). Multiple plasmid-borne copies of *expG* can also stimulate Rm1021 to produce EPS II (4). EPS II production by Rm1021 is not stimulated by low nitrogen levels, low sulfur levels, high osmolarity, and dehydration (152). EPS II synthesis in Rm1021 can also result from a null allele of the *mucR* gene (70, 151). MucR is a negative transcriptional regulator of

the *exp* genes. It is predicted to contain a C_2H_2 zinc finger motif and is highly homologous to the Ros protein from *Agrobacterium tumefaciens*. However, the EPS II produced by a *mucR*::Tn*5* mutant is limited to a HMW fraction and is not symbiotically active (59).

When strain Rm1021 carries an allele of the *expR* gene originally termed *expR101*, it produces forms of EPS II ranging from LMW to HMW (55, 59). This is the only known circumstance that causes Rm1021 to synthesize symbiotically active EPS II. It is now clear that the Rm1021 *expR* gene is disrupted by an insertion sequence (IS) element. The allele originally termed *expR101* resulted from an excision of this IS element that restored the *expR* reading frame to yield the $expR^+$ gene. The $expR^+$ gene product is homologous to RhiR from *R. leguminosarum* bv. *viciae*, SdiA from *Escherichia coli*, and a number of other *Vibrio fischeri* LuxR homologs (**Chapter 3**). In an $expR^+$ strain, high phosphate levels and high sodium chloride conditions limit EPS II production (86, 147).

K antigen. Little is currently known about the regulation of K antigen production, though it is known that *S. meliloti* modulates its K antigen in response to environmental factors including pH and temperature (112). Given the complexity of the regulation of other polysaccharides that can mediate alfalfa root nodule invasion (succinoglycan and EPS II), it seems likely that, as more is learned about the genetics of K antigen production, both positive and negative regulators of its synthesis will be identified.

Low-Molecular-Weight Forms of Succinoglycan, EPS II, and K antigen Appear to Function as Signals to the Host Plant

Three classes of experiments have led to the hypothesis that succinoglycan, EPS II, and K antigen are functioning as signal molecules that modulate some host plant function. First, the succinoglycan symbiotic function can be provided *in trans* to a polysaccharide-deficient,

nitrogen-fixation-proficient strain by a coinoculated succinoglycan-proficient, nitrogen-fixation-deficient strain, resulting in a productive symbiosis (73, 91). Second, genetic evidence suggests that low-molecular-weight (LMW) forms of all three polysaccharides appear to be the symbiotically active forms; perturbing the molecular weight distribution of the polysaccharides reduces symbiotic efficiencies (33, 59, 78, 113). Finally, in the case of succinoglycan and EPS II, it has been reported that picomolar quantities of purified LMW polysaccharide (but not heterologous polysaccharides) can partially rescue the nodule invasion defect of a polysaccharide deficient, invasion deficient strain (10, 59, 133).

Succinoglycan. In a glutamate-mannitol-salts (GMS) minimal medium, *S. meliloti* Rm1021 produces a roughly equal balance of two distinct molecular weight fractions of succinoglycan (77, 148): high-molecular-weight (HMW) succinoglycan, which has hundreds to thousands of repeating units per molecule, and low-molecular-weight (LMW) succinoglycan, which consists of only monomers, dimer, and trimers of the octasaccharide repeating unit (60, 138). Both genetic and biochemical evidence suggests that LMW succinoglycan is the symbiotically active form of succinoglycan. A purified sub-fraction of LMW succinoglycan, specifically the succinoglycan trimer **(Chapter 4)**, can partially restore the nodule invasion defect of a polysaccharide deficient strain, but heterologous polysaccharides can not (10, 133). Furthermore, almost all of the succinoglycan synthesized by a Rm1021 *exoH* null mutant is HMW succinoglycan, which is the apparent cause of the symbiotic defect in this strain (78).

EPS II. Although EPS II production by strain Rm1021 can result from phosphate limitation (152), from a mutation in *mucR* gene (70, 151), or from reversion of the *expR*::IS allele (in Rm1021) to $expR^{+}$ (55)**(Chapter 3)**, the EPS II molecular weight distribution is not the same in each case. Phosphate limitation results in the production of HMW EPS II (10, 133,

152), as does a null mutation in the *mucR* gene (59). In contrast, an *expR*$^+$ (formerly *expR101*) strain produces EPS II ranging from LMW to HMW (59). Since only the *expR*$^+$ strain produces production of symbiotically active EPS II, it appears that LMW EPS II is the symbiotically active fraction. This conclusion is supported by the observation that a LMW EPS II fraction containing oligomers with 15-20 disaccharide repeating units appears to be capable of partially restoring the nodule invasion to a polysaccharide deficient strain (59).

K antigen: Similarly, a LMW fraction of K antigen also appears to be the symbiotically active form. *S. meliloti* strain AK631, a derivative of Rm41that is incapable of producing succinoglycan and EPS II, produces K antigen in both LMW and a HMW forms (113). Strain AK631 is symbiotically proficient on alfalfa, but AK631 carrying a null allele of the *rkpZ* gene produces only HMW K antigen and is not able to invade alfalfa root nodules (25, 102, 141). This suggests that the symbiotically active form of K antigen produced by strain Rm41 is the LMW fraction.

Control of the Molecular Weight Distribution of Symbiotically Important *S. meliloti* Polysaccharides

As discussed above, a common theme that has emerged from both genetic and biochemical studies is that specific low-molecular-weight (LMW) fractions of succinoglycan, EPS II, and K-antigen appear to be the forms active in symbiosis. Perhaps, then, it is not surprising that *S. meliloti* has specific control systems for modulating the molecular weight distribution of these polysaccharides.

Succinoglycan. *S. meliloti* can produce LMW succinoglycan by direct synthesis of LMW oligomers or by cleaving HMW succinoglycan to LMW forms using glycanases. The products of the *exoP*, *exoQ*, and *exoT*, genes appear to be involved not only in succinoglycan

polymerization and export (109), but also in succinoglycan molecular weight control. Genetic evidence suggests that separate systems exist for synthesizing HMW and LMW succinoglycan: ExoP and ExoT cooperate in the synthesis of succinoglycan monomers, dimers and trimers, whereas ExoP and ExoQ cooperate to synthesize HMW succinoglycan (60). Rm1021 also produces ExoK and ExsH, secreted endo β-1,3-1,4 glycanases that cleave HMW succinoglycan to LMW forms (144). In addition to *exoK* and *exsH*, Rm1021 has a gene (*eglC*) predicted to encode a third succinoglycan glycanse (142). Though *eglC* does not appear to contribute to LMW succinoglycan production in Rm1021, it does contribute to LMW succinoglycan production in an independently isolated *S. meliloti* strain (124).

Several factors contribute to cleavage of succinoglycan chains by ExoK and ExsH. Environmental conditions can influence the secretion and activity levels of ExoK and ExsH (143). In addition, only nascent succinoglycan is susceptible to cleavage by ExoK and ExsH (143). Following synthesis, succinoglycan becomes refractory to cleavage, even by high concentrations of glycanases, in a time dependent manner. The nature of this refractory state is presently unknown, but presumably results from changes in succinoglycan confirmation and/or aggregation. The acetyl and succinyl modifications also influence the susceptibility of succinoglycan to ExoK and ExsH cleavage *in vitro* (145). Relative to succinoglycan isolated from Rm1021, succinoglycan chains lacking the acetyl modification are more susceptible to cleavage, whereas succinoglycan chains lacking the succinyl modification are less susceptible to cleavage. This also appears to be the case *in vivo*: a mutant that does not succinylate succinoglycan (*exoH154*::Tn*5*) produces a much higher HMW:LMW ratio than Rm1021, whereas a mutant that does not acetylate succinoglycan (*exoZ341*::Tn*5*) produces a lower HMW:LMW ratio than Rm1021. This data also fits nicely with the observation that in Rm1021

LMW succinoglycan is more highly succinylated than HMW succinoglycan (138). The acetyl and succinyl modifications might influence the susceptibility of succinoglycan oligomers to ExoK and ExsH by affecting the rate at which succinoglycan become refractory to cleavage or by changing the way the polysaccharide physically interacts with the enzymes.

Other factors also influence the molecular weight distribution of succinoglycan produced by *S. meliloti*. For example, the addition of sodium chloride to the growth medium causes an increase in the ratio of HMW:LMW succinoglycan (23). In addition to decreasing overall succinoglycan production (42), higher nitrogen levels favor the production of the LMW fraction of succinoglycan (44). In addition to its role in the regulation of succinoglycan production, the *syrM* gene product plays a complex role in succinoglycan molecular weight control. In a strain carrying a *syrM* null allele, the production of LMW succinoglycan is highly favored under low nitrogen condition (in contrast to the situation in *syrM*$^+$ cells), but high nitrogen conditions no longer favor the production of LMW succinoglycan (44).

EPS II. Although little is known about the control of EPS II molecular weight distribution, it seems reasonable to speculate that ExpR modulates certain molecular weight control mechanisms that lead to the production of symbiotically active LMW EPS II, mechanisms that are not operative when EPS II production is stimulated by a null allele of *mucR* or by low phosphate conditions. Considering that *S. meliloti* employs the ExoK and ExsH glycanases in succinoglycan molecular weight control (144) and that galactoglucanases that cleave polysaccharide chains with the same backbone as EPS II are produced by at least one other bacterium (96), it seems plausible that EPS II-degrading enzymes may also be produced by *S. meliloti*. It is also a distinct possibility that EPS II molecular weight control could be mediated at the level of *exp* gene expression, with each EPS II-inducing stimulus differentially

activating the transcription of various *exp* genes to produce distinct polysaccharide synthesis machines with varying processivities.

K antigen. The *rkpZ* gene, which is found in Rm41 but not in Rm1021, clearly plays an important role in the production of symbiotically active LMW K antigen in strain Rm41. Strain Rm1021 only produces a HMW K antigen, which is structurally distinct from that of Rm41 (110). However, introduction of the *rkpZ* gene influences the molecular weight distribution of Rm1021 K antigen, causing production of a LMW Rm1021 K antigen that can partially substitute for succinoglycan or EPS II in nodule invasion (113, 141). Similarly, introduction of *rkpZ* into *S. fredii* USDA257 causes an alteration in the molecular weight distribution of its K antigen. It has been proposed that RkpZ modifies the K antigen polymerization process by interacting with a K antigen chain length determinant system and promoting the export of smaller K antigen molecules (113).

Succinoglycan, EPS II, and K antigen Function with Varying Efficiencies to Mediate Infection Thread Initiation and Elongation.

Until recently, a rate-limiting factor in performing a detailed analysis of the dynamics and efficiency of infection thread growth was the difficulty in observing sufficient numbers of infection threads. This problem was solved by the development of a system in which *S. meliloti* strains expressing the green fluorescent protein (GFP) are used to inoculate alfalfa seedlings grown on microscope slides (53). The roots of the seedlings can be observed by fluorescence microscopy, allowing the localization and quantification of significant numbers of symbiotic events in the nodule invasion process. In order to invade a developing root nodule, rhizobia must first colonize curled root hairs (Fig. 4A). Some of the colonized, curled root hairs develop infection threads (Fig. 4B), some of which grow all the way down the root hair cell and

penetrate the root. (Fig. 4C). For each strain tested, the number of curled root hair cells colonized by *S. meliloti* can be quantified and the efficiency with which the strain in question initiates and extends infection threads from the colonized, curled root hairs can be measured. The initiation and extension of infection threads is referred to as "nodule invasion".

This GFP-based plant assay system was used to measure the efficiency of nodule invasion mediated by succinoglycan and examine the nature of the symbiotic defects of certain succinoglycan mutants (33). Strain Rm1021, which produces succinoglycan, is highly efficient at initiating and extending infection threads from colonized curled root hairs (>95% of colonized, curled root hairs develop extended infection threads). However, an Rm1021 *exoY210*::Tn*5* mutant, which fails to produce succinoglycan, initiates infection thread growth from only 10% of colonized curled root hairs and is completely deficient in infection thread extension. This indicates that succinoglycan is functioning to mediate infection thread initiation and extension. Overproduction of succinoglycan by the *exoR95*::Tn*5* and *exoS96*::Tn*5* mutants strongly interferes with the colonization of curled root hairs. Mutations that eliminate the acetyl (*exoZ341*::Tn*5*) and succinyl (*exoH154*::Tn*5*) modifications of succinoglycan perturb the molecular weight distribution of succinoglycan (see above) and markedly reduce the efficiency of infection thread growth.

A second GFP-based study compared and contrasted alfalfa nodule invasion mediated by succinoglycan, EPS II, and K-antigen (98)(**Chapter 2**). As is the case with succinoglycan, both EPS II and K antigen mediate infection thread initiation and extension. However, the three polysaccharides perform their function with markedly different efficiencies. K antigen is significantly less efficient than succinoglycan at mediating infection thread extension, and EPS II is markedly less efficient than succinoglycan at mediating both infection thread initiation and

extension. Additionally, many infection threads mediated by EPS II or K antigen had aberrant structures when compared to those mediated by succinoglycan. This suggests that the three polysaccharides are acting by related but not identical mechanisms to mediate alfalfa root nodule invasion by *S. meliloti*.

SYMBIOTIC POLYSACCHARIDES INVOLVED IN OTHER RHIZOBIAL SYMBIOSES

The *Rhizobium* sp. NGR234 - *Leucaena leucocephala* Symbiosis

An acidic exopolysaccharide produced by *Rhizobium* sp. NGR234 is crucial for the establishment of a symbiosis with *Leucaena leucocephala*. EPS-deficient (*exo*$^-$) NGR234 strains nodulate this host poorly, inducing disorganized callus structures with little or no bacterial colonization (31). The symbiotic defect of an NGR234 *exo* mutant can be partially rescued by the addition of small quantities of purified, wild type NGR234 EPS (but not heterologous saccharides), suggesting a specific signaling role for the NGR234 EPS in its symbiosis with *Leucaena leucocephala* (41).

The structure of the NGR234 EPS repeating unit (Fig. 1D) is similar to that of succinoglycan. The first five sugars of each repeating unit are identical, as are the linkages between them. This suggests that the genes involved in the synthesis of the NGR234 EPS may share significant homology to those involved in succinoglycan production by Rm1021. In fact, many of the genes required for the production of the two polysaccharides are functionally interchangeable, and the general organization of the *exo* regions are similar between the two species (149). NGR234 EPS and Rm1021 also share the ExoX-ExoY regulatory system for controlling the production of their EPS (61). ExoX regulation of NGR234 EPS production is highly similar to ExoX regulation of succinoglycan production in Rm1021 (Table 1).

Despite these similarities between the NGR234 *exo* genes and Rm1021 *exo* genes, there are clearly differences in some of the details of the production of the two exopolysaccharides. First, the side chain residues in the two repeating units are very different, suggesting that the products of distinct genes may catalyze the additions of these sugar residues. Secondly, a

plasmid containing the NGR234 *exo* region does not restore succinoglycan production to an Rm1021 strain with a deletion of a large portion of its *exo* cluster, suggesting that some genes required for succinoglycan production in Rm1021 are missing from the NGR234 *exo* region (62). Finally, though an NGR234 *exoY* mutant carrying a cosmid containing the Rm1021 *exo* region [but not the *galE* (*exoB*) gene] produces multimers of the succinoglycan repeating unit, an NGR234 *exo* region deletion mutant containing the same cosmid only produces succinoglycan monomers (62). This suggests that the NGR234 *exo* deletion mutant is missing some function involved in succinoglycan molecular weight control that is present in the wild type NGR234 strain.

Rhizobium leguminosarum Symbiotic Polysaccharides

There are three closely related biovars of *R. leguminosarum*. *R. leguminosarum* bv. *phaseoli* nodulates beans, *R. leguminosarum* bv. *trifolii* nodulates clovers, and *R. leguminosarum* bv. *viciae* nodulates peas, vetches, and lentils. It has been known for many years that *R. leguminosarum* bv. *trifolii* and *R. leguminosarum* bv. *viciae* produce acidic exopolysaccharides (Fig. 1E) required for the establishment of successful symbioses with clover and vetch, respectively, hosts that form indeterminate nodules (38). However, the EPS produced by *R. leguminosarum* bv. *phaseoli* appears to be dispensable for symbiosis with bean, which forms determinate nodules (38). For example, EPS-deficient Tn*5* mutants of *R. leguminosarum* bv. *trifolii* strains 24AR5 and SU794 are symbiotically deficient on clover, inducing the formation of uninfected nodules (28, 37). Also, a Tn*5* insertion that abolishes EPS production in *R. leguminosarum* bv. *phaseoli* 8002 does not affect symbiosis with beans, but

the same mutation in *R. leguminosarum* bv. *viciae* prevents nodulation of peas and *Vicia hirsuta* (20).

Genetic and molecular analyses of EPS production have been performed in *R. leguminosarum* bv. *viciae*, *R. leguminosarum* bv. *trifolii*, and *R. leguminosarum* bv. *phaseoli*, and the genetics of EPS production in all three strains are highly similar. The *pssA* gene has been characterized in *R. leguminosarum* bv. *viciae*, bv. *trifolii*, and bv. *phaseoli* (19, 20, 67, 136). PssA is homologous to the ExoY protein in *S. meliloti* Rm1021 (which, along with ExoF, catalyzes the first step in succinoglycan biosynthesis). Mutations in *pssA* eliminate or greatly reduce EPS production in *R. leguminosarum*. Alleles of *pssA* that severely reduce or eliminate EPS production in *R. leguminosarum* bv. *viciae* and *R. leguminosarum* bv. *trifolii* can delay effective nodulation, result in the formation of uninfected nodules, or completely prevent nodulation, depending on the host plant. The *pssC*, *pssD*, and *pssE* genes have been characterized in both *R. leguminosarum* bv. *trifolii* and bv. *viciae* (75, 136). The putative protein product of *pssC* is homologous to the *S. meliloti* ExoM and ExoW proteins, glucosyl transferases involved in succinoglycan production. A *R. leguminosarum* bv. *trifolii pssC* mutant strain, which produces only 27% as much EPS as the wild type parent, forms nitrogen-fixing nodules on Dutch white clover, though nodule appearance is delayed by 3-4 days. The predicted *pssD* and *pssE* gene products have homology to glycosyl transferases from both *Sphingomonas* S88 and *Streptococcus pneumoniae*. Null alleles of *pssD* cause *R. leguminosarum* bv. *trifolii* to form delayed, uninfected, non-nitrogen fixing nodules on clover and prevent nodule formation on vetch by *R. leguminosarum* bv. *viciae*. Insertion of a *lacZ* cassette in the *R. leguminosarum* bv. *trifolii pssE* gene does appear to impact the strain's symbiotic proficiency on clover. Because *pssD* and *pssE* are in the same complementation

group in *R. leguminosarum* bv. *viciae*, it has been proposed that they encode proteins that function together as a glycosyl transferase (136).

A number of additional *pss* genes have recently been described in *R. leguminosarum* bv. *viciae* (Genbank accession number AJ293261) (122). The *pssFGHIJS* genes are predicted to produce glucosyl transferases, and the products of the *pssK* and *pssL* genes are thought to be involved in polymerization of the EPS. The product of the *pssM* gene is predicted to function as a pyruvyl transferase, and the *pssR* gene product is thought to encode an acetyl transferase. As these genes and the functions of their products are analyzed, a model for how the *pss* genes cooperate to synthesize the *R. leguminosarum* EPS will likely emerge.

The regulation of EPS production in *R. leguminosarum* shares several similarities with succinoglycan production in *S. meliloti*. A gene analogous to the *exoR* gene in *S. meliloti* has been identified in *R. leguminosarum* bv. *viciae* (107). The *R. leguminosarum exoR* gene encodes a negative regulator of EPS synthesis that is highly homologous to *S. meliloti* ExoR. *R. leguminosarum* also contains a gene, *psiA*, whose product is functionally analogous to the *S. meliloti* ExoX protein (21, 22, 88). When present in multiple copies, the *R. leguminosarum psiA* gene (previously called *psi*) prevents EPS production. Multiple copies of *psrA* (previously called *psr*), a negative regulator of *psiA* production (22, 89), or multiple copies of *pssA*, which codes for a predicted glycosyl transferase (20), can overcome the EPS-inhibiting effect of multiple copies of *psiA*.

Similarly to *S. meliloti*, *R. leguminosarum* produces both HMW and LMW EPS fractions (146). *R. leguminosarum* also produces glycanases that cleave HMW EPS into LMW EPS. The products of *plyA* and *plyB* are predicted to encode proteins with homology to polysaccharide lyases (50). PlyB contributes substantially to the LMW EPS produced by *R.*

leguminosarum, but PlyA is only produced in small quantities in the free living state. Both PlyA and PlyB seem to require some EPS-related component to be active (154). While PlyA remains associated with the cell surface, PlyB is able to diffuse away from the cell. In addition to PlyA and PlyB, it is possible that *R. leguminosarum* may produce at least one additional EPS-cleaving glycananse (50).

The results of microscopy experiments suggest that, similar to *S. meliloti* polysaccharides, the *R. leguminosarum* EPS mediates infection thread growth on hosts that form indeterminate nodules (135). A *R. leguminosarum bv. viciae pssA* mutant that produces very small quantities of EPS can colonize curled root hairs, but rarely induces infection threads on vetch. A *R. leguminosarum bv. viciae pssD* mutant that produces no EPS can also colonize curled root hairs, but can only induce very rare, aborted infection threads. Also similar to the *S. meliloti*-alfalfa symbiosis, *R. leguminosarum* EPS function can be provided *in trans* to an EPS-deficient mutant by a second, polysaccharide proficient strain (20, 118) or by the application of purified wild type EPS (41). This suggests that *R. leguminosarum* EPS may be functioning as a specific signal to the host plant.

OUTLOOK

Are LMW *S. meliloti* polysaccharides functioning as signals to the host plant? Some of the most intriguing experiments reported to date are those in which small quantities of purified succinoglycan or EPS II are used to partially rescue the nodule invasion defect of a strain deficient in the production of symbiotic polysaccharides (10, 59, 133). These experiments provide the most direct evidence that *S. meliloti* polysaccharides are functioning as signals to the host plant and should allow precise polysaccharide structure-function relationships to be defined. Of particular interest is the issue of whether the non-carbohydrate modifications play a role in the activity of the polysaccharides or are used solely for polysaccharide molecular weight and biosynthetic control **(Chapter 4)**.

It is not yet clear what plant response could be mediated by *S. meliloti* polysaccharides. However, two attractive models are that bacterial polysaccharides are 1) modulating plant defense responses to facilitate invasion (95) or 2) signaling infection thread-specific rearrangements of the plant cytoskeleton (115). If indeed LMW *S. meliloti* polysaccharides are functioning as signals to the host plant, then one must wonder whether a receptor(s) exists that senses the presence of the polysaccharides. One attractive model is that LMW bacterial polysaccharides are perceived by a plant receptor(s) which then transduce the bacterial signal and mediate the appropriate response. If plant receptors for succinoglycan, EPS II, and K antigen exist, one intriguing structure-function issue is what features of these receptors allow recognition of only specific LMW oligomers.

Regulation of the production and molecular weight distribution of symbiotically important polysaccharides. The task of decoding the complex regulation of the production of symbiotic polysaccharides also remains. It will be interesting to determine how rhizobia

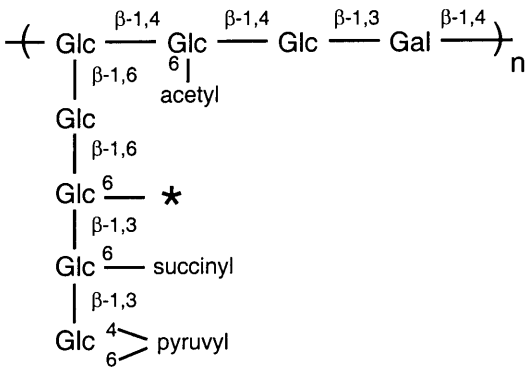
modulate symbiotically important polysaccharide synthesis and molecular weight distribution in response to environmental conditions. For example, the question of how multiple control circuits simultaneously regulate the production of EPS II needs to be addressed. It will also be of interest to elucidate how K antigen production is controlled and which currently undescribed genes are involved in the production of K antigen.

ACKNOWLEDGEMENTS

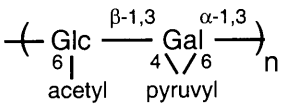
We would like to thank Brad Smith for his help in constructing figures. This work was supported by Public Health Service grant GM31030 from the National Institute of Health (NIH) to G.C.W. and NIH predoctoral training grant T32GM07287 (B.P.).

Figure 1. Structures of symbiotically important rhizobial polysaccharides. (A) The structure of the *S. meliloti* Rm1021 succinoglycan repeating unit (2, 108). The asterisk indicates the position of a second succinyl modification in some repeating units (138). (B) The structure of the *S. meliloti* Rm1021 EPS II repeating unit (55, 64). (C) The structure of the Rm41 K antigen repeating unit (110, 112). (D) The structure of the *Rhizobium* sp. NGR234 EPS repeating unit (40). (E) The repeating unit structure of the EPS produced by many *R. leguminosarum* strains (43, 84, 116). Non-stoichiometric acetate and 3-hydroxybutanoate modifications are not shown. Abbreviations: Glc, glucose; GlcA, glucuronic acid; Gal, galactose; Pse, pseudaminic acid = 5,7-diamino-3,5,7,9-tetradeoxynonulosonic acid. Figure adapted with permission from (98).

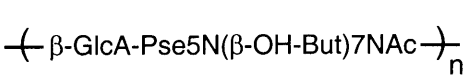
A)


β-1,4
β-1,4
β-1,3
β-1,4
Glc
Glc
Glc
Gal
n
β-1,6
6
acetyl
Glc
β-1,6
Glc 6 *
β-1,3
Glc 6 succinyl
β-1,3
Glc 4 pyruvyl
6

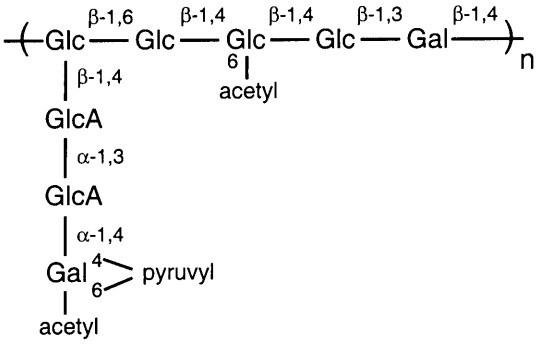
B)


β-1,3
α-1,3
Glc
Gal
n
6
4 6
acetyl
pyruvyl

C)


β-GlcA-Pse5N(β-OH-But)7NAc
n

D)


β-1,6
β-1,4
β-1,4
β-1,3
β-1,4
Glc
Glc
Glc
Glc
Gal
n
β-1,4
6
acetyl
GlcA
α-1,3
GlcA
α-1,4
Gal 4 pyruvyl
6
acetyl

E)

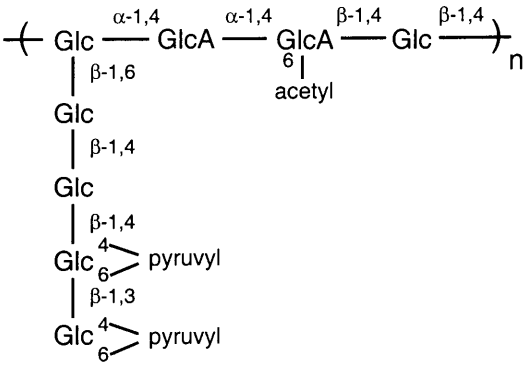

α-1,4
α-1,4
β-1,4
β-1,4
Glc
GlcA
GlcA
Glc
n
β-1,6
6
acetyl
Glc
β-1,4
Glc
β-1,4
Glc 4 pyruvyl
6
β-1,3
Glc 4 pyruvyl
6

Figure 2. (A) Schematic diagram of the *exo-exs* gene cluster from *S. meliloti* strain Rm1021. The *exo* and *exs* gene products are involved in succinoglycan production. *exo* genes are in grey, *exs* genes are in black. The thin black arrows represent transcriptional units. (B) Schematic diagram of the *exp* gene cluster from *S. meliloti* strain Rm1021. The *exp* genes are required for EPS II production in *S. meliloti* Rm1021. It was recently discovered that the *expA2* and *expA3* genes are actually one gene, designated here as *expA2/3* (11). The thin black arrows represent transcriptional units.

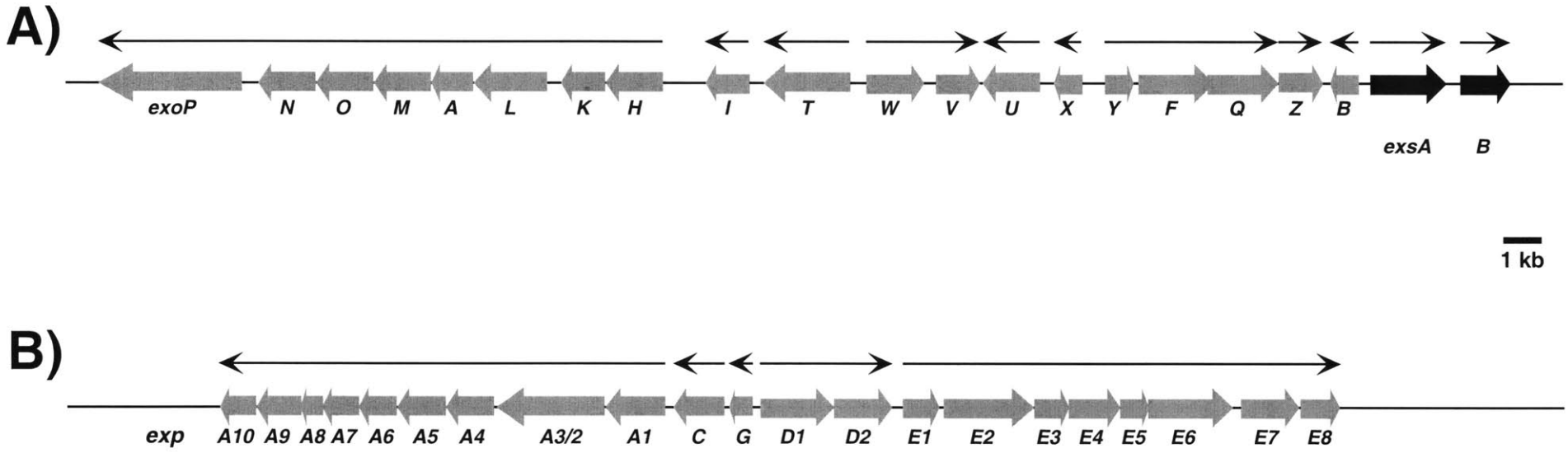

A)
exoP
N
O
M
A
L
K
H
I
T
W
V
U
X
Y
F
Q
Z
B
exsA
B
1 kb
B)
exp
A10
A9
A8
A7
A6
A5
A4
A3/2
A1
C
G
D1
D2
E1
E2
E3
E4
E5
E6
E7
E8

Figure 3. Biosynthetic model for succinoglycan production by *S. meliloti* Rm1021. The *exoC*, *exoN*, and *galE* (*exoB*) genes are involved in the synthesis of precursors. The succinoglycan repeating units are synthesized on a C-55 undecaprenyl lipid carrier. The individual sugar residues are transferred to the growing chain from UDP-glucose or UDP-galactose by a series of glycosyl transferases. The succinyl, acetyl, and pyruvyl modifications are transferred to the sugar backbone from acetyl-CoA, Succinyl-CoA, and phosphoenol pyruvate, respectively. Figure adapted with permission from (109).

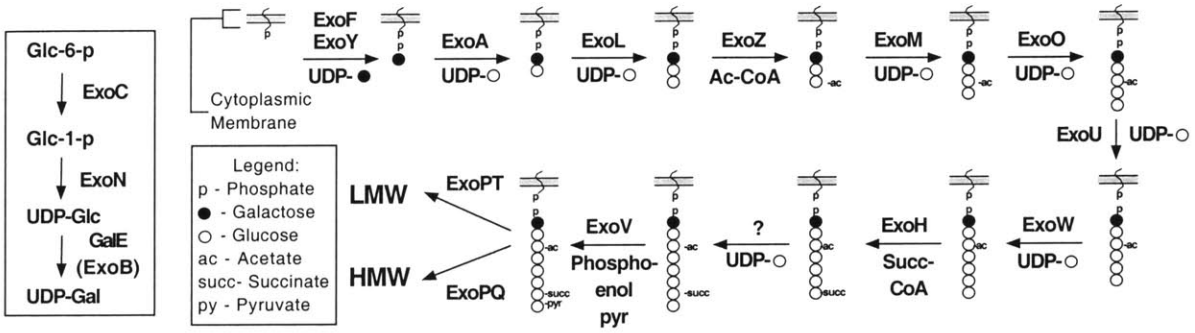

Glc-6-p
ExoC
Glc-1-p
ExoN
UDP-Glc
GalE
(ExoB)
UDP-Gal
Cytoplasmic
Membrane
ExoF
ExoY
UDP-●
ExoA
UDP-○
ExoL
UDP-○
ExoZ
Ac-CoA
ExoM
UDP-○
ExoO
UDP-○
ExoU
UDP-○
ExoW
UDP-○
ExoH
Succ-
CoA
?
UDP-○
ExoV
Phospho-
enol
pyr
ExoPT
LMW
ExoPQ
HMW
Legend:
p - Phosphate
● - Galactose
○ - Glucose
ac - Acetate
succ- Succinate
py - Pyruvate

Figure 4. Composite fluorescence micrographs of alfalfa root hair cells infected with GFP-expressing *S. meliloti* cells. The bacteria first colonize a curled root hair (A), forming a tight focus of bacterial cells. Infection threads (B and C) allow the rhizobia to proceed down the root hair cell and penetrate the root. The infection thread in panel (B) has aborted growth prior to reaching the base of the root hair cell, but the infection thread in panel (C) has extended to the base of the root hair cell. Figure adapted with permission from (98).

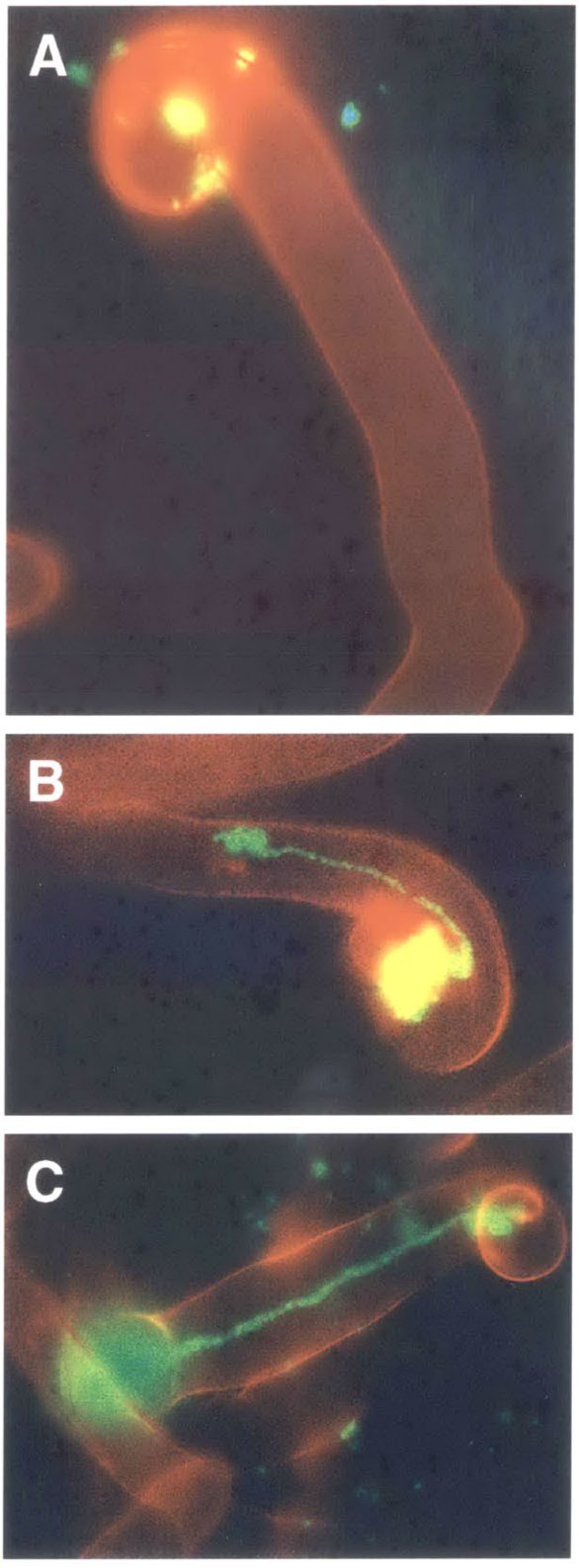

A
B
C

Table 1. Regulators of symbiotically important *S. meliloti* polysaccharides

Polysaccharide	Gene(s)	Specifics	Reg. level[a]	Ref.
Succinoglycan	*exoR*	negative regulator – null mutant makes ~11x more SG than wt. and does not reduce SG production under high nitrogen conditions	tc	(42, 104)
	exoS/chvI	positive 2 component regulatory system. ExoS96 is N-terminally truncated, constitutively active. *exoS96* mutant makes ~7.6x more SG than wt. ChvI is the response regulator.	tc	(32, 42)
	exoX	negative regulator – ExoX ≈ ExoY/F: wt SG levels. ExoX < ExoY/F: high SG levels. ExoX > ExoY/F: low SG levels	post-tl	(105, 149)
	mucR	positive regulator – null mutant makes less SG than wt. MucR binds upstream of *exoH* and *exoY* and is required for production of HMW SG	post-tc	(70, 151)
	exsB	negative regulator – null mutant makes ~3x more SG than wt. multiple copies reduce SG production ~5x	post-tc	(15)
	syrA, syrM	SyrA is a positive regulator – multiple copies induce SG production in *syrM*-dependent fashion. SyrM positively regulates *syrA* and is involved in SG molecular weight control.	tc[b],post-tl[c]	(8, 44, 92)
	exoD	null mutant makes ~2-4x less SG than wt in the absence of nitrogen and ~7x more SG than wt in the presence of nitrogen.	non-specific	(106)
	lon	protease mutant – thought that a *lon* null mutant does not degrade positive regulators of SG production	non-specific	(129)
EPS II	*expR*	positive regulator – the *expR101* allele of *expR* turns on production of LMW to HMW EPS II.	tc	(55)
	mucR	negative regulator – null mutant produces HMW EPS II (not symbiotically active)	tc	(70, 151)
	expG	positive regulator – multiple copies turn on production of EPS II. involved in *phoB*-mediated EPS II production. not involved in *mucR*-mediated EPS II production.	tc	(4, 120)
	phoB	positive regulator – required for production of very HMW EPS II in response to phosphate starvation	tc	(120)
	lon	protease mutant – thought that a *lon* null mutant does not degrade positive regulators of EPS II production	non-specific	(129)
	phoCDET	encodes a phosphonate uptake system. null mutants in this locus are phosphate starved, leading to EPS II production	non-specific	(7)

a – tc, transcriptional; tl, translational

b – *syrM* regulation of *syrA* is transcriptional

c – *syrA* regulation of succinoglycan production is thought to be post-translational

Chapter 2

Alfalfa Root Nodule Invasion Efficiency is Dependent on

Sinorhizobium meliloti Polysaccharides*

* **Pellock, B. J., Cheng, H.-P., and G. C. Walker.** 2000. Alfalfa root nodule invasion efficiency is dependent on *Sinorhizobium meliloti* polysaccharides. J. Bacteriol. 182:4310-4318.

ABSTRACT

The soil bacterium *Sinorhizobium meliloti* is capable of entering into a nitrogen-fixing symbiosis with *Medicago sativa* (alfalfa). Particular low molecular weight forms of certain polysaccharides produced by *S. meliloti* are crucial for establishing this symbiosis. Alfalfa nodule invasion by *S. meliloti* can be mediated by any one of three symbiotically important polysaccharides: succinoglycan, EPS II, or K-antigen (also referred to as KPS). Using green fluorescent protein (GFP)-labeled *S. meliloti* cells, we have shown that there are significant differences in the details and the efficiencies of nodule invasion mediated by each polysaccharide. Succinoglycan is highly efficient in mediating both infection thread initiation and extension. However, EPS II is significantly less efficient than succinoglycan at mediating both invasion steps, and K-antigen is significantly less efficient than succinoglycan at mediating infection thread extension. In the case of EPS II-mediated symbioses, the reduction in invasion efficiency results in stunted host plant growth relative to plants inoculated with succinoglycan or K-antigen-producing strains. Additionally, EPS II and K-antigen mediated infection threads are 8-10 times more likely to have aberrant morphologies than those mediated by succinoglycan. These data have important implications for understanding how *S. meliloti* polysaccharides are functioning in the plant-bacterium interaction, and models are discussed.

INTRODUCTION

The establishment of the symbiotic relationship between the soil bacterium *Sinorhizobium meliloti* (also referred to as *Rhizobium meliloti*) and its host plant, *Medicago sativa* (alfalfa) is a complex process involving the exchange of a series of signals between the plant and bacteria (24, 36, 83, 114). Flavonoid compounds released into the soil by alfalfa attract rhizobia and stimulate bacterial production of Nod factors, lipochitooligosaccharides that trigger root hair curling and the formation of root nodules on the host plant. *S. meliloti* cells trapped in curled root hairs invade the developing root nodule via tubes known as infection threads and are ultimately released into the nodule where they differentiate into nitrogen-fixing bacteroids.

Polysaccharides produced by *S. meliloti* are critical for the establishment of a productive plant-bacteria symbiosis. Bacterial mutants that fail to produce certain polysaccharides are substantially impaired in their ability to invade developing root nodules and thus primarily yield root nodules devoid of bacteria and bacteroids (55, 79, 94, 113). The wild type *S. meliloti* laboratory strain Rm1021 is capable of producing two symbiotically important exopolysaccharides, termed succinoglycan and EPS II. Both succinoglycan and EPS II can be produced in symbiotically active forms (i.e.- forms sufficient to mediate nodule invasion). In culture, Rm1021 produces succinoglycan alone; EPS II is not produced at detectable levels. EPS II production by Rm1021 can occur in the presence of the *expR101* mutation (55), a *mucR*::Tn*5* mutation (70), or very low phosphate conditions (152). However, symbiotically active EPS II is only produced in an *expR101* derivative of strain Rm1021 (59, 86). Rm41, an independently isolated wild type *S. meliloti* strain, is also able to produce

succinoglycan. However, it also produces a symbiotically active form of a capsular polysaccharide, termed K-antigen (also known as KPS), which can substitute for succinoglycan and EPS II in mediating the nodule invasion step of symbiosis (102, 111, 113). Rm1021 lacks the ability to produce symbiotically active K-antigen (113) and is therefore dependent on EPS production for root nodule invasion.

Intriguingly, succinoglycan, EPS II, and K-antigen are structurally diverse polysaccharides. Succinoglycan is a polymer of an octasaccharide repeating unit composed of one galactose and seven glucose residues with acetyl, succinyl, and pyruvyl modifications (Fig. 1A) (2, 108), whereas EPS II is a polymer of a glucose-galactose disaccharide repeating unit modified with acetyl and pyruvyl substituents (Fig. 1B) (55, 64). K-antigen has a disaccharide repeating unit containing glucuronic acid and a modified pseudaminic acid residue (Fig. 1C) (112). It is currently unclear how these three structurally distinct polysaccharides are each able to mediate root nodule invasion by *S. meliloti*, though it appears that low molecular weight forms of all three polysaccharides are the symbiotically active forms (10, 59, 138). In the case of succinoglycan, the symbiotically active form is the trimer of the octasaccharide repeating unit (138), and in the case of EPS II, it is the class of oligosaccharides consisting of 15-20 disaccharide repeating units (59).

Accumulating evidence suggests that low molecular weight polysaccharides are acting as signaling molecules that trigger developmental responses in the host plant or regulate host defense responses. First, small quantities of purified, low molecular weight succinoglycan or EPS II can partially rescue the nodule invasion defect of a strain producing no symbiotically active polysaccharide (10, 59, 133, 138). Second, polysaccharide function can be provided *in trans* to a strain producing no symbiotically active polysaccharide by a second, polysaccharide

proficient strain (73, 91). Finally, strains carrying mutations that perturb the molecular weight distribution of succinoglycan or EPS II have a reduced nodule invasion ability (33, 78).

Recently, our laboratory (33) has refined a previously described system (53) for examining the kinetics and efficiency of nodule invasion events in the alfalfa-*S. meliloti* symbiosis. Briefly, alfalfa plants growing on glass microscope slides coated with nitrogen-free media are inoculated with *S. meliloti* constitutively expressing the green fluorescent protein (GFP) from a stably-maintained plasmid vector. Early events in symbiotic nodulation, including formation of colonized, curled root hairs, initiation of infection threads, and extension of infection threads are visualized (Fig. 1D) and quantified using fluorescence microscopy. Using this technique, we previously (33) examined the role of succinoglycan in symbiosis and determined that succinoglycan production by strain Rm1021 is important for infection thread initiation and absolutely required for infection thread extension. Additionally, we determined that the presence of the acetyl and succinyl modifications of succinoglycan are important for infection thread extension and that overexpression of succinoglycan profoundly reduces the efficiency of colonized, curled root hair formation.

To gain further insight into the mechanism by which three *S. meliloti* polysaccharides of such strikingly different structures can promote the common biological outcome of nodule invasion, we used our GFP-based approach to analyze nodule invasion by *S. meliloti* derivatives that produced only EPS II or K-antigen. Our work has revealed that succinoglycan, EPS II, and K-antigen do not function equally well in mediating root nodule invasion. Succinoglycan is more efficient than K-antigen and much more efficient than EPS II in mediating the growth infection threads on alfalfa. In addition, we have observed distinct morphologies for infection threads promoted by different polysaccharides. This suggests that

succinoglycan, EPS II, and K-antigen are acting by related but not identical mechanisms to promote nodule invasion.

MATERIALS AND METHODS

Bacterial strains and growth media.

The strains used in this study are listed in Table 1. Strain Rm9011 was constructed by generalized transduction (49) of the *expA3*::*lacZ* (Gm^r) allele from strain RmAR1011 (16) into Rm9000. Strains were grown in Luria-Bertani (LB) liquid medium supplemented with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$. Antibiotics were used at the following concentrations: streptomycin, 500 μg/ml; neomycin, 200 μg/ml; gentamycin, 50 μg/ml; spectinomycin, 100 μg/ml; tetracycline, 10 μg/ml. All strains used for fluorescence microscopy analyses contained pHC60 (33), a stably-maintained plasmid that constitutively expresses GFP.

Nodulation assays.

Standard plant assays were performed as described (79), and each plant was inoculated with one milliliter of a cell suspension with an $O.D._{600}$ of 0.05. Plant height means and nodule percentage means were calculated from data from several separate inoculations. At least 60 total plants inoculated with each strain were scored. The error ranges represent the standard error of the mean computed using mean values from groups of five to ten plants.

Nodulation of alfalfa (*M. sativa* cv. Iroquois) by *S. meliloti* for GFP analyses was analyzed on microscope slides as described by Cheng and Walker (33). To determine the kinetics of nodule invasion, at least three separate sets of 12 plants were inoculated with each strain and examined by fluorescence microscopy for 12 days. The overall efficiencies of nodule invasion for each strain were calculated from data collected on day 12 of the experiments. Infection threads that had a wide, irregular region in excess of double the width of another

section of the thread were scored as aberrant. Additionally, any infection thread with more than one densely packed pocket of bacteria along its length was scored as aberrant.

Fluorescence microscopy.

Alfalfa seedlings inoculated with GFP-expressing *S. meliloti* strains were examined at 100X magnification (10X eyepiece, 10X objective) using a Zeiss fluorescence microscope model Axioskop (Carl Zeiss, Inc., Thornwood, NY) equipped with a FITC filter set . Image acquisition was performed at 400X magnification (10X eyepiece, 40X objective) using a Zeiss fluorescence microscope model Axioplan2 equipped with a integrated 3-chip cooled CCD color camera (model DEI-750T; Optronics Engineering). Images of GFP-expressing *S. meliloti* cells were obtained using a filter set consisting of a 460- to 500-nm bandpass exciter, a 505-nm longpass dichroic filter, and a 510-nm longpass emitter (model 41012; Chroma, Brattleboro, VT). Images of root hair cells were obtained using a filter set consisting of a 510- to 560-nm bandpass exciter, a 565-nm longpass dichroic filter, and a 582- to 647-nm bandpass emitter (model 41002; Chroma). Images were captured using Scion Image 1.62 (public domain) installed on a Power Macintosh 8600/200 computer. Composite images were generated using Adobe Photoshop 4.0 software.

Acetylene reduction assays.

Acetylene reduction assays were performed as follows: 1.5 ml of acetylene gas (generated by the reaction of water and calcium carbide) was injected into stoppered 15 ml tubes containing alfalfa root systems from four week old plants inoculated with various *S. meliloti* strains. The tubes were incubated for 10 hours before analysis. A 100 μl sample

from each tube was analyzed for the presence of acetylene and ethylene using a Shimadzu GC-9A gas chromatograph equipped with a Porapak N (Supelco, Bellafonte, PA) molecular sieve column (6 feet x 1/8 inch, mesh size 80/100) and a flame ionization detector. The flow rate of the nitrogen carrier gas was set at 40 ml/minute. The injector temperature was 100°C, and the column temperature was 65°C. Under these conditions, ethylene typically eluted after 60 seconds, while acetylene eluted after 90 seconds. Following the incubation and gas analyses, all pink nodules were removed and weighed. The amount of ethylene produced per pink nodule fresh weight was calculated for Rm1021, Rm9000, Rm8530, Rm41, and AK631. No acetylene reduction was observed for root systems from plants that had been inoculated with water alone, Rm7210, Rm9011, or PP674.

RESULTS

Alfalfa plants inoculated with succinoglycan-producing *S. meliloti* strains grow better than those inoculated with a strain producing EPS II alone. Over the course of many separate inoculations in standard plant assays scored four weeks after inoculation, it became clear that the mean plant height of alfalfa (*M. sativa* cv. Iroquois) inoculated with a succinoglycan-producing *S. meliloti* strain (Rm1021) was noticeably greater [8.0cm (±1.4cm)] than the average height of plants inoculated with Rm9000 [3.7cm (±0.8cm)], an Rm1021 derivative producing only EPS II. Additionally, 93.0% (±2.7%) of the nodules on plants inoculated with the strain producing succinoglycan were pink, nitrogen-fixing nodules, whereas only 55.3% (±3.8%) of the nodules induced by a strain producing EPS II alone were pink four weeks after inoculation. Since pink nodules are symbiotically successful, this suggested that the strain producing EPS II alone was forming nitrogen-fixing nodules at a reduced rate. We did not observe a significant difference in either plant height [8.8cm (±1.6cm) for Rm41 vs. 8.37cm (±1.9cm) for AK631] or percentage of nodules that were pink [90.4% (±3.3%) for Rm41 vs. 89.1% (±3.9%) for AK631] on plants inoculated with AK631, a strain that produces K-antigen alone, or its wild type parent, Rm41, which produces both succinoglycan and K-antigen. Very similar standard plant assay results were obtained regardless of whether alfalfa plants were inoculated with strains carrying the GFP plasmid [pHC60 (33), the pHC60 parent plasmid [pHC41 (33)], or containing no plasmid (data not shown).

To determine whether the stunted growth of the EPS II-inoculated plants relative to those inoculated with succinoglycan or K-antigen producing strains was due to a deficiency in the ability of the EPS II-producing strain to fix nitrogen, we used acetylene reduction assays to

compare the nitrogen-fixing capacity of pink nodules taken from four week old alfalfa plants inoculated with *S. meliloti* strains that differed with respect to the polysaccharides they produced. Pink nodules from plants inoculated with strains producing various polysaccharides (Rm1021, Rm9000, Rm8530, Rm41, or AK631) reduced acetylene to ethylene with similar efficiencies in terms of the amount of ethylene generated per pink nodule fresh weight (data not shown). This suggested that the above differences in plant condition at four weeks were not due to an inherent decrease in the nitrogen-fixing capacity/potential of the EPS II-producing strain relative to the other strains examined. Additionally, the total number (both pink and white) of nodules induced by the EPS II-producing strain was comparable to the quantity of nodules induced by the succinoglycan or K-antigen producing strains (data not shown), and the kinetics of nodule appearance were similar in all strains examined (data not shown). This suggested that the stunted growth of the EPS II-producing strain was not due to a defect in the strain's ability to induce nodule formation. Taken together with the difference in the percentage of pink nodules, there results suggested that there might be a difference in nodule invasion mediated by EPS II and nodule invasion mediated by succinoglycan or K-antigen.

EPS II-mediated nodule invasion is less efficient than succinoglycan mediated-nodule invasion. To determine whether the differences observed in the standard plant assays were the result of an invasion defect in the EPS II-producing strain, we used GFP-labelled *S. meliloti* strains to examine the kinetics (Fig. 2) and efficiency (Fig. 3) of nodule invasion in the symbioses mediated by succinoglycan, EPS II, and K-antigen. At least thirty-six plants inoculated with each strain were scored daily for the appearance of colonized curled root hairs, initiated infection threads, and extended infection threads (Fig. 2). Plants inoculated with the

succinoglycan-producing strain, Rm1021, developed colonized curled root hairs, initiated infection threads, and extended infection threads beginning on day four, and the numbers of all three events increased rapidly until day eight or nine at which time they reached a plateau (Fig. 2A). A high percentage (>98%) of colonized curled root hairs initiated infection threads, and most of the infection threads (>92%) were extended to the base of the root hair (Fig. 3). As noted previously (33), nearly all (>95%) of the nodules induced by GFP-expressing Rm1021 fluoresced very brightly throughout when viewed under the fluorescence microscope.

The EPS II-producing strain (Rm9000) also developed colonized curled root hairs on day four, but, in contrast to Rm1021, substantial numbers of initiated infection threads and extended infection threads did not appear until day five (Fig. 2A). Furthermore, relative to the succinoglycan-producing strain, the EPS II-producing strain exhibited a 40-50% reduction in both the total number of colonized curled root hairs and the percentage of colonized curled root hairs that initiated infection threads (Fig. 3A,B). Strikingly, less than 20% of colonized curled root hairs on plants inoculated with the EPS II-producing strain developed extended infection threads compared to the >90% seen with succinoglycan (Fig. 3C). Also, in contrast to the high percentage (>95%) of bright nodules induced by succinoglycan-producing strains, a significant proportion (~50%) of the nodules induced by Rm9000 were dark on day 12 when viewed under the fluorescence microscope, suggesting that these nodules had not been filled with bacteria. These analyses clearly indicate that nodule invasion mediated by EPS II proceeds less efficiently than nodule invasion mediated by succinoglycan.

In the course of our GFP analyses of succinoglycan and EPS II-mediated symbioses, we observed that infection threads formed on plants inoculated with the *S. meliloti* strain producing EPS II alone frequently had unusual morphologies relative to those induced by succinoglycan-

producing strains. Infection threads induced by succinoglycan-producing strains almost always maintained a constant width and were located in the centers of the root hairs (Fig. 4A). This regular infection thread phenotype was observed in 96.4% (±1.2%) of infection threads observed on plants inoculated with succinoglycan-producing strains. In the rare instances that succinoglycan-mediated infection threads aborted prior to reaching the base of the root hair, they usually had, at most, one densely populated pocket of bacteria located at the terminus of the aborted infection thread (Fig. 4B). In contrast, we observed that 39.5% (±3.5%) of EPS II-mediated infection threads had aberrant morphologies compared to typical succinoglycan-mediated infection threads. Infection threads that had a wide, irregular region in excess of double the width of another section of the thread and threads that had more than one densely packed pocket of bacteria along their length were classified as aberrant. A majority (66%) of aberrant EPS II-mediated infection threads exhibited irregular width variation (Fig. 4E), though some (34%) had constant widths and had two or more densely packed pockets of bacteria along their lengths (Fig. 4D). Just over half (53%) of aberrant EPS II-mediated infection threads aborted before reaching the base of the root hair cell.

***expR101*-induced EPS II production does not compromise the efficiency of succinoglycan-mediated nodule invasion.** The fact that EPS II is less efficient than succinoglycan at mediating nodule invasion raised the question of whether EPS II production either by a succinoglycan-producing strain or by a coinoculated second strain could interfere with succinoglycan-mediated nodule invasion. However, strain Rm8530 (Rm1021 *expR101* *exo*$^{+}$), which produces both succinoglycan and EPS II, was able to invade nodules with virtually the same kinetics, efficiency, and infection thread morphology as strain Rm1021

which produces succinoglycan alone (data not shown). When alfalfa plants were coinoculated with a 1:1 ratio of GFP-expressing Rm1021 cells (which produce only succinoglycan) and GFP-expressing Rm9000 cells (which produce only EPS II), we also observed Rm1021-like nodule invasion kinetics, nodule invasion efficiency, and infection thread morphology. This indicates that neither *expR101*-induced production of EPS II in a succinoglycan-producing strain nor EPS II production by a coinoculated strain interfere with the efficiency of succinoglycan in mediating nodule invasion.

A *S. meliloti* strain producing K-antigen alone is less proficient at mediating infection thread extension than a strain producing both succinoglycan and K-antigen. Rm41, an independently isolated *S. meliloti* strain, can produce symbiotically active forms of both succinoglycan and K-antigen. In GFP-based plant assays, Rm41 performed similarly to Rm1021, the strain that makes succinoglycan alone, in terms of its ability to colonize curled root hairs, initiate infection threads, and extend infection threads (Figs. 2B, 3). Based on the results of our standard plant assays, we expected that K-antigen-mediated nodule invasion would proceed with approximately the same kinetics and efficiency as observed for strain Rm41 (Figs. 2B, 3). We were therefore surprised to observe a slight delay relative to Rm41 in the appearance of extended infection threads on plants inoculated with AK631, an Rm41 derivative that produces only K-antigen (Fig. 2B). Additionally, although AK631 was able to colonize curled root hairs and initiate infection threads with approximately the same efficiency as Rm41, it exhibited a sizable reduction in the efficiency of infection thread extension relative to Rm41; fewer than 35% of colonized curled root hairs on a plant inoculated with strain AK631 developed extended infection threads (Fig. 3). However, this reduced efficiency of

infection thread extension did not appear to negatively impact the results of standard plant assays using the K-antigen producing strain. Nearly all (>95%) of the nodules induced on plants inoculated with AK631 fluoresced brightly throughout when viewed under the fluorescence microscope on day 12. This suggested that, despite the reduced occurrence of extended infection threads, most nodules were associated with a successful infection event (not all infection threads are nodule-associated).

Similar to EPS II-mediated infection threads, 40.7% (±3.4%) of the infection threads formed by an *S. meliloti* strain producing only K-antigen were aberrant, compared to only 6.4% (±3.35%) on plants inoculated with Rm41. Aberrant K-antigen-mediated infection threads almost always (96%) consisted of threads of constant widths having two or more densely packed pockets of bacteria along the length of the thread (Fig. 4F,G). Interestingly, almost half (45.5%) of aberrant K-antigen-mediated infection threads were extended to the base of the root hair cell (Fig. 4F,G), suggesting that the infection thread had aborted/stalled then restarted several times during the extension process, resulting in the formation of several densely packed pockets of bacteria along the length of the thread.

As was the case with EPS II production in a succinoglycan-producing strain, the production of K-antigen by Rm41 did not appear to interfere with the high efficiency of succinoglycan-mediated nodule invasion (Figs. 2B, 3). Additionally the efficiencies of nodule invasion and the infection thread morphologies that we observed in Rm41/AK631 and Rm1021/AK631 coinoculations (data not shown) were similar to those seen in inoculations using Rm1021 alone (Figs. 2A, 3) or Rm41 alone (Figs. 2B, 3).

EPS II and K-antigen-producing strains prevented from producing any symbiotically active polysaccharides are not defective in colonization of curled root hairs. Previously we observed that Rm7210 (Rm1021, *exoY210*::Tn*5*), a succinoglycan-deficient mutant of Rm1021, was highly compromised in its ability to initiate and extend infection threads but retained its ability to colonize curled root hairs (33). To determine whether either the *expR101* mutation in the EPS II-producing strain Rm9000 or the *galE* (*exoB*) mutation in the K-antigen-producing strain AK631 resulted in a reduced ability to colonize curled root hairs, we compared the kinetics and efficiency of nodule invasion of Rm9011 [Rm9000 *expA3*::*lacZ* (Gm^r)], an EPS II-deficient derivative of Rm9000, and PP674 (AK631 *rkpA*::Tn*5*), a K-antigen-deficient derivative of AK631, to that of Rm7210 (Rm1021 *exoY210*::Tn*5*). All three strains performed similarly in terms of their infection kinetics (data not shown) and overall nodule invasion efficiencies (Fig. 3). Rm7210, Rm9011, and PP674 were able to colonize curled root hairs approximately as well as Rm1021 and Rm41, but were substantially compromised in their abilities to initiate infection threads and completely deficient in infection thread extension; most of the symbiotic events observed on plants inoculated with *S. meliloti* strains deficient in the production of succinoglycan, EPS II, and K-antigen consisted of colonized, curled root hairs with no infection threads (Fig. 4C). These analyses demonstrated that neither the *expR101* mutation nor the *galE* (*exoB*) mutation significantly reduced the colonized, curled root hair formation efficiencies of strains carrying them. As expected, these data indicated that, like succinoglycan in our previous investigation (33), EPS II and K-antigen mediate infection thread initiation and extension in symbiosis.

DISCUSSION

Using *S. meliloti* strains constitutively expressing GFP from a stably-maintained plasmid vector, we have shown that there are clearly defined quantitative and qualitative differences in the abilities of succinoglycan, EPS II, and K-antigen to mediate *M. sativa* cv. Iroquois (alfalfa) root nodule invasion. K-antigen is less efficient than succinoglycan at mediating infection thread extension, whereas EPS II is less efficient than succinoglycan at mediating both infection thread initiation and extension. Constitutive GFP expression did not appear to substantially impact the symbiotic efficiencies of the strains used in this study.

The differences in the efficiencies of nodule invasion mediated by succinoglycan, EPS II, and K-antigen suggest that the three polysaccharides seem to act through related but different mechanisms. Specific low molecular weight forms of succinoglycan or EPS II provided at low levels are sufficient to promote nodule invasion (10, 59, 138), and it appears that low molecular weight forms of K-antigen are similarly the symbiotically active form (113). For this reason, it seems probable that succinoglycan, EPS II, and K-antigen are acting as signals to the plant, likely by signaling via a plant receptor or receptors. In one model (Fig. 5A), one plant receptor is able to recognize all three polysaccharides. In this three-polysaccharide - one receptor model, the differences in nodule invasion-promoting activity among the three polysaccharides would most likely result from varying interactions of three structurally diverse polysaccharides with the single receptor. In an alternate class of models (Fig. 5B,C), a distinct receptor is proposed to exist for each polysaccharide. Though it is formally possible that each of the three receptors could signal a completely distinct signal transduction pathway (Fig. 5C), it would seem that a more plausible version of this type of

model would be one in which the signals from the three receptors feed into a common signal transduction pathway involved in nodule invasion (Fig. 5B). In three polysaccharide - three receptor models, the varying efficiencies of nodule invasion mediated by succinoglycan, EPS II, or K-antigen would most likely result from inherent differences in the signal transduction pathways that are specific to each receptor. The fact that both strains producing EPS II alone and strains producing K-antigen alone induce distinct aberrant infection threads seems easiest to explain by a model in which there are three separate receptors that influence a common invasion pathway in somewhat different fashions (Fig. 5B).

The model that we presently favor (Fig. 5B) is schematically simple, but, if one considers what is known about the perception of Nod factor by host legumes, it is very likely that signal transduction pathways involved in the recognition of three structurally diverse signal polysaccharides are far more complicated than those represented in our figure. A core set of genes involved in Nod factor production is conserved among the rhizobia, and the various Nod factors produced by rhizobial strains adhere to common structural themes. Even so, isolation of candidate Nod factor receptors has proven very challenging [see (93) for review], partially due to the identification of multiple candidates with varying affinity for Nod factors, the possibility that lipochitooligosaccharides are a general class of developmental signaling molecules, and the fact that slightly different Nod factor molecules appear to control different aspects of the host's response to Nod factor. Because succinoglycan, EPS II, and K-antigen have very different structures and each has at least one distinct cluster of genes involved in its synthesis (16, 55, 71, 72, 82), it seems very likely that the perception of each of these three polysaccharides by a host plant is at least as complex as the plant's perception of Nod factors.

Though succinoglycan, EPS II, and K-antigen mediate infection thread initiation and extension, it is not clear which plant functions are being modulated. One attractive possibility is that rhizobial polysaccharides function as signals that direct cytoskeletal movements in the root hair cell which stimulate infection thread initiation and maintenance (115). Another intriguing possibility is that bacterial polysaccharides could function as signals that affect plant defense responses (95). The aberrant infection thread structures seen on plants inoculated with strains producing EPS II alone or K-antigen alone could result from less efficient modulation of plant cytoskeletal movements or less efficient modulation of host defense responses. Many aberrant infection thread events consist of several densely packed pockets of bacteria along the length of the infection thread (Fig. 4F), and some aberrant threads are wider than typical succinoglycan-mediated infection threads (Fig. 4E). Because those few succinoglycan-mediated infection threads that abort prior to reaching the base of the root hair cell tend to have one such pocket of bacteria at the point of termination (Fig. 4B), those threads that have two or more such pockets along their lengths may have arisen from multiple rounds of infection thread stalling/abortion and reinitiation. Both stalling/abortion or a widening of the infection thread could either be a result of inefficiently modulated plant defenses acting to halt or slow bacterial invasion or a consequence of inefficient modulation of plant cytoskeletal components.

The decrease in nodule invasion efficiency of a strain producing EPS II alone is manifested in reductions (relative to plants inoculated with succinoglycan-producing strains) in both the mean plant height and the percentage of root nodules that are pink after a four week growth period. However, plants inoculated with a strain producing K-antigen alone do not manifest stunted phenotypes, despite the observed reduction in efficiency of nodule invasion. The stunting seen in plants inoculated with the strain producing EPS II alone appears to be a

result of its invasion deficiency rather than a deficiency in the ability of the strain to induce root nodule formation or a defect in the innate ability of the strain to fix nitrogen. Our interpretation of this is that the reduced invasion efficiency of the strain producing EPS II alone (reduced infection thread initiation and extension) results in a delay in the appearance of symbiotically successful, nitrogen-fixing nodules. In contrast, the reduction in infection thread extension efficiency seen with the strain producing K-antigen alone, though measurable, is not severe enough to result in a delay in the establishment of a productive symbiosis. Though pink nodules from plants inoculated with the EPS II-producing strain have the same nitrogen-fixing capacity per unit of fresh weight as pink nodules from plants inoculated with strains producing succinoglycan or K-antigen, fewer symbiotically effective (pink) nodules are present on plants inoculated with strains producing EPS II alone.

Since a number of *S. meliloti* strains produce more than one symbiotically important polysaccharide (80, 101, 147), it seems possible that certain bacterial polysaccharides may be more efficient in mediating root nodule invasion on specific hosts or more efficient under specific environmental conditions. Thus, production of more than one symbiotically important polysaccharide by *S. meliloti* may provide a strain with a selective advantage, allowing the strain to interact as efficiently as possible under a variety of conditions with many cultivars or ecotypes of legumes that it can nodulate. Succinoglycan may be the most efficient polysaccharide of the three at mediating root nodule invasion on *M. sativa* cv. Iroquois under our assay conditions, but EPS II or K-antigen may be more efficient than succinoglycan on another host plant or under different environmental conditions. It is also possible that some hosts of *S. meliloti* only have receptors for a subset of the three known symbiotically important polysaccharides produced by the bacterium. Either possibility could explain our lab's previous

observation (55) that, while succinoglycan is able to mediate nodule invasion on alfalfa, white clover, yellow clover, fenugreek, and the one ecotype of *Medicago truncatula* tested, EPS II is able to mediate nodule invasion on alfalfa, but not the other hosts.

Given the general importance of rhizobial polysaccharides in the establishment of a successful symbiosis, the data presented in this work may be useful for thinking about the role(s) of rhizobial polysaccharides in determining host range and efficiencies of symbioses other than that between *S. meliloti* and alfalfa. For example, *Bradyrhizobium japonicum* strain 2143 and two derivative strains are capable of producing three exopolysaccharides that appear to be involved in the efficiency of their symbioses with *Glycine max* (68), and *B. japonicum* strain USDA 123 produces two structurally distinct polysaccharides, one when outside the nodule and the second when inside the nodule (3). Additionally, the symbiotic defects of EPS-deficient mutants of *B. japonicum* strain 110*spc*4 are host dependent, differing markedly on the hosts *Glycine max* and *G. soja* (97), and there are many other rhizobia for which production of specific polysaccharides is important for symbiosis, including *Rhizobium* sp. NGR234 (31), *R. leguminosarum* bv. *viciae* (38), *R. leguminosarum* bv. *trifolii* (117, 126), and *R. loti* (65). Thus, the conclusions from this work are likely to be broadly applicable to analyses of bacterial polysaccharides that mediate interactions with host plants .

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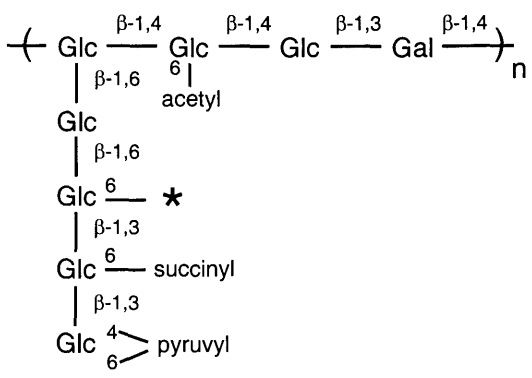
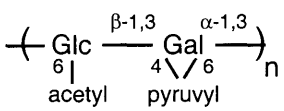
Figure 1. (A) Structure of the succinoglycan repeating unit. The (*) indicates the position of a second succinyl modification (138) in some repeating units. (B) The structure of the EPS II repeating unit. (C) The structure of the K-antigen repeating unit. Pse = pseudaminic acid = 5,7-diamino-3,5,7,9-tetradeoxynonulosonic acid. (D) Schematic diagram of a curled root hair and the three indices scored in GFP-based plant assays: a colonized curled root hair (CCRH), a CCRH with initiated infection thread (IT), and a CCRH with extended infection thread [adapted with permission from (33)].

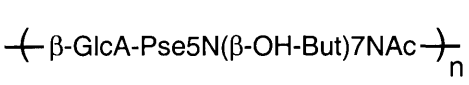
A)


β-1,4
β-1,4
β-1,3
β-1,4
Glc
Glc
Glc
Gal
n
β-1,6
6
acetyl
Glc
β-1,6
Glc
6
*
β-1,3
Glc
6
succinyl
β-1,3
Glc
4
6
pyruvyl

B)


β-1,3
α-1,3
Glc
Gal
n
6
4
6
acetyl
pyruvyl

C)


β-GlcA-Pse5N(β-OH-But)7NAc
n

D)

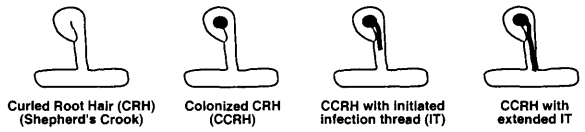

Curled Root Hair (CRH)
(Shepherd's Crook)
Colonized CRH
(CCRH)
CCRH with initiated
infection thread (IT)
CCRH with
extended IT

Figure 2. Kinetics of colonized curled root hair (CCRH) formation, infection thread (IT) initiation, and IT extension on alfalfa plants inoculated with various *S. meliloti* strains. Sets of at least 36 plants were inoculated with (A) Rm1021 [succinoglycan (SG)-producing strain] or Rm9000 (EPS II-producing strain) or with (B) Rm41 (succinoglycan and K-antigen / KPS-producing strain) or AK631 (K-antigen / KPS-producing strain). The number of CCRHs, initiated ITs, and extended ITs per plant were recorded for 12 days. The CCRH count includes those with initiated and extended ITs, and the initiated IT count includes extended ITs. Standard error of the mean calculations were performed on the mean daily values from at least three groups of 12 plants. The standard errors for each time point were non-overlapping.

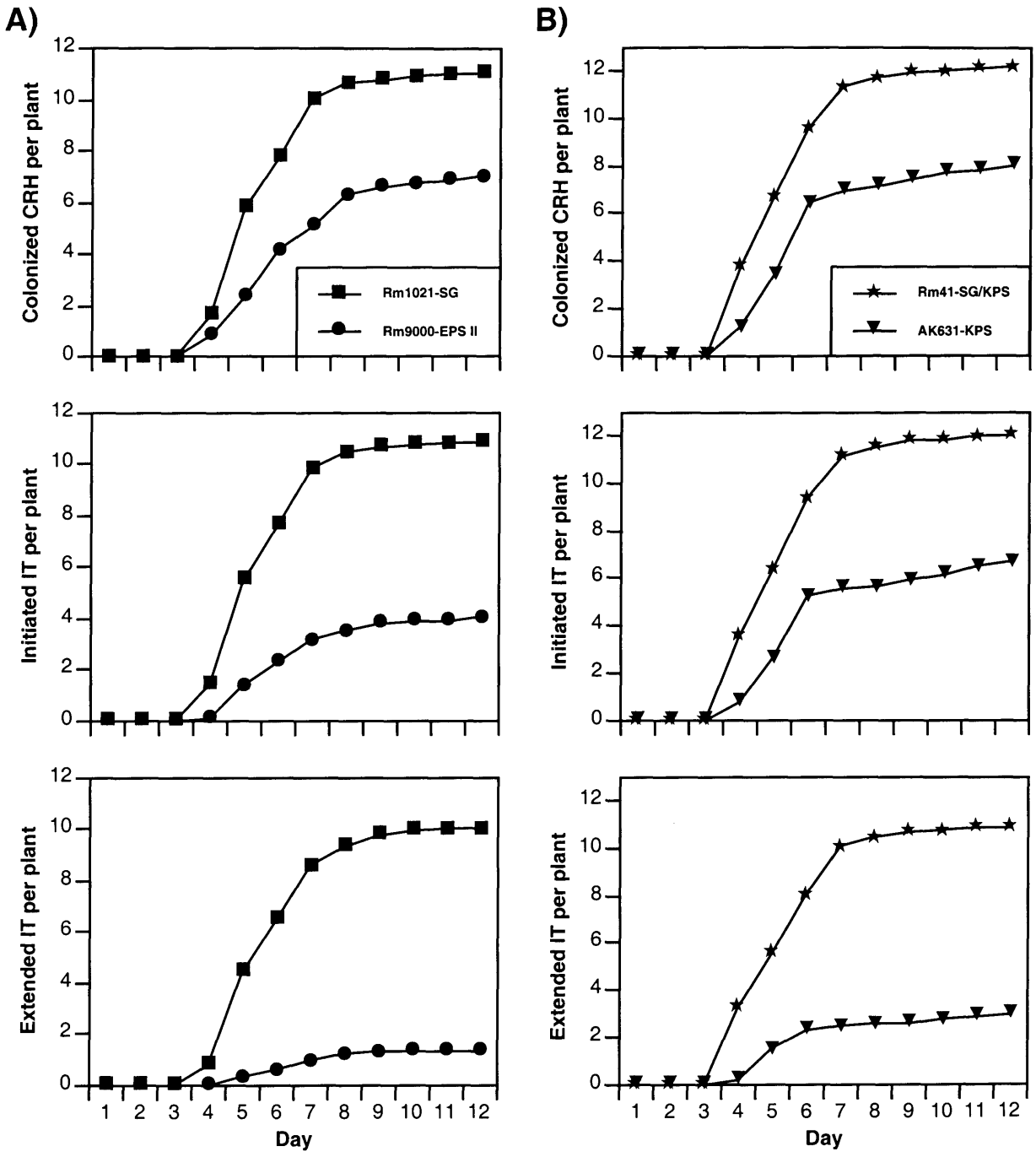

A)
B)
Colonized CRH per plant
Initiated IT per plant
Extended IT per plant
Rm1021-SG
Rm9000-EPS II
Rm41-SG/KPS
AK631-KPS
Day
0
2
4
6
8
10
12
1 2 3 4 5 6 7 8 9 10 11 12

Figure 3. Efficiencies of nodule invasion by various *S. meliloti* strains. Sets of at least 36 plants were inoculated with different strains and scored on day 12 for numbers of colonized curled root hairs (CCRHs), numbers of initiated infection threads (ITs), and numbers of extended ITs. (A) The relative efficiencies of CCRH formation were computed by normalizing the numbers of CCRHs to Rm1021 (for Rm7210, Rm9000, and Rm9011) or to Rm41 (for AK631 and PP674). (B) The efficiency of infection thread initiation is the percentage of curled root hairs colonized by a particular strain that initiate an infection thread. (C) The efficiency of infection thread extension is the percentage of curled root hairs colonized by a particular strain that have infection threads that are extended (reach the base of the root hair cell). The error bars represent the standard error of the mean computed using the mean values from at least three groups of twelve plants.

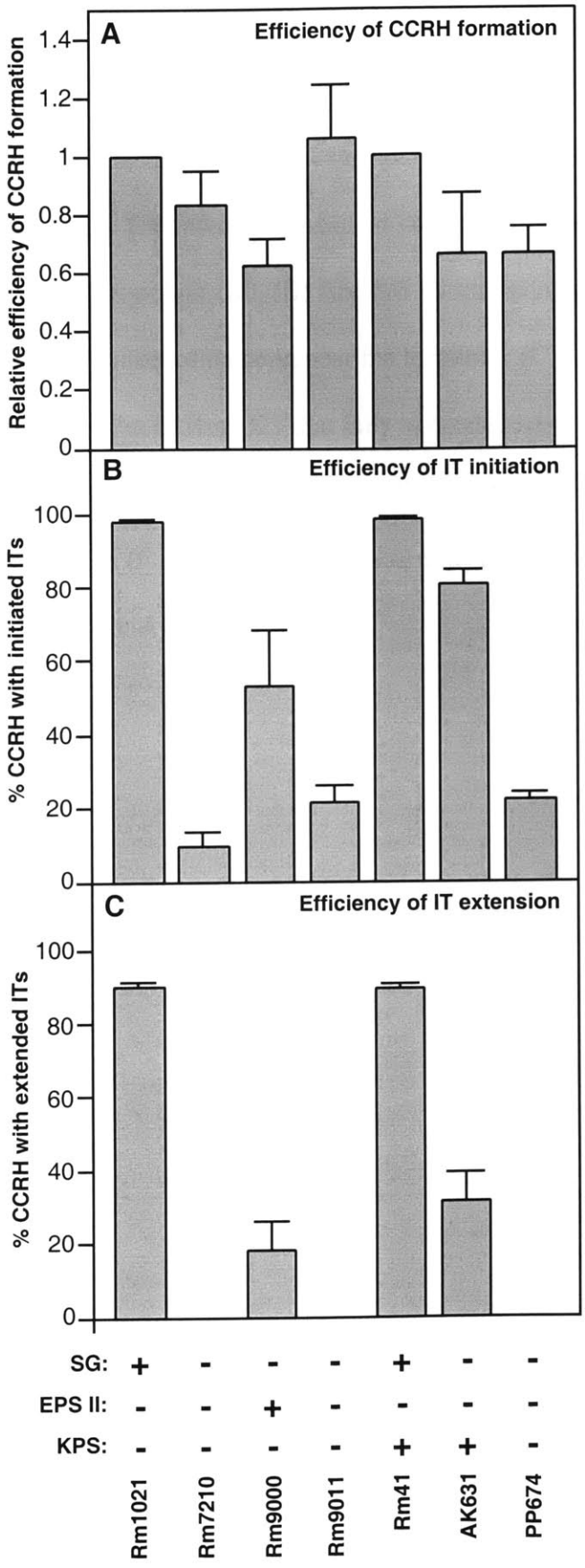

A
Efficiency of CCRH formation
Relative efficiency of CCRH formation
1.4
1.2
1
0.8
0.6
0.4
0.2
0
B
Efficiency of IT initiation
% CCRH with initiated ITs
100
80
60
40
20
0
C
Efficiency of IT extension
% CCRH with extended ITs
100
80
60
40
20
0
SG: + - - - + - -
EPS II: - - + - - - -
KPS: - - - - + + -
Rm1021
Rm7210
Rm9000
Rm9011
Rm41
AK631
PP674

Figure 4. Fluorescence microscopy analyses of infection thread formation mediated by various *S. meliloti* polysaccharides. All images are composite images of GFP-expressing *S. meliloti* cells (green) and root hair cells (red). (A) Typical succinoglycan-mediated extended infection thread (IT) formed by Rm1021. The infection thread extends from the colonized, curled root hair (CCRH) to the base of the root hair cell. (B) Aborted succinoglycan-mediated infection thread with a densely packed pocket of bacteria near the terminus. (C) CCRH formed by Rm7210, an *exoY210*::Tn*5* mutant of Rm1021 that fails to produce a symbiotically active polysaccharide. (D and E) Aborted, aberrant EPS II-mediated infection threads present on plants inoculated with Rm9000. (F and G) Extended, aberrant, K-antigen-mediated infection threads on plants inoculated with AK631.

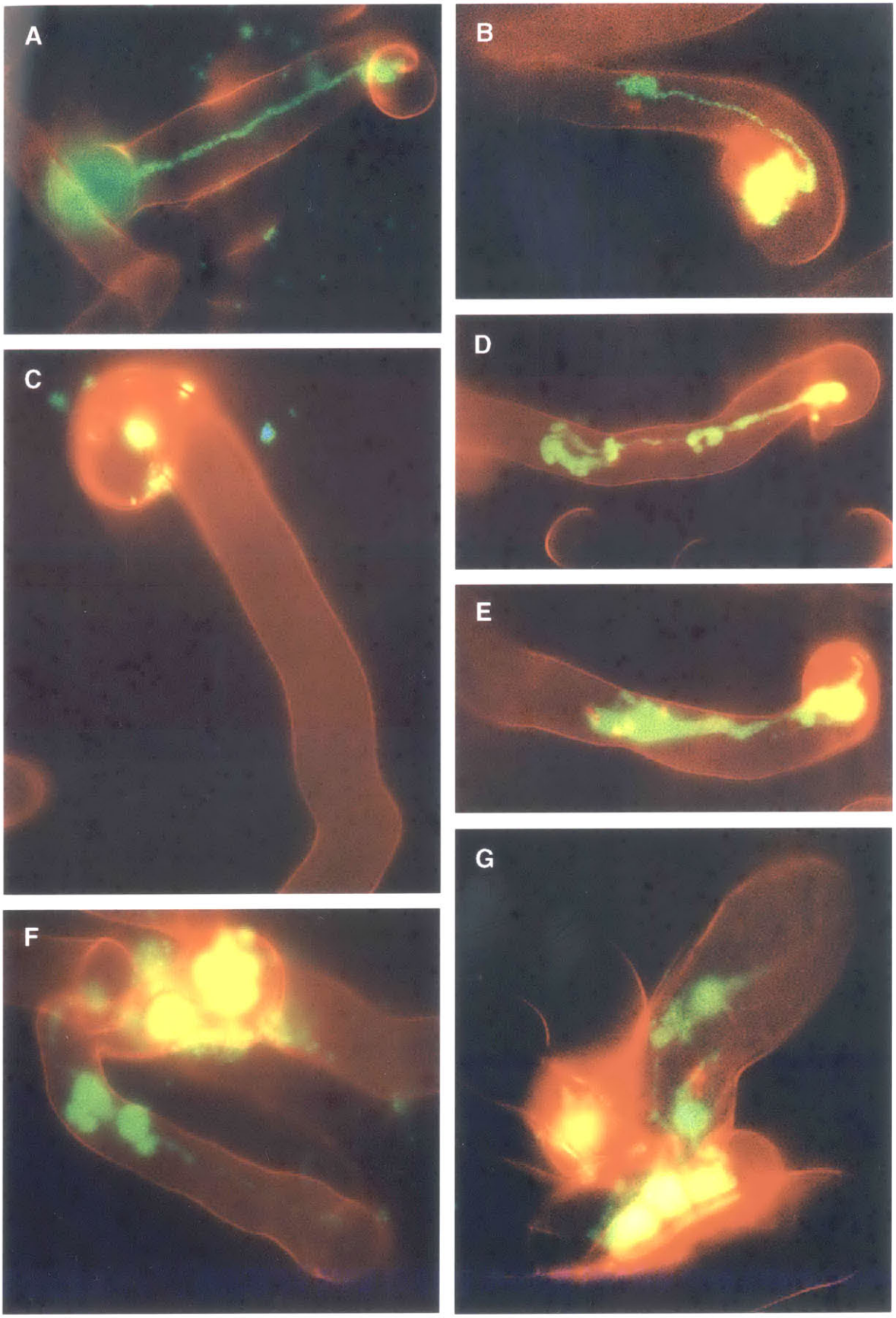

A
B
C
D
E
F
G

Figure 5. Models for the perception of succinoglycan (SG), EPS II, and K-antigen (KPS) by a host plant and subsequent signal transduction resulting in nodule invasion by *S. meliloti*. (A) A three polysaccharide - one receptor model. (B) A three polysaccharide - three receptor model in which the three signal transduction pathways feed into a common signal transduction pathway. (C) A three polysaccharide - three receptor model in which the three signal transduction pathways are independent of one another.

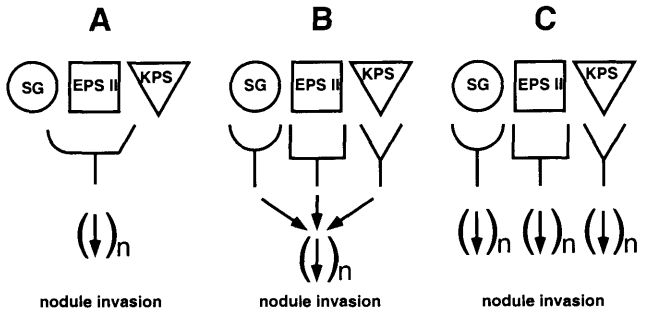

A
B
C
SG
EPS II
KPS
SG
EPS II
KPS
SG
EPS II
KPS
nodule invasion
nodule invasion
nodule invasion

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

Strain	Genotype	SG[a]	EPS II[a]	KPS[a,b]	Reference
Rm1021	SU47 Sm^r	+	-	-	(85)
Rm7210	Rm1021 *exoY210*::Tn*5*	-	-	-	(79)
Rm9000	Rm1021 *expR101 exoY210*::Tn*5*	-	+	-	(59)
Rm8530	Rm1021 *expR101*	+	+	-	(55)
Rm9011	Rm1021 *expR101 exoY210*::Tn*5 expA3*::*lacZ* (Gm^r)	-	-	-	This study
Rm41	Sp^r	+	?[c]	+	(101)
AK631	Rm41 *exoB631*	-	-	+	(6)
PP674	AK631 *rkpA*::Tn*5*	-	-	-	(102)

a: "+" indicates production of a symbiotically active form of the polysaccharide. "-" indicates that the strain does not produce a symbiotically active form of the polysaccharide.

b: KPS = K-antigen.

c: It is not known whether Rm41 produces EPS II.

Chapter 3

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

* Manuscript in preparation

ABSTRACT

The production of complex extracellular polysaccharides 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, *S. meliloti* wild type strain Rm1021 produces only symbiotically active 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 cloned and characterized the *expR101* mutation from *S. meliloti*. The *expR101* mutation, a spontaneous, dominant mutation, results from a 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, the *Escherichia coli* SdiA protein, and a number of other *Vibrio fischeri* LuxR homologs. We have also investigated whether the *expR* gene product mediates a quorum sensing response in *S. meliloti*.

INTRODUCTION

The soil bacterium *Sinorhizobium meliloti* induces the formation of symbiotic root nodules on the host plant *Medicago sativa* (alfalfa). *S. meliloti* cells invade developing root nodules via extended invaginations of root hair cell membranes called infection threads. Once the infection threads have penetrated the nodule, *S. meliloti* cells are individually surrounded by host membrane and released into the nodule cells where they differentiate into bacteroids, the nitrogen fixing form of the bacteria.

Extracellular polysaccharides produced by *S. meliloti* are crucial for establishing a successful, nitrogen-fixing symbiosis with alfalfa. *S. meliloti* mutants that are unable to produce certain polysaccharides are defective in nodule invasion and primarily induce the formation of symbiotically ineffective root nodules that are devoid of bacteria and bacteroids (55, 79, 111). Any one of three *S. meliloti* polysaccharides, succinoglycan, EPS II, or K antigen, can mediate alfalfa root nodule invasion. Using GFP-expressing *S. meliloti* strains, we have recently demonstrated that each these three polysaccharides functions to mediate infection thread initiation and extension on alfalfa (33, 98). However, under laboratory conditions, there are quantitative and qualitative differences in the manner in which succinoglycan, EPS II, and K antigen function (98). This suggests that certain polysaccharides are able to function more efficiently under different conditions, which may provide a strain that produces 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, 108). EPS II has a galactoglucan repeating unit

modified with acetyl and pyruvyl moieties (55, 64). The K antigen repeating unit is a disaccharide containing glucuronic acid and a modified pseudaminic acid residue (112). Despite the structural diversity of these three polysaccharides, genetic and biochemical evidence strongly suggest that low molecular weight forms of each polysaccharide are the symbiotically active forms (i.e., the forms able to promote nodule invasion) (10, 59, 113, 133, 138).

Under non-starvation conditions, the wild type laboratory *S. meliloti* strain Rm1021 produces measurable quantities of succinoglycan, but it 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 (55), by a null allele of the *mucR* gene (70, 151), or by growth in very low phosphate conditions (152). However, only the *expR101* mutation, a spontaneous mutation in Rm1021 that results in a mucoid colony morphology, stimulates production of 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 alone (59, 86), whereas *expR101*-induced EPS II ranges from high molecular weight to low molecular weight, the fraction active in promoting nodule invasion (59).

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 (55). The *exp* gene cluster has been sequenced (16), revealing 21 *exp* genes which are organized into six operons, including the *expA* (nine genes), *expC* (one gene), *expD* (two genes), *expE* (eight genes), and *expG* (one gene) transcriptional units. However, among the predicted *exp* gene products are several proteins whose roles in EPS II production are not easily explained. In addition, more predicted glycosyltransferases (five) are present than might have been expected to synthesize a

polysaccharide with only two sugars in the repeating unit. Thus, it is currently unclear which *exp* gene products are directly involved in the synthesis of EPS II. Furthermore, it is not yet 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* (70).

Prior to this work, the nature of the *expR101* mutation was not known. The *expR101* locus had been mapped to the ~3.4 Mb *S. meliloti* main chromosome (55). Additionally, cotransduction analyses indicated that the *expR* locus was ~66% transductionally linked to one allele of the *ndvB* gene and ~7% transductionally linked to the *trp-33* locus (54). 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 *expR*, a gene whose predicted protein product is significantly similar to members of the LuxR family of proteins. Because LuxR and many of its homologs are involved in a process known as quorum sensing (the modulation of gene expression in response to density-dependent bacterial signals), we have analyzed whether the production of symbiotically active EPS II by *S. meliloti* strain Rm1021 is regulated by density-dependent signals accumulated in the bacterial growth medium.

MATERIALS AND METHODS

Bacterial strains and culture media.

The strains used in this study are listed in Table 1. Strains were either grown at 30°C (*S. meliloti*) or 37°C (*E. coli*) in LBMC medium [Luria-Bertani (LB) (123) liquid or agar supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$] or in TYC medium [TY (18) liquid or agar supplemented with 12 mM $CaCl_2$]. Antibiotics were used at the following concentrations: streptomycin, 500 μg/ml; neomycin, 200 μg/ml; gentamycin, 50 μg/ml for *S. meliloti*, 5 μg/ml for *E. coli*; spectinomycin, 100 μg/ml; tetracycline, 10 μg/ml; kanamycin, 25 μg/ml.

Alfalfa nodulation assays.

Alfalfa nodulation assays were performed as described (79). Each plant was inoculated with 1 ml of a cell suspension with an $O.D._{600}$ of 0.05. Plants were scored four 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 (5) from *S. meliloti* strain Rm10006 (Rm1021 *expR101* ΩTn*5-233* #3-15) (55), partially restricting the genomic DNA with *Eco*RI (New England Bioloabs (N.E.B.), Beverly, MA), and ligating 20-35 kb genomic DNA fragments to *Eco*RI-restricted pLAFR1 (51) using T4 DNA ligase (N.E.B.). The resultant recombinant cosmid DNA was delivered into *E. coli* strain HB101 (Gibco BRL, Gaithersburg, MD) using the Gigapack III-XL kit (Stratagene, La

Jolla, CA). Tetracycline resistant HB101 cells (those carrying recombinant cosmids) were screened for their ability to confer resistance to spectinomycin and kanamycin, the antibiotic resistance markers located on Tn*5-233* (35) 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* ORF [nucleotides 4229 to 4237 in the total published sequence (66)]. This *ndvB* allele confers the expected symbiotic defect (46), but not a hypoosmotic adaptation defect (45).

Diagnostic polymerase chain reaction (PCR) analysis of the *expR* region and DNA sequencing.

Both strands of the *expR* region were sequenced using two templates, pBSKSII+ (Stratagene) subclones of the *expR* region and PCR products generated from genomic DNA templates using oligonucleotide primers (generated by Gibco BRL) specific to the *expR* region. DNA sequencing was performed by the Molecular Biology Core Facility at Dartmouth College using reaction products generated 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 units 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% DMSO frozen permanent strain stock suspended in 100 μl of water. Primers were used at a concentration of 1 μM, and dNTPs (Pharmacia Biotech, Piscataway, NJ) were used at a concentration of 200 μM. The PCR

program used was as follows: (1) 95°C for 5 minutes, (2) 94°C for 30 seconds, (3) 65°C for 30 seconds, (4) 72°C for 5 minutes, (5) Go to step (2) 29 times, 6) Hold at 4°C.

Construction of the *expR*::*lacZ* fusion and β-galactosidase activity assays.

The genomic *expR* null allele and *lacZ* transcriptional fusion was generated as follows. A 2.2 kb PCR product with a *Xba*I restriction site engineered at the *expR* 5' end was generated using the *expR101* locus from Rm8530 genomic DNA as a template and the primer and dNTP 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 using a PCR purification kit (Qiagen, Valencia, CA). Following restriction with *Xba*I and *Hin*dIII, the PCR product was ligated to *Xba*I / *Hin*dIII restricted pK19*mob*GII (69) using T4 DNA ligase (N.E.B.), generating pK19*expR*. The knockout construct was generated by inserting a 4.0kb *lacZ*-Gm *Sph*I cassette from pAB2001 (17) 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*::*lacZ*-Gm was mobilized into Rm8530, and homologous recombinant candidates were identified by gentamycin resistance, kanamycin sensitivity, and white color on plates containing X-gluc. 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.

β-galactosidase activity assays were performed as described (87). Assays for each strain tested were performed on five independent cultures. Error margins represent one standard deviation. Cells from overnight cultures were diluted to an $O.D._{600}$ of 0.2 and outgrown for 4 hours. After the outgrowth period, cultures had reached an $O.D._{600}$ of between 0.5 and 0.8, at which point the β-galactosidase activity assays were performed. Conditioned LBMC medium was generated by growing cultures of Rm1021 or Rm8530 (Rm1021 *expR101)* to an O.D.600 of greater than 4.5 and then filter sterilizing the culture supernatants.

Conditioned medium experiments were performed on cultures consisting of 90% conditioned medium and 10% fresh medium (experimental group) and cultures consisting of 90% 0.85% saline and 10% fresh medium (control group). For conditioned medium experiments, exponentially growing cells were diluted to an $O.D._{600}$ of between 0.025 and 0.050 and thereafter, β-galactosidase activity was measured hourly.

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 was unlinked to the Tn*5* insertion, indicating that the *expR101* mutation has arisen spontaneously (55). Our previous attempts to complement or suppress the mucoid colony morphology of the *expR101*-containing strain using cosmids from a Rm1021 pLAFR1 genomic library (51) were unsuccessful (56, 99), suggesting either that the *expR101* mutation was dominant or that the Rm1021 genomic library was incomplete. Therefore we decided to attempt a direct cloning approach to isolate the *expR101* mutation.

To clone the *expR101* mutation, we exploited a transposon insertion [termed ΩTn*5-233* #3-15 (55)] that was ~95% linked to the *expR101* mucoid colony phenotype in generalized transductions using ϕM12 (49). We constructed a pLAFR1 cosmid library in *E. coli* using genomic DNA from Rm10006 (Rm1021 *expR101* ΩTn*5-233* #3-15) and screened for cosmid clones that carried the antibiotic resistance markers present in Tn*5-233*. Southern blot analysis indicated that one cosmid clone, called p1-37, carried ΩTn*5-233* #3-15 and greater than 10 kb of chromosomal DNA flanking either side of the transposon (data not shown). Because a ϕM12 cotransduction frequency of ~95% in *S. meliloti* usually indicates that two loci are separated by 5 to 10 kb, cosmid p1-37 was selected for further analysis.

Cosmid p1-37 contains the *expR101* mutation and induces a mucoid colony morphology in *S. meliloti* strain Rm1021 by inducing EPS II production. To determine

whether the *expR101* mutation was present on p1-37, we mobilized p1-37 into the wild type strain Rm1021 and examined the colony morphology of the resultant transconjugant. On both LBMC agar and TYC agar plates (see Materials and Methods) Rm1021 carrying p1-37 had a mucoid colony morphology (Fig. 1B), whereas Rm1021 carrying pLAFR1, the parental vector for p1-37, had a dry colony morphology (Fig. 1A). To determine whether this mucoid colony morphology was due to EPS II production and/or succinoglycan production, we mobilized p1-37 and pLAFR1 into Rm7210 (Rm1021 *exoY210*::Tn*5*), a strain incapable of producing succinoglycan, and into Rm10001 (Rm1021 *expA3*::Tn*5*), a strain incapable of producing EPS II. Rm7210/p1-37 had a mucoid colony morphology (Fig. 1B), whereas Rm7210/pLAFR1 colonies had a dry colony morphology (Fig. 1A). In contrast, both Rm10001/p1-37 and Rm10001/pLAFR1 had a dry colony morphology (Fig. 1A). Taken together, these results strongly suggested that we had cloned the *expR101* mutation on p1-37, that the *expR101* mutation is dominant to the *expR* allele in strain Rm1021, and that the mucoid 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. Using an ability to induce a mucoid colony morphology in Rm7210 as an assay for the presence of the *expR101* mutation, we subcloned *Kpn*I and *Hin*dIII restriction fragments from p1-37 into pSW213, a broad host range IncP vector (30). We then mobilized the p1-37 subclones into Rm7210 and examined the colony morphology of the resultant strains on LBMC and TYC plates. A 5 kb *Kpn*I fragment from p1-37 and a 6 kb *Hin*dIII fragment from p1-37 induced mucoid colony phenotype in Rm7210, whereas Rm7210 containing the pSW213 vector had a dry colony morphology. A ca. 3.2 kb *Kpn*I-*Hin*dIII fragment common to both mucoid colony-inducing

subclones above, pK5 and pH6 (Fig. 2), was also able to induce a mucoid colony phenotype in Rm7210 when subcloned into pSW213 (pKH3.2). Convinced that we had isolated the *expR101* mutation on a reasonably sized DNA fragment, we determined the DNA sequence of this 3274 bp *Kpn*I-*Hin*dIII fragment and compared the DNA sequences and predicted protein sequences to the Genbank database using a BLAST search (1).

Sequence analysis of the 3274 bp *Kpn*I-*Hin*dIII fragment revealed the 5' ends of the *ndvA* (128) open reading frame (ORF) and the 5' end of an ORF predicted to encode pyruvate carboxylase flanking one complete ORF, which we have designated *expR* (Fig. 2). The *expR* protein product was predicted to be 246 amino acids and showed significant homology to the *Rhizobium leguminosarum* bv.*viciae* RhiR protein (34), the *Escherichia coli* SdiA protein (139), and a number of other *Vibrio fischeri* LuxR (47, 48) homologs (Fig. 3). The predicted ExpR sequence contained all seven amino acid residues conserved in most LuxR homologs (52) .

To test whether the *expR* ORF on the 3274 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 the 1.4 kb *Sph*I fragment (Fig. 2) internal to the 3274 bp *Kpn*I-*Hin*dIII fragment, creating pKH3.2Δ*Sph*I. Removal of this fragment of *expR* eliminated the ability of the 3274 bp fragment to induce a mucoid colony phenotype.

The *expR* ORF in *S. meliloti* Rm1021 is disrupted by an insertion sequence (IS) element. To determine the difference between the *expR101* mutant and the wild type *expR* locus, we designed primers in the *expR* region and performed PCR using genomic DNA as a template to generate template for DNA sequencing. We were surprised to find that, though the

PCR product from the *expR101* mutant was the expected size (0.9 kb), the PCR product that we obtained from the wild type Rm1021 using the same primer pair was 2.2 kb (Fig. 4A). When we sequenced the 2.2 kb PCR product from Rm1021, we discovered that the *expR* ORF in Rm1021 was disrupted (Fig. 4B) by a previously described 1319 bp insertion sequence, IS*Rm*2011-1 (76, 125). Previous analyses indicated that eight copies of IS*Rm*2011-1 exist in the Rm1021 genome (125). We confirmed this observation for Rm1021 using both *Eco*RI-restricted genomic DNA and *Eco*RV-restricted genomic DNA (Fig. 5). However, in Rm8530 (Rm1021 *expR101*) we detected only seven copies of IS*Rm*2011-1 (Fig. 5), suggesting that the *expR*-disrupting copy of IS*Rm*2011-1 in *expR101* strains had been eliminated rather than reinserting in another location in the genome.

IS*Rm*2011-1 is very closely related to IS*Rm*1, a second *S. meliloti* IS element that creates a 5 bp target site duplication upon insertion (121, 140). Similarly, 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 as *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. In order 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 needed for colony mucoidy in 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 LMW EPS II in Rm9026 culture supernatants using Dionex high performance anion exchange chromatography (HPAEC) coupled with highly sensitive, pulsed amperometric detection (PAD) (data not shown).

Growth in conditioned media does not activate transcription of the *expA* operon or stimulate EPS II production. LuxR and many or its homologs activate the transcription of target genes in response to extracellular factors that function as cell density indicators [reviewed in (52)]. Many members of the LuxR family respond to acyl-homoserine lactone molecules synthesized by a member of the LuxI family. To ascertain whether ExpR activates the transcription of the *exp* genes in response to an extracellular factor(s) produced by *S. meliloti*, we measured the transcription of the *expA3*::*lacZ*-Gm fusion in Rm9011 (Rm1021

expR$^+$ (formerly *expR101*) *exoY210*::Tn*5* *expA3*::*lacZ*-Gm) in both unconditioned LBMC medium and LBMC medium that had been conditioned by growth of *S. meliloti* cells to stationary phase (see Materials and Methods). Following the initial dilution of the cells, the transcription of the *expA3* gene in Rm9011 in conditioned medium was virtually identical to that observed in the control medium at all time points (data not shown). We also performed an experiment in which we measured EPS II production over time by Rm9000 (*expR*$^+$ (formerly *expR101*) *exoY210*::Tn*5*) in both conditioned medium and control medium. The amounts of EPS II produced in both media were very similar at all time points. Under the conditions tested, it appears that the ability of ExpR to activate transcription of the *exp* genes and to cause EPS II production is not dependent on the accumulation of extracellular factors in the medium.

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 and performed diagnostic PCR on the *expR* region 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 for Rm1021, Rm2011, Rm5000, and RCR2011) as well as 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, though 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 agrees completely with the previously published *ndvA* region sequence data (128).

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 as *expR101*. In this report we have shown that the widely used laboratory strain Rm1021 and several related strains have a copy of the insertion sequence IS*Rm*2011-1 (76, 125) within the coding region of a gene (*expR*) that encodes a LuxR homolog. The "*expR101* mutation" evidently resulted from a precise excision of this insertion sequence, 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 as *expR*$^+$. Rm1021 and related strains carry the *expR102*::IS*Rm*2011-1 allele.

ExpR is clearly required in some positively acting form for the production of symbiotically active LMW EPS II. However, either the introduction of a null allele of the *mucR* gene (70, 151) or growth under low phosphate conditions (152) result in the production of HMW EPS II that is not symbiotically active (59, 86). It is not yet entirely clear what is responsible for these differences in EPS II molecular weight distribution, although it has already been shown that EPS II synthesis in response to phosphate starvation does not require either *mucR* function (120). In addition, we can now infer that a functional *expR* gene is not required for EPS II production under low phosphate conditions or for EPS II production by strain carrying a null allele of *mucR*. Thus, it seems likely that 1) introduction of the *expR*$^+$ allele (formerly *expR101*), 2) inactivation of *mucR*, and 3) phosphate limitation differentially

activate a gene or genes involved in the control of the molecular weight distribution of EPS II production.

To date, we have not observed an *expR*$^+$-mediated increase of *exp* transcription in response to conditioned medium as might be expected for a LuxR homolog. It may be the case that ExpR does not sense a density dependent signal or that the *expR* gene product is a constitutively active, positive regulator of *exp* gene expression. If ExpR does respond to an acyl-homoserine lactone molecule, it is likely produced by the one gene present in the *S. meliloti* Rm1021 genome predicted to encode a LuxI homolog (11). Since there are nine LuxR homologs present in the *S. meliloti* Rm1021 genome (11), it could be that, similar to the situation in *R. leguminosarum* bv. *viciae*, there is a complex regulatory cascade involving multiple LuxR homologs. If such a regulatory web exists in *S. meliloti*, our assay conditions may not be specific enough to allow us to detect an ExpR-mediated change in *exp* gene transcription.

There are a number of examples of insertion sequence elements modulating polysaccharide phase variation in bacteria. Reversible insertion of IS1301 in *siaA*, a sialic acid biosynthesis gene, converts *Neisseria meningitidis* from a sialic acid capsule-producing form to a capsule-deficient form, a phenotypic change that modulates infectivity (63). In *Pseudoalteromonas atlanticus*, IS492 mediates reversible inactivation of EPS production, a phase variation important in biofilm formation (9). Stationary phase cultures of *Xanthomonas oryzae* pv. *oryzae* exhibit a high frequency of phase variation mediated by reversible insertion of IS*Xo*1 and IS*Xo*2 into *gumM*, a gene involved in the synthesis of xanthan gum, a virulence determinant (103). *Staphylococcus epidermis* phase variation from biofilm forming variant to a biofilm negative form is mediated by reversible IS256 insertion into *icaA*, *icaC*, genes involved

in synthesis of polysaccharide intercellular adhesins (153). It is possible that *S. meliloti* is using the IS*Rm*2011-1 insertion in *expR* as a genetically plastic switch for controlling EPS II production, but under laboratory conditions, the mucoid colony phenotype of *expR*$^+$ strains appears to be highly stable. Additionally, it has been reported that *S. meliloti* IS element genomic fingerprint patterns of are highly stable over long periods of time (125) under laboratory conditions. Consistent with this observation, our IS*Rm*2011-1 fingerprinting analysis revealed a hybridization pattern identical to that seen previously, and the hybridization patterns of closely related strains were identical (Fig. 5). Nevertheless, we cannot rule out the possibility that, in the field, IS elements are mediating phase variation with regard to symbiotically important polysaccharides in *S. meliloti*.

It is not clear when the *S. meliloti expR* ORF was disrupted by IS*Rm*2011-1 in the lineage involving Rm1021, but it almost certainly occurred in strain SU47, as all the daughters of SU47 also have an IS*Rm*2011-1-disrupted *expR* ORF. It is possible that strain SU47, which has a dry colony morphology (Fig 1A), is descended from a field isolate that also had a dry colony morphology. However, given that many strains of rhizobia isolated from the field have a very mucoid colony appearance, the *expR* disruption may have occurred under laboratory conditions. Since it is easier to isolate single colonies of strains with a non-mucoid colony morphology, a strain with the IS element disrupting the *expR* gene may have been picked for further genetic analysis. It is not known what stimulus induced the excision of the IS*Rm*2011-1 from the *expR* ORF, but one possibility is that environmental stress (possibly from starvation conditions) or some other condition such as the Tn*5* mutagenesis that preceded the isolation of the strain carrying the *expR*$^+$ allele.

The results of this study raise a number of intriguing questions about the regulation of the production of symbiotically active EPS II. It will be of interest to investigate which, if any, non-*exp* genes are regulated by the *expR* gene product. In addition, genes that are involved in regulating the expression of *expR* may exist. The question of how ExpR is functioning to regulate *exp* gene expression remains open, as does the question of what differences exist in the regulation of EPS II production by the *expR*$^{+}$ allele, a null allele of *mucR*, and very low phosphate conditions.

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Figure 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.

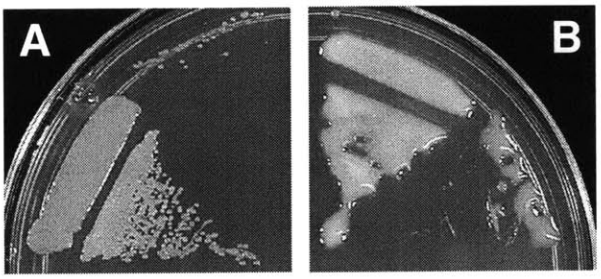
A
B

Figure 2. Schematic representation of the *expR* gene region from a Rm1021 *expR*$^{+}$ (formerly *expR101*) strain. Two subclones of p1-37, pK5 and pH6, share a 3274 bp *Kpn*I-*Hin*dIII fragment that induces a mucoid colony phenotype in Rm1021 and carries the *expR101* mutation. The *ndvA* and *pyc* ORFs are not completely contained on the 3274 bp *Kpn*I-*Hin*dIII fragment; only the 5' ends of these genes are present, indicated by double slashes. Deletion of the 1.4 kb *Sph*I fragment internal to the 3274 bp *Kpn*I-*Hin*dIII fragment eliminates the ability of the 3274 bp fragment to induce mucoid colony morphology. Restriction enzyme site key: K, *Kpn*I; H, *Hin*dIII; S, *Sph*I.

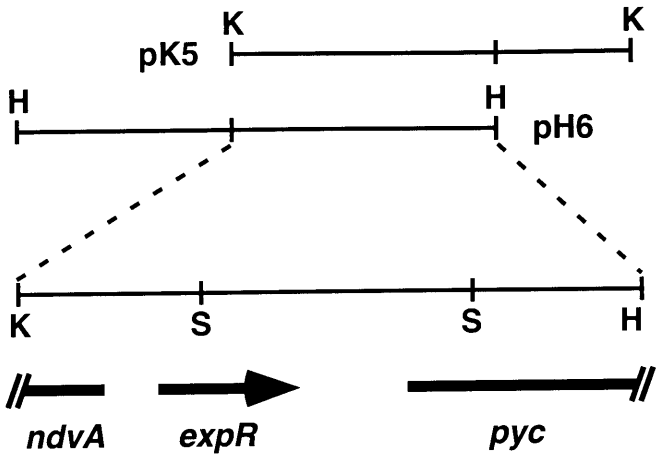
K
K
pK5
H
H
pH6
K
S
S
H
ndvA
expR
pyc

Figure 3. Alignment of ExpR with homologs *E. coli* SdiA, *R. leguminosarum* bv.*viciae* RhiR, and *V. fischeri* LuxR. Alignment was perfromed using the Jotal-Hein algorithm and a PAM250 weight table. Residues boxed in black are those that are within three distance units of the consensus. Alignment was performed using Lasergene software (DNASTAR, Madison, WI). The asterisks indicate the seven amino acid residues conserved in many LuxR homologs.

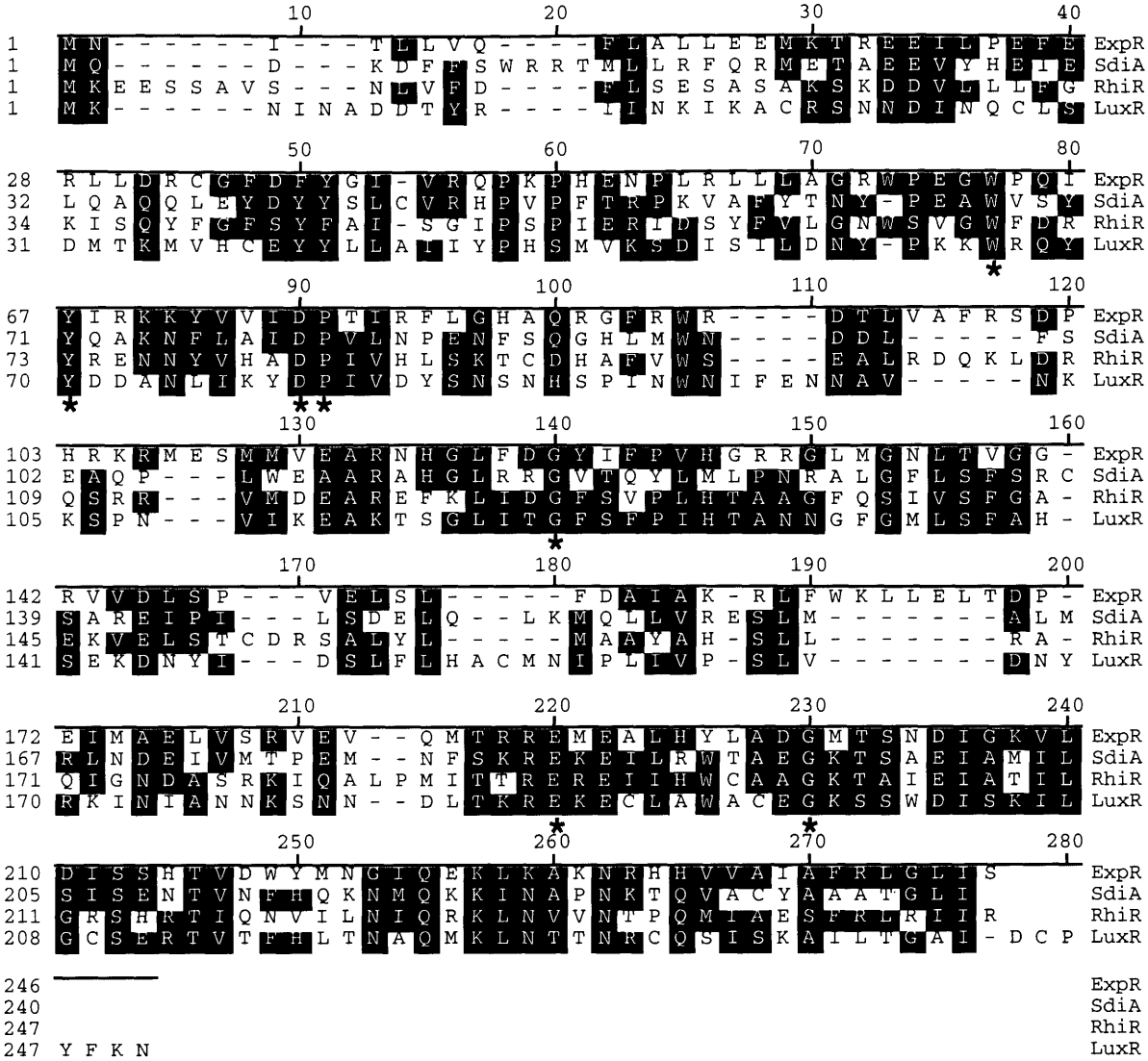
ExpR
SdiA
RhiR
LuxR

Figure 4. The *expR* ORF in *S. meliloti* strain Rm1021 is disrupted by a 1319 bp IS element. (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 lane (M) contains 1Kb Ladder (Gibco BRL). (B) Schematic representation of the *expR* ORFs from Rm1021 and Rm8530. The scale bars represent the diagnostic PCR products produced from each strain.

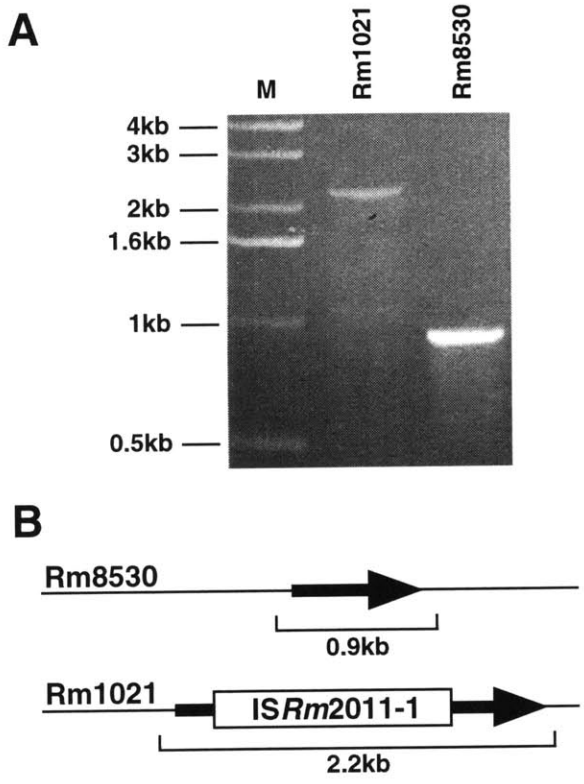
A
M
Rm1021
Rm8530
4kb
3kb
2kb
1.6kb
1kb
0.5kb
B
Rm8530
0.9kb
Rm1021
IS*Rm*2011-1
2.2kb

Figure 5. Autoradiographs of Southern blots of *S. meliloti* DNA probed with a sequence specific for IS*Rm*2011-1. (A) IS*Rm*2011-1 fingerprint of *Eco*RI-restricted genomic DNA from Rm1021 and Rm8530 (Rm1021 *expR*$^+$ (formerly *expR101*)). (A) 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.

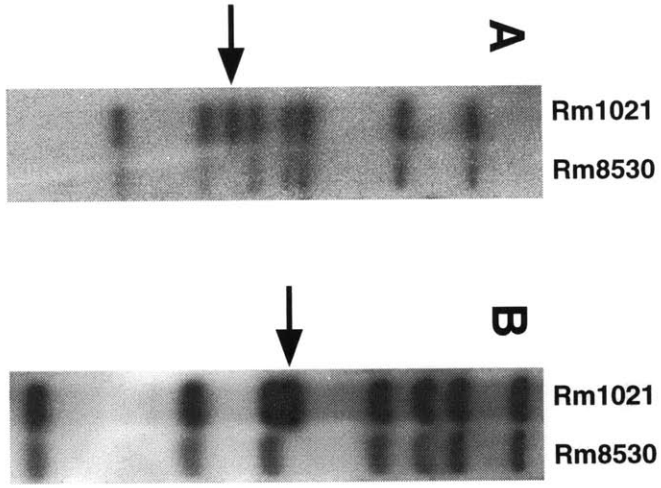
A
Rm1021
Rm8530
B
Rm1021
Rm8530

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

Strain name	Strain genotype	Reference
Rm1021	SU47 *str-21 expR102*::IS*Rm*2011-1	(85)
Rm7210	Rm1021 *exoY210*::Tn*5*	(79)
Rm8530	Rm1021 *expR*$^+$ (formerly *expR101*)	(55)
Rm10006	Rm1021 *expR*$^+$ ΩTn*5-233* #3-15	(55)
Rm9000	Rm1021 *expR*$^+$ *exoY210*::Tn*5*	(59)
Rm9011	Rm1021 *expR*$^+$ *exoY210*::Tn*5 expA3*::*lacZ*-Gm	(98)
Rm10002	Rm1021 *expA3*::*lacZ*-Gm	(55)
Rm9025	Rm1021 *expR103*::*lacZ*-Gm	This study
Rm9026	Rm8530 *expR103*::*lacZ*-Gm *exoY210*::Tn*5*	This study
Rm9028	Rm8530 *expR103*::*lacZ*-Gm	This study

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

Strain name	Reference	Size of *expR* PCR product	Status of *expR* ORF
Rm1021	(85)	2.2 kb	*expR102*::IS*Rm*2011-1
Rm8530	(55)	0.9 kb	*expR* ORF intact – *expR101*
SU47	(137)	2.2 kb	*expR102*::IS*Rm*2011-1[a]
Rm2011	(27)	2.2 kb	*expR102*::IS*Rm*2011-1[a]
Rm5000	(49)	2.2 kb	*expR102*::IS*Rm*2011-1[b]
RCR2011	(119)	2.2 kb	*expR102*::IS*Rm*2011-1[b]
Rm41	(101)	0.9 kb	*expR* ORF intact[c]
YE-2Sl	(147)	0.9 kb	*expR* ORF intact[d]
102F34	(39)	0.9 kb	*expR* ORF disrupted – 11 bp Δ

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-2Sl.
d – YE-2Sl *expR* has a sequence distinct from those of Rm1021 and Rm41.

Chapter 4

Structure-Function Analyses of the Symbiotically Important Low-Molecular-Weight Succinoglycan of *Sinorhizobium meliloti*

ABSTRACT

The production of succinoglycan by *Sinorhizobium meliloti* strain Rm1021 is required for successful nodule invasion on the host plant alfalfa. Succinoglycan is a complex, acidic exopolysaccharide with an octasaccharide repeating unit modified with acetyl, succinyl, and pyruvyl moieties. Rm1021 produces succinoglycan in distinct high-molecular-weight (HMW) and low-molecular-weight (LMW) forms. Previously, it was reported that LMW succinoglycan, specifically the a succinoglycan tetramer, was capable of partially restoring the formation of nitrogen-fixing nodules by a succinoglycan-deficient, invasion-deficient derivative of strain Rm1021. Subsequent to determining that LMW succinoglycan consists of monomers, dimers, and trimers of the repeating unit, we have demonstrated that the succinoglycan trimer is in fact the species active in promoting nodule invasion. We have also tested the biological activity of differentially succinylated succinoglycan trimer molecules and used a GFP-based plant assay system to determine whether LMW succinoglycan is promoting nodule invasion by stimulating the initiation and extension of infection threads.

INTRODUCTION

A successful *S. meliloti*-alfalfa symbiosis is the result of a complex, multi-step interaction between the host plant and the bacterial symbiont (24, 36, 83, 94, 114). Polysaccharides produced and secreted by *S. meliloti* are crucial for establishing a successful symbiosis with alfalfa. *S. meliloti* strain Rm1021 produces a complex exopolysaccharide called succinoglycan which is crucial for the successful invasion of alfalfa root nodules (79). Derivatives of strain Rm1021 that are deficient in succinoglycan production primarily induce the formation of uninfected, non-nitrogen-fixing root nodules that are devoid of bacteria and bacteroids. Strain Rm1021, a succinoglycan-proficient strain, is highly efficient at inducing infection thread growth. Greater than 95% of colonized, curled root hairs infected by Rm1021 develop infection threads that extend to the base of the infected root hair cell (33, 98). In contrast, succinoglycan-deficient strains of *S. meliloti* are defective in the initiation and extension of infection threads; strain Rm7210 (Rm1021 *exoY210*::Tn*5*), which does not produce any succinoglycan, is able to colonize curled root hairs, but only ~10% of these colonized curled root hair develop infection threads, all of which abort growth prior to reaching the base of the root hair cell (33, 98).

Succinoglycan is a complex exopolysaccharide with an octasaccharide repeating unit (Fig. 1). The succinoglycan repeating unit is composed of one galactose residue and seven glucose residues and contains acetyl, succinyl, and pyruvyl modifications (2, 108). *S. meliloti* strain Rm1021 produces two distinct molecular weight fractions of succinoglycan: a high-molecular-weight (HMW) fraction and a low-molecular-weight (LMW) fraction (77, 148). HMW succinoglycan molecules contain hundreds of repeating units, whereas the LMW

fraction consists of monomers, dimers, and trimers of the octasaccharide repeating unit (138). *S. meliloti* can produce LMW succinoglycan both by direct synthesis (60) and by the action of ExoK and ExsH, endo β-1,3-1,4 glycanases that cleave HMW succinoglycan to LMW forms (144).

Taken together, several lines of evidence suggest that LMW succinoglycan is the symbiotically active fraction and that it functions as a signal to the host plant. 1) Succinoglycan function can be provided *in trans* to a succinoglycan deficient, nitrogen fixation proficient strain by a coinoculated succinoglycan proficient, nitrogen fixation deficient strain, resulting in a productive symbiosis (73, 91). Second, genetic evidence suggests that LMW succinoglycan is the symbiotically active form; a strain that produces almost all of its succinoglycan in the HMW form is severely compromised in nodule invasion (33, 78). Finally, prior to this work, the most direct evidence that LMW succinoglycan is acting as a signal to the host plant comes from two sets of experiments in which small quantities of purified LMW succinoglycan partially rescue the nodule invasion defect of a succinoglycan-deficient, invasion-deficient *S. meliloti* strain (10, 133). In one study, LMW succinoglycan was shown to contain the nodule invasion-promoting activity (133). In the second study, the most highly charged succinoglycan oligomer (estimated at the time to be a succinoglycan tetramer) was identified as the active species (10).

A recent structural characterization of succinoglycan (138) carried out by Lai-Xi Wang, with whom I closely collaborated, indicated that both LMW and HMW succinoglycan have similar degrees of acetylation (~0.7 acetyl residues per repeating unit) and pyruvylation (~1.0 pyruvyl residues per repeating unit). However, it was found that LMW succinoglycan is more highly succinylated that HMW succinoglycan and that each succinoglycan repeating unit can

contain 0, 1, or 2 succinyl modifications (Fig. 1). HMW succinoglycan has an average of 0.93 succinyl residues per repeating unit, whereas succinoglycan trimers, dimers, and monomers have an average of 1.25, 1.40, and 1.43 succinyl residues per repeating unit, respectively. Hence, LMW succinoglycan oligomers exhibit a great deal of structural heterogeneity with respect to their succinyl modification: *S. meliloti* produces succinoglycan monomers with 0 to 2 succinyl modifications, succinoglycan dimers with 0 to 4 succinyl modifications, and succinoglycan trimers with 0 to 6 succinyl modifications.

In this study, we have confirmed that the largest LMW succinoglycan oligomer found in culture supernatants (the trimer) can partially rescue the nodule invasion defect of a succinoglycan-deficient derivative of strain Rm1021. We have also tested whether the degree of succinylation of the succinoglycan trimer affects the ability of the molecule to perform in this activity assay. Finally, we have modified a GFP-based nodulation assay in an attempt to determine whether the succinoglycan trimer can rescue the infection thread growth defect of a succinoglycan-deficient *S. meliloti* strain.

MATERIALS AND METHODS

Production, purification, and chromatographic analysis of succinoglycan.

S. meliloti strain Rm1021 was grown in glutamate-D-mannitol-salts (GMS) medium (pH 7.0) supplemented with biotin, thiamine, and trace elements as described (148). One liter quantities of GMS medium in 2-liter Erlenmeyer flasks were inoculated with 0.85% saline-washed cells from a 10 ml overnight LBMC (Luria-Bertani (LB) medium (123) supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$) culture of Rm1021 (85) and were incubated for 5 days at 30°C with shaking (250 rpm). Cultures were centrifuged at 20,000 x g for 30 minutes, and the culture supernatants were lyophilized. The dried material was then resuspended in 200 ml of 100 mM NaCl. HMW succinoglycan was precipitated by the addition of 3 volumes of ethanol and collected by centrifugation at 6,000 x g for 30 minutes. The LMW succinoglycan that remained in the supernatant was precipitated by the addition of another 7 volumes of ethanol and collected by centrifugation.

LMW succinoglycan monomers, dimers, and trimers were separated by gel filtration chromatography (Bio-Gel P-6 fine mesh, Bio-Rad, Hercules, CA) and purified as described (138). The differentially succinylated forms of the succinoglycan trimer were separated by anion-exchange chromatography (DEAE Sephadex A-25 (Sigma-Aldrich, St. Louis, MO) and purified as described (138). Carbohydrate quantities were measured using the anthrone-sulfuric acid method (81). Dionex high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) was performed as described (138).

Standard plant assay rescue experiments.

Medicago sativa cv. Iroquois seeds were obtained from Agway (Plymouth, IN). The seeds were surface sterilized by a 15 minute immersion in a 50% bleach solution and then rinsed three times in sterile, Milli-Q water. Surface-sterilized seeds were germinated in the dark for three days on 1% agar plates. Spot inoculations were performed as described (59) with the following modifications: 0.1 - 10 μg (glucose equivalent) of each succinoglycan sample was mixed with 5000-7000 succinoglycan-deficient *S. meliloti* cells (0.85% saline-washed cells from an overnight LBMC culture) in a final volume of 4 μl. This inoculum was applied directly to the seedling root in the region of emerging root hairs (4 μl of aqueous solution applied to a root in this way momentarily beads on the top of the root before wicking around and beneath the tip of the root). The plants were scored after 5 weeks for the presence of pink nodules. Nodules that were ambiguous in terms of their phenotype when viewed with the unaided eye were further examined using a dissecting microscope. Scoring was routinely performed in a blinded fashion to eliminate experimenter bias.

GFP-based plant assay rescue experiments.

GFP-based plant assays were conducted as described (33) with the following modifications for use in assaying the biological activity of LMW succinoglycan molecules. Each plant was inoculated with 10 μl of a cell suspension with an $O.D._{600}$ of 0.05 in which 1-10 μg of succinoglycan was dissolved. The dialysis tubing used to cover the seedling root was gently shaken prior to application to remove excess water that would dilute the inoculum. The plant roots were observed starting on the third day after inoculation. Final counts of the numbers of colonized, curled root hairs and infection threads were performed on the tenth day after inoculation.

Deacylation of succinoglycan.

Succinoglycan trimer was deacylated by treatment in 10 mM KOH for 6 hours at 20°C. The solution was then neutralized with 100 mM HCl, dialyzed against water, and then lyophilized.

RESULTS

Variability in these activity assays. The nodule invasion rescue experiments described below are subject to variability from experiment to experiment. In some iterations of the experiments, up to 70% of the plants co-inoculated with an invasion-deficient *S. meliloti* strain and symbiotically active succinoglycan have at least one nitrogen-fixing nodule. In other iterations of the same experiment, we do not observe rescue activity above background (10-15%). Typically, however, when we do see activity, the results fall somewhere between the two extremes.

Our results are consistent, however, in that we only see activity with the succinoglycan trimer. We have never observed significant nodule invasion-promoting activity associated with HMW succinoglycan, succinoglycan monomers, or succinoglycan dimers. The results presented below are compiled from multiple, independent experiments in which we have observed activity. We have also repeated these results with succinoglycan fractions purified from independent cultures. Thus, we believe that the results and conclusions presented are valid, despite the experiment to experiment variability that we have seen.

Although we have not yet succeeded identifying the source of the experiment to experiment variability, it is unlikely to be a result of the strains we have used, as all cultures have been inoculated from frozen permanent stocks. In addition, the seeds we have used are all from the same batch. Finally, using identical succinoglycan trimer from the same stock (which is kept frozen between uses), we have seen the experiment to experiment variability. Since the variability seems to affect all of the plants, it would appear to result from some as yet uncontrolled environmental factor and not some variability in inoculating individual seedlings.

The succinoglycan trimer is the symbiotically active species. Battisti et al. had previously reported that a succinoglycan tetramer was the symbiotically active species (10). However, in the course of a structural analysis of LMW succinoglycan, we demonstrated that *S. meliloti* LMW succinoglycan is composed of monomers, dimers, and trimers of the octasaccharide repeating unit and that no tetramers are present.

To develop an assay for further structure-function analyses involving succinoglycan and to reconcile the contradiction between our structural data and that previously reported, we purified succinoglycan produced by *S. meliloti* strain Rm1021 using gel filtration chromatography (Fig. 2A) and attempted to rescue the nodule invasion defect of Rm7210 (Rm1021 *exoY210*::Tn*5*)(79), a succinoglycan-deficient strain, using this material (Table 1). Five weeks after inoculation, only 14.3% of plant inoculated with strain Rm7210 alone had at least one pink, nitrogen-fixing nodule, a background consistent with previous reports (95). Purified HMW succinoglycan was not able to restore nodule invasion, but we did see partial rescue with unfractionated LMW succinoglycan. When we tested individual LMW succinoglycan oligomers for activity, only the succinoglycan trimer was able to restore significant levels of nodule invasion above background (Table 1). Therefore it appears that the succinoglycan trimer is the species active in promoting nodule invasion.

The smallest amount of purified succinoglycan trimer that gave the most robust rescue activity was 200 picomoles (1 μg glucose equivalent), though we did observe some activity using 20 picomoles of trimer. The addition of larger quantities of LMW succinoglycan (up to 2 nanomoles) did not improve the quality of the rescue we observed. Thus, for these and subsequent standard plant assay rescue experiments we have used 1 μg glucose equivalent of each fraction tested.

The succinoglycan succinyl and acetyl modifications are not necessary for the nodule invasion-promoting activity of succinoglycan trimer. In the course of purifying and analyzing LMW succinoglycan oligomers for use in our nodule invasion rescue assays, it became clear that LMW succinoglycan oligomers were highly heterogeneous with regard to their succinyl modifications (138). Individual succinoglycan trimers containing from 0 to 6 succinyl modifications could be detected in a trimer fraction purified by gel filtration chromatography (Fig. 2B). We were able to purify sufficient quantities of succinoglycan trimers with 1 through 6 succinyl modifications to perform subsequent invasion rescue assays, but we were unable to isolate sufficient quantities of trimer lacking succinyl modifications. Consequently, we generated non-succinylated trimer molecules for use in our rescue experiments by base-treating a gel filtration purified trimer fraction, a treatment that removes both the succinyl and acetyl modifications from the polysaccharide backbone but leaves the pyruvyl modification.

To determine whether the degree of succinylation was important for the biological activity of the succinoglycan trimer, we performed a series of nodule invasion rescue experiments using the differentially succinylated trimers (Table 2). In these experiments, only 10% of the plants inoculated with the succinoglycan-deficient, invasion-deficient strain (Rm7210) developed one or more pink, nitrogen-fixing nodule. All of the differentially succinylated trimer molecules, including the completely de-acylated trimer, appeared to be able to partially restore nodule invasion, suggesting that neither the succinyl modification nor the acetyl modification are required for promoting nodule invasion.

The efficiency of nodule invasion rescue is low. Though we observed a partial restoration of nodule invasion mediated by succinoglycan trimers in standard plant assays, the overall proportion of nodules that were pink on any given plant that had at least one pink nodule was low. Typically, a 5-week-old plant inoculated with *S. meliloti* strain Rm1021 developed 25-30 total nodules, almost all of which were pink, and 100% of plants were successfully infected (data not shown). In these rescue experiments, plants with at least one pink nodule usually had 1 to 4 pink nodules. This suggested that the rescue that we observed was just sufficient for us to be able to detect the response by the criteria used.

Two types of pink nodules are present on plants with rescued infections. When scoring plant roots for the presence of pink nodules, we observed two distinct pink nodule phenotypes. The first class of pink nodules were large, elongated, and had a dark pink color, very similar to those observed when alfalfa were inoculated with the wild type strain Rm1021. Plants that had a dark pink nodule always had some dark green, healthy growth, indicating that significant nitrogen fixation was occurring. The second class of pink nodules were small, elongated, and light pink in color. Plants containing light pink nodules but no dark pink nodules were often less healthy than those plants that had at least one dark pink nodule. This suggested that the light pink nodules were fixing less nitrogen than the dark pink nodules or had begun but then ceased fixing nitrogen.

In most cases, the light pink nodules that we observed were present on plants that were treated with the succinoglycan-deficient strain and symbiotically active succinoglycan. Only a small percentage (8%) of pink nodules found on plants inoculated with the invasion-deficient strain alone were light pink. Of the pink nodules present on the plants treated with the

succinoglycan trimer, a significant number (63%) were light pink. This suggested that the light pink nodules were largely the result of rescued nodule invasion events.

To determine whether the bacteria present in pink nodules that we observed in our rescue assays had reverted to a succinoglycan-producing state, we isolated *S. meliloti* from both light pink and dark pink nodules. We typically recovered more bacteria from dark pink nodules (10-100 fold more) than the light pink nodules, suggesting that the dark pink nodules contained more viable bacteria than the light pink nodules. All of the bacteria isolated from both types of pink nodules were succinoglycan-deficient, indicating that these pink nodules were not the result of an invasion by succinoglycan-proficient revertants (data not shown). To be certain that the bacteria from the rescued pink nodules had not acquired a suppressor mutation that allowed invasion (for example, a mutation affecting EPS II or K antigen), we inoculated alfalfa plants with these bacteria. The rescued pink nodule bacteria were still defective in nodule invasion, indicating that they had not acquired a mutation that suppressed their symbiotic defect (data not shown).

The succinoglycan trimer promotes the initiation and extension of infection threads. To determine whether the increase in the number of pink nodules that we observed in our rescue experiments was accompanied by an increase in infection thread initiation and extension, we modified a GFP-based system (33, 53) for quantifying events in nodule invasion (i.e.- colonization of curled root hairs and the initiation and extension of infection threads) for use as a biological activity assay for succinoglycan. When we inoculated alfalfa seedlings with a succinoglycan-deficient strain (which was also deficient in production of the ExoK and ExsH glycanases – see discussion) that constitutively expressed GFP (Rm8834 = Rm1021 *exoY exsH*

exoK), we saw an infection thread growth defect consistent with what had been previously observed for succinoglycan deficient strains (33, 98): only 17.2% of colonized, curled root hairs initiated infection thread growth, and less than 2% of the colonized, curled root hairs had infection threads that were extended to the base of the root hair cell (Table 3). In contrast, when strain Rm8834 was coinoculated with gel filtration-purified succinoglycan trimer, nearly half of the colonized curled root hairs initiated infection thread growth, and nearly one quarter of the colonized, curled root hairs developed extended infection threads (Table 3). Thus, it appears that, in addition to promoting the formation of nitrogen-fixing nodules, purified succinoglycan trimer is capable of promoting the initiation and extension of infection threads.

These GFP experiments were also subject to the variability discussed above. To date, we have performed 4 nodule invasion rescue experiments in our GFP-based plant assay system. In two of the experiments we have seen activity associated with the succinoglycan trimer (the data presented in Table 3). In the other two iterations of the experiment, we have not observed nodule invasion-promoting activity associated with the succinoglycan trimer.

DISCUSSION

The results of these experiments strongly suggest that the succinoglycan trimer is the active species in promoting alfalfa root nodule invasion by *S. meliloti* (Table 1) and that the succinyl and acetyl modifications are dispensable for this activity (Table 2). In addition, it appears that the succinoglycan trimer can promote the initiation and extension of infection threads (Table 3). The fact that we have observed an increase in infection thread growth mediated by the succinoglycan trimer suggests that the trimer is promoting the formation of pink nodules via an infection thread-dependent invasion process and not by stimulating some other mode of invasion.

We have seen partial rescue of nodule invasion using as little as 20 picomoles of succinoglycan trimer, which, along with the low-molecular-weight nature of the succinoglycan trimer suggests that the succinoglycan trimer is acting as a signal to the host plant. The amount of succinoglycan required to promote nodule invasion activity is consistent with the quantity (picomoles to nanomoles) of purified Nod factor, a signal to the host plant, that can induce root hair curling and induction of cortical cell divisions (132). Thus, we propose that the succinoglycan trimer is acting as a signal to the host plant that induces the formation of infection threads.

If the succinoglycan trimer is indeed functioning as a signal to the host plant, it could be acting to modulate a number of plant responses. Two attractive possibilities are that succinoglycan signals the plant for the purpose of 1) modulating plant defense responses to facilitate invasion (95) or 2) signaling infection thread-specific rearrangements of the plant cytoskeleton (115). If the succinoglycan trimer is a signal to the host plant, then it is likely that

some sort of a receptor that senses the presence of succinoglycan exists which then transduces the signal and mediates the appropriate response.

A majority of the pink nodules that result from a rescued infection appear to be only partially infected (light pink nodules), a nodule phenotype observed previously by Urzanqui and Walker (133) in succinoglycan rescue experiments. From our previous GFP analyses (33, 98) of the nodule invasion process, we know that succinoglycan function is required both in the colonized, curled root hairs (for infection thread initiation) and in the infection thread (for infection thread extension). Thus, nodules that are not completely infected could be a result of this requirement for succinoglycan deep within the infection thread, a point that is not easily reached by exogenously applied succinoglycan. It is also possible that the exogenously applied succinoglycan is not present at the optimal time for highly efficient nodule invasion. This complication in the delivery of exogenously applied succinoglycan could result in the premature abortion of some of the rescued infection threads, resulting in nodules that are partially infected. In addition, nodules that appear to have ceased fixing nitrogen could result from the inability of rescued infection threads to continue growing, thereby depriving the continuously replicating cells of an indeterminate nodule of *S. meliloti* cells.

Because the succinyl and acetyl modifications appear to be dispensable for the nodule invasion-promoting activity of succinoglycan, it would appear that their primary function is in succinoglycan molecular weight control. Almost all of the succinoglycan produced by a *S. meliloti* strain defective in adding the succinyl modification to succinoglycan is HMW polysaccharide (78), whereas a strain that fails to acetylate succinoglycan produces a significantly greater proportion of its succinoglycan as LMW material relative to a wild type strain (145). This genetic data is supported by biochemical data that indicate that the succinyl

modification increases the susceptibility of succinoglycan to the ExoK and ExsH glycanases, whereas the acetyl modification decreases the susceptibility of succinoglycan to the ExoK and ExsH glycanases (145).

It is currently unclear whether HMW succinoglycan plays some as yet unidentified role in the *S. meliloti*-alfalfa symbiosis, as it is not able to promote root nodule invasion. However, it seems highly likely that HMW succinoglycan is involved in some aspect of the free-living state of *S. meliloti*. For example, as with polysaccharides produced by other bacteria, HMW succinoglycan could protect the cells against physiological stress or could play a role in biofilm formation.

The question of why we see biological activity in some rescue experiments and not in others remains unresolved at the writing of this thesis. It is possible that there is some as yet unidentified environmental factor that is responsible for the variability. For example, variations in the relative humidity coupled with the fact that root hair cells are very sensitive to humidity and are very fragile could be introducing variability into these experiments. Whatever the case, we would like to develop a more consistent assay 1) to reduce the amount of experimentation necessary to determine whether a particular polysaccharide fraction is active and 2) to mitigate any doubt that arises as a result of the variability and are actively exploring how the response in our rescue assays can be improved.

To date we have explored a number of variations in our nodule invasion rescue assays in an attempt to improve the response in our rescue assays. However, none of these individual changes have overcome the experiment to experiment variability or significantly improved rescue. In the course of our experiments we have varied a number of conditions in our assays: 1) Because our analyses have shown that LMW succinoglycan oligomers are good substrates

for cleavage by the ExoK and ExsH glycanases, we have recently attempted to rescue nodule invasion in a succinoglycan-deficient strain that is unable to produce both ExoK and ExsH. 2) We have used purified agar (Sigma-Aldrich) in the place of Bacto-Agar (Difco). 3) We have added inhibitors of ethylene production [aminoethoxyvinyl glycine (AVG) and silver sulfate] to the plant growth media to alleviate any possible negative effects from production of this plant hormone. 4) We have buffered the plant growth media to neutral pH. 5) We have subjected plants to a "vacuum infiltration" treatment immediately following inoculation (2 minutes under house vacuum). We are actively continuing our experimentation with changes in our protocols in an attempt to improve the response that we see in the rescue assays.

Should we develop a more consistent biological activity assay for succinoglycan, there are a number of questions we plan to address. Though the succinyl and acetyl modifications seem to be dispensable for the nodule-promoting activity, it remains to be seen whether the pyruvyl modification is required for the biological activity of succinoglycan. We would also like to test each of the differentially succinylated trimer molecules in a GFP-based rescue assay to confirm that each molecule can stimulate infection thread growth.

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Figure 1. (A) The structure of the succinoglycan repeating unit. The asterisk indicates the position of a second succinyl modification in some repeating units (138).

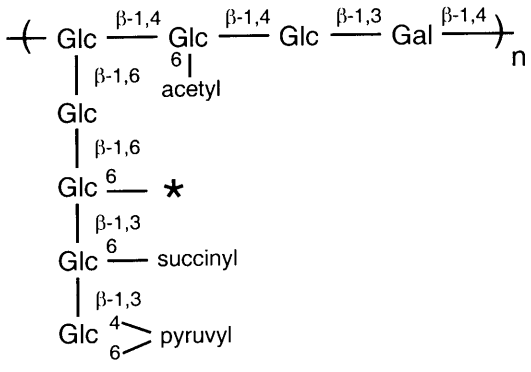
Glc
β-1,4
Glc
β-1,4
Glc
β-1,3
Gal
β-1,4
n
6
acetyl
β-1,6
Glc
β-1,6
Glc
6
*
β-1,3
Glc
6
succinyl
β-1,3
Glc
4
6
pyruvyl

Figure 2. (A) Bio-Gel P-6 gel filtration chromatography of succinoglycan secreted by *S. meliloti* strain Rm1021. Peak 1 is HMW succinoglycan (void volume). LMW succinoglycan is contained in peaks 2, 3, and 4 and correspond to trimers, dimers, and monomers, respectively, of the octasaccharide repeating unit. (B) Dionex high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) analysis of succinoglycan trimers purified by gel filtration chromatography (peak 2 in part (A) above). A PA-100 column used for this separation.

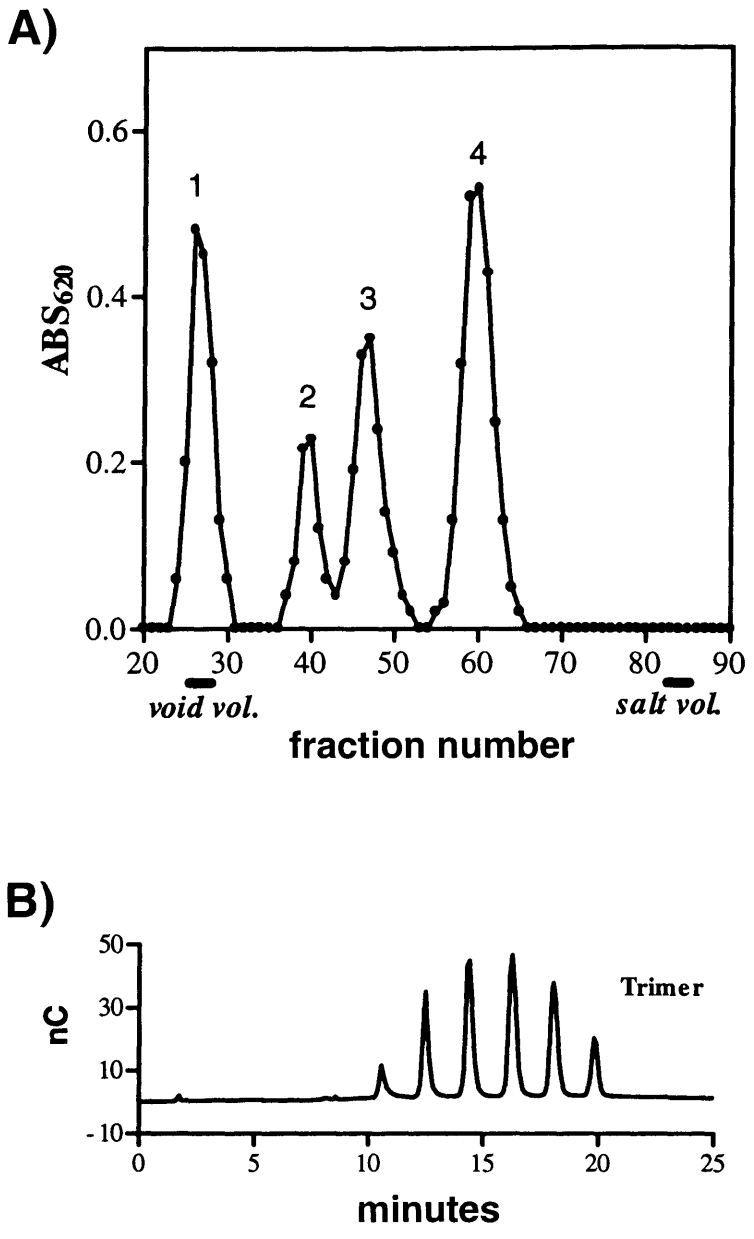
A)
0.6
0.4
0.2
0.0
ABS620
1
2
3
4
20
30
40
50
60
70
80
90
void vol.
salt vol.
fraction number
B)
50
30
10
-10
nC
Trimer
0
5
10
15
20
25
minutes

TABLE 1. Results[a] of standard plant assay rescue experiments using various succinoglycan fractions

Treatment	% rescue[b] (# of plants tested)
Rm1021	100% (15)
exoY[c] alone	14.3% (35)
exoY + 1 μg HMW SG[d]	10% (20)
exoY + 1 μg LMW SG[e]	45.9% (37)
exoY + 1 μg SG monomer	22.5% (31)
exoY + 1 μg SG dimer	22.5% (31)
exoY + 1 μg SG trimer	58.8% (51)

a – these are the combined results of three experiments
b – % of plants with at least one pink nodule at 5 weeks after inoculation
c – Rm7210 (Rm1021 *exoY210*::Tn*5*)
d – SG = succinoglycan
e – consists of succinoglycan monomers, dimers, and trimers

TABLE 2. Results[a] of standard plant assay rescue experiments using differentially succinylated succinoglycan trimers

Treatment	% rescue[b] (# of plants tested)
Rm1021	100% (12)
exoY[c] alone	10% (40)
exoY + 1 μg SG T-0[d]	46.2% (39)
exoY + 1 μg SG T-1[e]	42.1% (38)
exoY + 1 μg SG T-2	30.1% (39)
exoY + 1 μg SG T-3	46.2% (39)
exoY + 1 μg SG T-4	37.5% (40)
exoY + 1 μg SG T-5	43.6% (39)
exoY + 1 μg SG T-6	45.% (40)

a – these are the combined results of two experiments
b - % of plants with at least one pink nodule at 5 weeks after inoculation
c – Rm7210 (Rm1021 *exoY210*::Tn*5*)
d – T-0 is de-acylated trimer
e – for T-1 to T-6, the number indicates the number of succinyl modifications

TABLE 3. Results[a] of GFP-based plant assay rescue experiments

Treatment	# of plants	#CCRHs[b]	%CCRH w/IT[c]	%CCRH w/ExIT[d]
Rm1021	8	65	98.5%	87.7%
exoY[e]	20	116	17.2%	1.7%
exoY + 4 μg SG trimer[f]	20	87	49.4%	24.1%

a – these are the combined results of two experiements
b – number of colonized, curled, root hairs
c – per cent of colonized, curled root hairs with infection threads
d – per cent of colonized, curled root hairs with extended infection threads
e – Strain used was Rm8834 (Rm1021 *exoY exoK exsH*)
f – 4 μg of material was used to account for dilution when dialysis membrane is added

Chapter 5

Conclusions

My dissertation research has focused on three topics involving the roles played by symbiotically important *Sinorhizobium meliloti* polysaccharides in the symbiosis with alfalfa. 1) This work has advanced the field's understanding of the exact point in symbiosis where these polysaccharides function and of the relative efficiencies with which succinoglycan, EPS II, and K antigen mediates alfalfa root nodule invasion. 2) In my work I have characterized a *S. meliloti* regulatory gene that controls the production of symbiotically active EPS II. This has advanced the understanding of the regulatory mechanisms that *S. meliloti* uses to control the production and molecular weight distribution of its symbiotically important polysaccharides. 3) I have performed a number of structure-function analyses of succinoglycan which have strengthened the evidence that low-molecular weight succinoglycan functions as a signal to the host plant. These analyses have also provided some insight into the role played by the succinoglycan acetyl and succinyl modifications.

The work presented in **Chapter 2** has a number of implications for how the field views symbiotically important *S. meliloti* polysaccharides. First, this work demonstrated that succinoglycan, EPS II, and K antigen function with significantly different efficiencies to mediate alfalfa root nodule invasion. Previously, it was thought that these three polysaccharides were iso-functional with regard to their symbiotic efficiencies on alfalfa. The fact that EPS II and K antigen-mediated infection threads frequently have aberrant morphologies suggests that the three polysaccharides are acting by related, but not identical, mechanisms to mediate alfalfa root nodule invasion by *S. meliloti*. That the three polysaccharides function with significantly different efficiencies in the symbiosis with alfalfa raises the question of why many strains of *S. meliloti* produce more than one symbiotically active polysaccharide (80, 101, 147). Production

of more than one symbiotically important polysaccharide by *S. meliloti* may provide a strain with a selective advantage, allowing the strain to interact as efficiently as possible under a variety of conditions with many cultivars or ecotypes of legumes that it can nodulate.

The work presented in **Chapter 3** describes the cloning and characterization of a *S. meliloti* gene that controls the production of symbiotically active EPS II. The "*expR101* mutation" turned out to be a result of a precise excision of an IS element from a gene (*expR*) predicted to encode a LuxR homolog. The excision event appears to have restored the gene to its original status prior to the insertion. This is the only known condition that induces the production of symbiotically active EPS II in *S. meliloti* Rm1021. This result highlights the role of IS elements as vehicles for phenotypic variation in bacterial populations, as there are a number of examples of IS elements that modulate bacterial polysaccharide phase variation. This result also raises a number of questions, including how ExpR functions to turn on EPS II production. Though ExpR is a LuxR homolog, it does not (to date) appear to mediate a response to an accumulated extracellular factor. However, there are nine genes in *S. meliloti* predicted to encode LuxR homologs (11). ExpR could be part of a complex regulatory web involving multiple LuxR homologs, so it is possible that we did not perform our conditioned media experiments under conditions that would allow us to see a response. It will be of interest to determine whether any non-*exp* genes are regulated by ExpR and to search for genes that regulate *expR* expression. The question of why *expR*-mediated *exp* expression results in the production of symbiotically active EPS II when other inducers of EPS II production do not also remains open.

The work presented in **Chapter 4** demonstrates that the symbiotically active form (i.e.- the form that can promote nodule invasion) of succinoglycan is the trimer. Furthermore, it indicates that the succinyl and acetyl modifications of succinoglycan are dispensable for this activity. This suggests that a primary role for these modifications is in succinoglycan molecular weight control. I have also shown that the succinoglycan trimer can promote the initiation and extension of infection threads. Because very small quantities of a low-molecular weight molecule can promote nodule invasion, it is likely that succinoglycan functions as a signal to the host plant. Two attractive models are that succinoglycan is 1) modulating plant defense responses to facilitate invasion (95) or 2) signaling infection thread-specific rearrangements of the plant cytoskeleton (115). If indeed *S. meliloti* polysaccharides are functioning as signals to the host plant, it seems likely that plant receptors exist which sense the polysaccharides and transduce the signal. One intriguing issue raised by the potential existence of receptors for these polysaccharides is how the receptors recognize specific oligomers of the low-molecular-weight polysaccharides.

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