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A *Sinorhizobium meliloti* Lipopolysaccharide Mutant Altered in Cell Surface Sulfation

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The *Rhizobium*-legume symbiosis involves the formation of a novel plant organ, the nodule, in which intracellular bacteria reduce molecular dinitrogen in exchange for plant photosynthates. Nodule development requires a bacterial signal referred to as Nod factor, which in *Sinorhizobium meliloti* is a β-(1,4)-linked tetramer of *N*-acetylglucosamine containing *N*-acyl and *O*-acetyl modifications at the nonreducing end and a critical 6-*O*-sulfate at the reducing end. This sulfate modification requires the action of three gene products: *nodH*, which catalyzes the sulfonyl transfer, and *nodPQ*, which produce the activated form of sulfate, 3′-phosphoadenosine-5′-phosphosulfate. It was previously reported that *S. meliloti* cell surface polysaccharides are also covalently modified by sulfate in a reaction dependent on NodPQ. We have further characterized this unique form of bacterial carbohydrate modification. Our studies have determined that one of the *nodPQ* mutant strains used in the initial study of sulfation of cell surface harbored a second unlinked mutation. We cloned the gene affected by this mutation (referred to as *lps-212*) and found it to be an allele of *lpsL*, a gene previously predicted to encode a UDP-glucuronic acid epimerase. We demonstrated that *lpsL* encoded a UDP-glucuronic acid epimerase activity that was reduced in the *lps-212* mutant. The *lps-212* mutation resulted in an altered lipopolysaccharide structure that was reduced in sulfate modification in vitro and in vivo. Finally, we determined that the *lps-212* mutation resulted in a reduced ability to elicit the formation of plant nodules and by altered infection thread structures that aborted prematurely.

In order to acquire reduced nitrogen, many leguminous plants enter into symbiotic associations with bacteria, culminating in the formation of a novel plant organ, the nodule, in which intracellular bacteria reduce molecular dinitrogen to ammonia. In a compatible symbiotic interaction, the bacteria trigger an alteration in the growth of the plant root hairs, resulting in a curled structure that entraps a microcolony of the bacteria. This curled root hair is the site for a plant cell wall-encapsulated ingrowth of the root hair, referred to as an infection thread. The infection thread, filled with proliferating bacteria, extends to the base of the root hair cell and then penetrates the root, allowing bacterial entry into the plant. Concurrent with the development of the infection thread, the cells in the root cortex dedifferentiate, leading to new cell division and the consequent formation of the nodule. The infection thread branches and penetrates the developing nodule, delivering the bacteria, which are then released into the plant cytoplasm. These intracellular bacteria undergo a series of developmental changes and signal transduction events to induce the expression of nitrogenase, which catalyzes the reduction of molecular dinitrogen to ammonia (4, 5, 18, 20, 31, 41).

Symbiotic nitrogen-fixing relationships between legumes and the genera *Rhizobium, Bradyrhizobium, Mesorhizobium,* and *Sinorhizobium* (collectively called rhizobia) are mediated by chemical signals exchanged between the symbiotic partners (21, 32). The plant produces a chemical signal (usually in the form of flavonoid molecules) that is perceived by the bacterium and activates transcription of the *nod* genes. Most of the known *nod* gene products catalyze synthesis of an oligosaccharide signal, referred to as Nod factor. All known Nod factors consist of β-(1,4)-linked *N*-acetylglucosamine residues which are *N*-acylated at the nonreducing end (9–11, 15, 17, 38). To this Nod factor structure are added host-specific modifications, which in *Sinorhizobium meliloti* consist of a 16:2 *N*-acyl group and 6-*O*-acetyl group at the nonreducing end of the molecule and a 6-*O*-sulfate modification at the reducing end (24).

The presence of the sulfate modification on Nod factor is absolutely required for the establishment of the symbiosis on alfalfa and is dependent on the products of three genes, *nodH, nodP,* and *nodQ*. The *nodH* gene product catalyzes the sulfonyl transfer to the Nod factor backbone (13, 33), while the *nodP* and *nodQ* gene products form a sulfate-activating complex which catalyzes the conversion of sulfate to 3′-phosphoadenosine-5′-phosphosulfate (34, 35), an activated form of sulfate used by all known carbohydrate sulfotransferases. Two copies of *nodPQ* exist in *S. meliloti* (36). One copy (referred to $nodP_1Q_1$) is present on pSymA, a large (1.35-Mb) symbiotic plasmid which carries the majority of the *nod* genes. An additional copy of *nodPQ* (referred to as $nodP_2Q_2$) is present on pSymB, a distinct large (1.68-Mb) symbiotic megaplasmid. The genes $nodP_1Q_1$ and $nodP_2Q_2$ are functionally redundant in that both copies have to be inactivated to impair nodule formation on alfalfa (36).

In addition to sulfate modification of the *S. meliloti* Nod factor, sulfuryl modifications are also carried on polysaccharides that constitute the *S. meliloti* cell surface (6). Sulfate modification of cell surface polysaccharides is increased in the

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
Rm1021	SU47 Sm^r	27
JT402	Tn*5* located between *nodE* and *nodG*	39
JSS12	*nodP$_1$Q$_1$*::Tn*5-233 lps-212*	36
JSS12Tr	*nodP$_1$Q$_1$*::Tn*5-233*	36
JSS14	*nodP$_2$Q$_2$*::Tn*5*	36
DKR1	*lps-212,* Tn*5* located in noncoding region between *nodE* and *nodG*	This study
DKR58	*lps-212* pMS03	This study
DKR59	*lps-212* pDKR59	This study
DKR60	*lps-212* pDKR60	This study
DKR61	*lps-212* pDKR61	This study
DKR62	*lpsL*::pDKR62	This study
DKR63	*rkpK*::pDKR63	This study
JT210	*nodH*::Tn*5*	39
Plasmids		
pMB393	Broad-host-range plasmid pBBS containing pBluescript multicloning site	2
pMS03	Broad-host-range plasmid pMB393 containing constitutive *trp* promoter	This study
pDKR59	pMS03/*lpsL*	This study
pDKR60	pMS03/*lpsL212*	This study
pDKR61	pMS03/*rkpK*	This study
pDKR62	pVO155/*lpsL*	This study
pDKR63	pVO155/*rkpK*	This study
pXLGD4	pRK290/*hemA*::*lacZ*	1
pVO155	Integrational vector	28
pTB93G	pMB393/GFP	16

presence of plant inducers of *nod* gene transcription (19) and is dependent upon NodPQ (6). To understand the mechanism and symbiotic function of this rare form of sulfate modification, we investigated the role of *nodPQ* in the sulfation of cell surface polysaccharide. During these studies, we identified an unlinked mutation in the *nodP$_1$Q$_1$* mutant background used in the previous studies of cell surface sulfation (6). We characterized this unlinked mutation, *lps-212*, and found it to be an allele of the gene *lpsL*. We demonstrate that the *lpsL* gene encodes a UDP-glucuronic epimerase activity that is reduced in the *lps-212* mutant. The *lps-212* mutation alters the structure of *S. meliloti* lipopolysaccharide (LPS) and reduces its sulfation in vitro and in vivo. Finally, the *lps-212* mutant is symbiotically compromised, exhibiting at least a 10-fold reduction in nitrogen fixation.

MATERIALS AND METHODS

Bacterial strains and media. All strains used were derivatives of *Sinorhizobium meliloti* 1021 and are described in Table 1. All strains were grown in Luria-Bertani (LB) (8) or M9 medium (26) with the antibiotic concentrations described previously (28).

Strain construction. Strain DKR1 was constructed by using N3 phage grown on strain JT402 (39) to transduce strain JSS12 to neomycin resistance. Neomycin-resistant transductants which were spectinomycin sensitive (due to replacement of the *nodP$_1$Q$_1$*::Tn*5-233* with wild-type sequence) were screened for altered sulfation and LPS structure.

Plasmid construction. Plasmid pDKR59 was constructed by PCR amplification of *lpsL* from strain Rm1021 with primers 5′-TCTGCGAAAGCTTCCCGACCCTGGA-3′ and 5′-TGCAATTGGGTACCGAAGCACGCGC-3′. The PCR product was cloned into the Topo2.1 plasmid with the TopoTA cloning kit (Invitrogen). The resulting plasmid was then digested with *Kpn*I and *Cla*I, and the insert was isolated from an agarose gel with a gel extraction kit (Qiagen) and ligated with plasmid pMS03 digested with the same enzymes. Plasmid pDKR60 was constructed in the same manner as pDKR59, but chromosomal DNA prepared from strain DKR1 was used to amplify *lpsL*.

Plasmid pDKR61 was constructed by PCR amplification of *rkpK* with primers 5′-TGTGCGAAGCTTGTGCTTCCGCACC-3′ and 5′-GGCAGAGGGATCCCCGTGCAGCTTC-3′. The PCR product was then cloned into pCR2.1 as described above. The plasmid was then digested with *Kpn*I and *Cla*I, and the insert was isolated and cloned into pMS03 digested with the same enzymes. Plasmid pMS03 was constructed by digestion of plasmid pTE3 with *Hin*dIII. The insert containing the *trp* promoter was isolated as described above and ligated into pMB393 (3) digested with the same enzymes.

Plasmid pDKR62 was constructed by PCR amplification of an internal fragment of *lpsL* from strain Rm1021 with primers 5′-TATGTCGACGCGAACCTCGT-3′ and 5′-ATTTCTAGAGCGCCATGTCCGGCCG-3′. The PCR product was cloned into pCR2.1 as described above. The plasmid was then digested with *Sal*I and *Xba*I, and the insert was isolated and ligated with pVO155 (28) digested with the same enzymes. Plasmid pDKR63 was constructed by PCR amplification of an internal fragment of *rkpK* with primers 5′-AGAAAGTCGGTACCAATGTGCAGGA-3′ and 5′-TACTTGGATCCGCGGATATCGCCGC-3′. The PCR product was cloned into pCR2.1 as described above. The plasmid was then digested with *Sal*I and *Bam*HI, and the insert was isolated and then ligated into pVO155 digested with the same enzymes.

Preparation of extracts for LPS analysis. Extracts were prepared according to Reuhs et al. (30) with the following modifications. The cells from 1.5 ml of log-phase (optical density at 600 nm, 0.5) culture were centrifuged at 8,000 × *g* and resuspended in 1 ml of water. The cells were again centrifuged at 8,000 × *g*, and the pellet was resuspended in 0.15 ml of solution A (0.05 M Na_2HPO_4, 0.005 M EDTA, pH 7). To the cell suspension was added 0.15 ml of 90% phenol, and the sample was vortexed and then incubated at 65°C for 15 min, followed by incubation on ice for 10 min. Samples were then centrifuged at 8,000 × *g* for 10 min, and the aqueous phase was removed and dried under vacuum. The pellet was resuspended in sample loading buffer and fractionated by deoxycholate-polyacrylamide gel electrophoresis (PAGE) as described previously (30). The polysaccharides were then visualized by silver staining (Bio-Rad) or phosphorimaging (Bio-Rad).

Preparation of cell surface protein extracts. Cultures of 100 ml of cells were grown in LB medium until the cultures reached stationary phase, and the cells were then collected by centrifugation at 600 × *g*. The cell pellets were resuspended in 3 ml of buffer A (25 mM Tris-HCl [pH 7.5] containing 5 mM 2-mercaptoethanol and 10% glycerol) and disrupted by two passes through a Bionebulizer (Glasco), and the cell debris was removed by centrifugation at 6,000 × *g*. A cell surface extract was then prepared by centrifugation at 100,000 × *g* for 30 min in a tabletop ultracentrifuge (Beckman). The resulting pellet was resuspended in 100 μl of buffer A, and the protein concentration was determined by a modified Bradford assay (Bio-Rad).

In vitro cell surface sulfation assay. From 2.5 to 10 μg of membrane extract was combined with 5 μCi of $^{35}SO_4$-labeled 3′-phosphoadenosine-5′-phosphosulfate (prepared as described previously [13, 25, 34]), and 2 μl of 5× buffer B (50 mM Tris-HCl [pH 8], 30 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol) in a total reaction volume of 10 μl. The mixture was then allowed to incubate for 30 min at 30°C, and the reaction was stopped by incubation for 2 min at 100°C. Sodium dodecyl sulfate (SDS) sample buffer, prepared as described above was then added, and the samples were heated at 95°C for 5 min and fractionated on an SDS–12.5% PAGE gel. The gel was dried, and the incorporation of $^{35}SO_4$ into LPS was then determined by quantitation of the radioactivity in the gel with a Bio-Rad phosphorimager.

UDP-glucuronic acid epimerase assay. [U-^{14}C]uridine 5′-diphosphoglucuronic acid (10 μCi of 225 mCi/mmol; ICN) was incubated with 2 to 10 μg of clarified soluble cell extracts that had been desalted by passage through a G-25 spin column (Pharmacia). The reaction was allowed to proceed for 30 min at 37°C. The extracts were then spotted on fluorescent polyethyleneimine-cellulose plates (Baker) and developed in 0.3 M lithium chloride. The migration of the labeled material was monitored by autoradiography or phosphorimaging, (Bio-Rad). The migration of UDP-glucose, UDP-glucuronic acid, and UDP-galacturonic acid was determined by comparison to unlabeled standards (Sigma) that were visualized by long-wave UV light.

UDP-glucose dehydrogenase assay. UDP-glucose dehydrogenase was assayed spectrophotometrically as described previously (19).

Nodulation assay. The ability of wild-type and *lps-212* mutants to initiate formation of nodules on alfalfa was assayed as described previously (12). Briefly, alfalfa seeds were sterilized by shaking in 70% ethanol for 45 min, followed by shaking for 45 min in 20% hypochlorite. The seeds were then washed four times

with sterile H_2O, allowed to imbibe H_2O overnight, and germinated on an inverted petri dish in the dark. The seedlings were then transferred to slant agar tubes containing BNM agar (12). The plants were allowed to grow in the tubes for 4 days and then were inoculated with bacterial strains which had been cultured to log phase (optical density at 600 nm, 0.5) in TY medium and then diluted 1:200 in BNM liquid medium. A total of 5 ml of medium was poured over the plants and then removed from the tube. At various times, inoculated plants were observed under a dissecting scope, and the number of nodules was counted. Each experiment was performed with replicate tubes.

Nitrogenase assay. Nitrogen fixation was quantitated in plants 28 days after inoculation via the acetylene reduction assay (40). Ethylene-acetylene separation and quantitation were carried out on a Shimadzu GC-8A1F gas chromatograph with a Porapak N column and flame ionization detector. The amount of ethylene produced was calculated by integration of the peak and converted to nanomoles of ethylene formed per plant by comparison to a standard curve developed from injected standard amounts of ethylene.

LacZ staining of bacteria in infection threads. The progress of infection was monitored by visualization of the bacteria within the infection thread as described previously (1, 3). Briefly, wild-type and *lps-212* mutant strains were engineered to express β-galactosidase by introduction of plasmid pXLGD4 containing *lacZ* under the control of the constitutive *hemA* promoter. The plants were inoculated with the mutant strains as described above, and the infection was allowed to proceed for either 7 days or 28 days. Sections of the plant root containing nodules were then removed and fixed with 1.25% glutaraldehyde in 200 mM sodium cacodylic acid (pH 7.2) under vacuum for 30 min, followed by incubation under normal atmospheric pressure for an additional hour. The plants were washed four times for 15 min with 200 mM sodium cacodylic acid buffer (pH 7.2) and then placed in 1 ml of 200 mM sodium cacodylate (pH 7.2)–5 mM potassium ferrocyanide–5 mM sodium ferricyanide–0.8% X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside). The samples were allowed to stain overnight in the dark and were then washed twice with 200 mM sodium cacodylate (pH 7.2), followed by treatment for 5 min with 20% hypochlorite. Finally, the plants were washed two times with 200 mM sodium cacodylate (pH 7.2) and then photographed under visible light with a Nikon Optiphot microscope with a 20× objective.

RESULTS

Isolation of *S. meliloti* mutant with altered cell surface sulfation. A previous study reported that carbohydrate residues on the *S. meliloti* cell surface are covalently modified by sulfate in a reaction dependent on NodPQ (6). To further characterize this modification in vivo, we examined the sulfate modification of LPS in strains having null mutations in either $nodP_1Q_1$ or $nodP_2Q_2$. Our results were consistent with the previous studies (6), demonstrating that NodPQ are required for sulfation of cell surface carbohydrates (D. H. Keating, M. G. Willits, and S. R. Long, unpublished data).

However, we observed that LPS purified from exponentially growing strain JSS12 (*nodP*$_1$*Q*$_1$::Tn*5-233 lps-212*), fractionated by deoxycholate-PAGE and visualized by silver staining, was structurally distinct from wild-type LPS in that the LPS core region migrated at a higher relative mobility than wild-type LPS core (Fig. 1A). We found that exponentially growing *S. meliloti* produces primarily rough LPS (comprised of lipid A and LPS core) and lacks detectable O-antigen. Insertions that inactivated $nodP_2Q_2$ did not produce this altered form of LPS (Fig. 1A). Consistent with the observed changes in LPS structure, strain JSS12 was also found to be sensitive to the detergent deoxycholate (D. H. Keating, M. G. Willits, and S. R. Long, unpublished data), a common characteristic of mutants altered in LPS biosynthesis (19, 22). These results indicated that strain JSS12 produced an altered form of LPS.

Altered form of LPS found in JSS12 is genetically separable from the *nodP*$_1$*Q*$_1$::Tn*5-233* insertion. Transduction via phage N3 of the *nodP*$_1$*Q*$_1$::Tn*5-233* insertion from strain JSS12 into strain Rm1021 yielded strain JSS12Tr, which no longer showed the structural alteration of LPS seen in JSS12 (Fig. 1A). In order to demonstrate conclusively that the mutation leading to the altered LPS was unlinked to *nodP*$_1$*Q*$_1$::Tn*5-233*, we used strain JT402, which harbors a Tn*5* tightly linked to *nodP*$_1$*Q*$_1$ (39). Strain JT402 was used as a donor in phage N3-mediated transduction, selecting for the antibiotic resistance of the insertion (in a noncoding position adjacent to *nodP*$_1$*Q*$_1$), and the resulting transductants were then scored for retention or loss of the *nodP*$_1$*Q*$_1$::Tn*5-233* insertion.

A significant portion (24%) no longer harbored the *nodP*$_1$*Q*$_1$::Tn*5-233* insertion and were presumed to result from replacement of the *nodP*$_1$*Q*$_1$::Tn*5-233* region with the wild-type sequence. The transductants that no longer contained the *nodP*$_1$*Q*$_1$::Tn*5-233* (and were wild type for *nodP*$_1$*Q*$_1$) retained the altered LPS phenotype, as judged by silver-stained PAGE of LPS preparations (Fig. 1A) and by deoxycholate sensitivity (D. H. Keating, M. G. Willits, and S. R. Long, unpublished data). One of these strains, DKR1, was chosen for use in subsequent experiments. These data demonstrate that strain

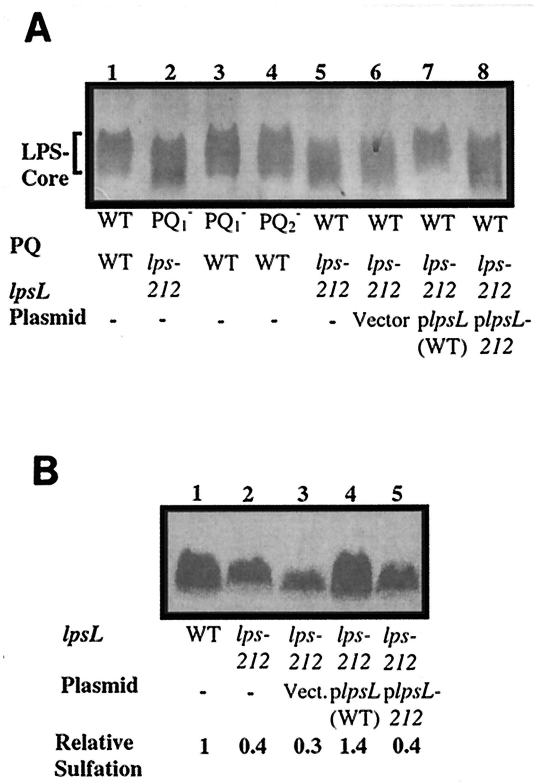

FIG. 1. Analysis of LPS from wild-type and mutant strains. (A) Silver-stained deoxycholate-PAGE gel of purified LPS core from *S. meliloti*. Lane 1, strain Rm1021 (wild type [WT]); lane 2, strain JSS12 (*nodP*$_1$*Q*$_1$::Tn*5-233 lps-212*); lane 3, JSS12Tr (*nodP*$_1$*Q*$_1$::Tn*5-233)*; lane 4, JSS14 (*nodP*$_2$*Q*$_2$::Tn*5*); lane 5, DKR1 (*lps-212)*; lane 6, DKR58 (*lps-212* pVector); lane 7, DKR59 (*lps-212* p*lpsL*); lane 8, DKR60 (*lps-212* p*lpsL212*). (B) In vivo sulfate labeling of *S. meliloti* LPS core. Wild-type and *lps-212* strains were cultured in the presence of 10 μCi of $Na_2{}^{35}SO_4$; the LPS was extracted, fractionated by deoxycholate-PAGE, and analyzed by phosphorimaging as described in Materials and Methods. Lane 1, Rm1021 (wild type [WT]); lane 2, DKR1 (*lps-212*); lane 3, DKR58 (*lps-212* pVector); lane 4, DKR59 (*lps-212* p*lpsL*); lane 5, DKR60 (*lps-212* p*lpsL212*).

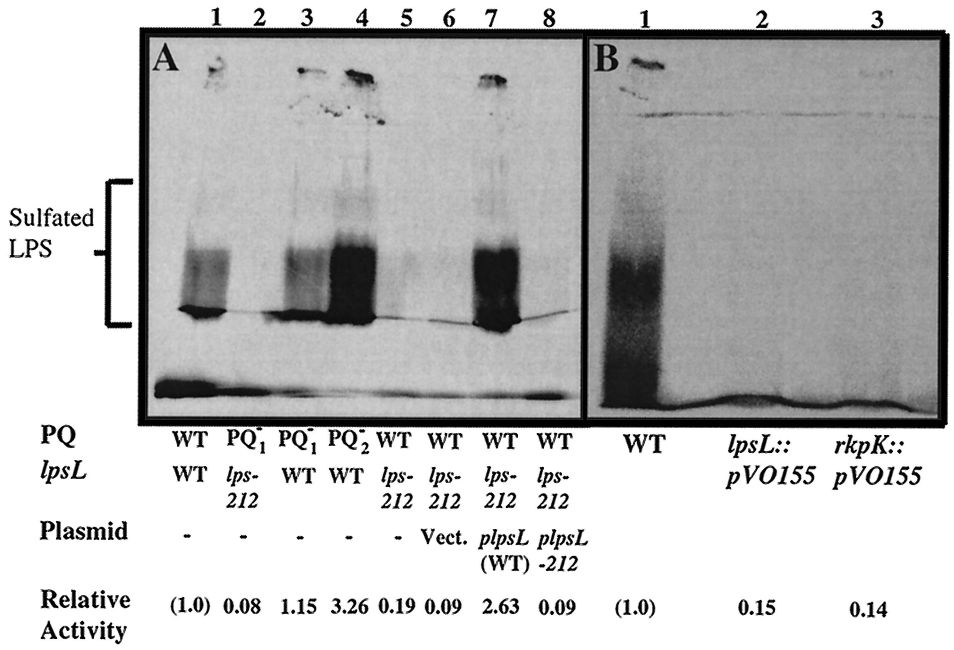

FIG. 2. In vitro LPS sulfation of wild-type *S. meliloti* and *lps-212* mutants. (A) In vitro sulfation activity of wild-type and mutant extracts. Extracts were prepared and assayed for in vitro polysaccharide sulfotransferase activity as described in Materials and Methods. Lane 1, strain Rm1021 (wild type [WT]); lane 2, JSS12 (*nodP$_1$Q$_1$*::Tn*5-233 lps-212*); lane 3, JSS12Tr (*nodP$_1$Q$_1$*::Tn*5-233)*; lane 4, JSS14 (*nodP$_2$Q$_2$*::Tn*5)*; lane 5, DKR1 (*lps-212)*; lane 6, DKR58 (*lps-212* pVector); lane 7, DKR59 (*lps-212* p*lpsL*); lane 8, DKR60 (*lps-212* p*lpsL212*). (B) In vitro sulfation of mutants lacking either *rkpK* or *lpsL*. Lane 1, Rm1021 (wild type [WT]); lane 2, DKR62 (*lpsL*::pVO155); lane 3, DKR63 (*rkpK*::pVO155).

JSS12 harbors two mutations, *nodP$_1$Q$_1$*::Tn*5-233* and an additional mutation (referred to as *lps-212*) that affects the structure of LPS.

***lps-212* mutant is deficient in sulfate modification of LPS.** Our finding that strain JSS12 contained two distinct mutations brought into question whether the decreased sulfation seen in strain JSS12 (6) resulted from the *nodP$_1$Q$_1$*::Tn*5-233* insertion or from the presence of the *lps-212* mutation. Strain JSS12Tr (which contains *nodP$_1$Q$_1$*::Tn*5-233* in an otherwise wild-type background) showed a reduced amount of sulfation in vivo (D. H. Keating, M. G. Willits, and S. R. Long, unpublished data). However, strain DKR1 (which is wild type for *nodPQ*) also exhibited a reduction in the relative amount of sulfation when analyzed on deoxycholate-PAGE (Fig. 1B).

***lps-212* mutant is deficient in cell surface sulfation in vitro.** To understand the role of the *lps-212* mutation in LPS sulfate modification, we developed an in vitro sulfation assay. This assay uses previously established protocols to prepare 3′-phosphoadenosine-5′-phosphosulfate labeled with ^{35}S at high specific activity (13, 25, 34). This radiolabeled 3′-phosphoadenosine-5′-phosphosulfate was then used as the donor for a recently discovered sulfate transfer activity (D. H. Keating and S. R. Long, unpublished results), which is membrane associated and modifies carbohydrates present in the *S. meliloti* cell surface fraction.

When wild-type Rm1021 was assayed, an abundant sulfation activity was observed (Fig. 2). Strains JSS12Tr (*nodP$_1$Q$_1$*::Tn*5-233*) and JSS14 (*nodP$_2$Q$_2$*::Tn*5*) were also found to be wild type for this activity, demonstrating that NodPQ (which produces 3′-phosphoadenosine-5′-phosphosulfate) is not required for in vitro sulfation when 3′-phosphoadenosine-5′-phosphosulfate is provided (Fig. 2A). However, strains JSS12 and DKR1 were found to be strongly deficient in this activity (Fig. 2A). JSS12 and DKR1 have in common the alteration in LPS structure, implying that the mutant form of LPS associated with *lps-212* is unable to undergo sulfation in vitro.

Cloning of *lps-212*. The deoxycholate-sensitive phenotype of strain DKR1 was used to clone the *lps-212* gene by complementation. A medium containing 0.6 mg of deoxycholate per ml impaired the growth of the *lps-212* mutant (D. H. Keating, M. G. Willits, and S. R. Long, unpublished data). A cosmid library containing ca. 20-kb pieces of the Rm1021 genome was mated into DKR1, and tetracycline-resistant exconjugants were selected on deoxycholate-containing medium. Colonies which grew on this medium were then screened for complementation, as judged by silver-stained PAGE of cell surface extracts, and in vitro carbohydrate sulfation activity. Surprisingly, none of the colonies initially selected on this medium (20 colonies screened) were found to complement the altered LPS phenotype of DKR1. Replating all of the colonies initially selected for growth on deoxycholate-containing medium resulted in only about 1% survival during subsequent challenge with deoxycholate. Of these, the majority (9 of 10 colonies tested) retained deoxycholate resistance in subsequent replating experiments and displayed wild-type LPS (as judged by silver-stained PAGE of polysaccharide preparations) and wild-type sulfation activity in vitro (D. H. Keating, M. G. Willits, and S. R. Long, unpublished data). Three of these colonies were kept for further analysis.

***lps-212* is an allele of *lpsL*.** The nucleotide sequence of the complementing clones was determined. Two of the sequenced clones carried identical inserts, whereas the third clone had a

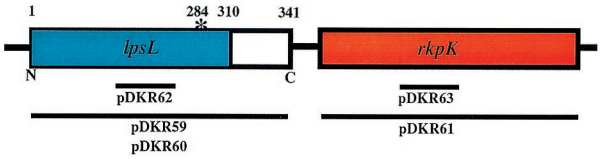

FIG. 3. *S. meliloti* genomic region containing *lpsL* and *rkpK*. The site of the frameshift mutation is denoted with an asterisk. The unshaded region corresponds to the part of LpsL truncated as the result of the premature termination resulting from the frameshift mutation. The numbers correspond to the amino acid positions of the open reading frame. The lines placed below the open reading frame depiction correspond to the regions of *lpsL* and *rkpK* carried on the plasmids used for complementation and gene inactivation.

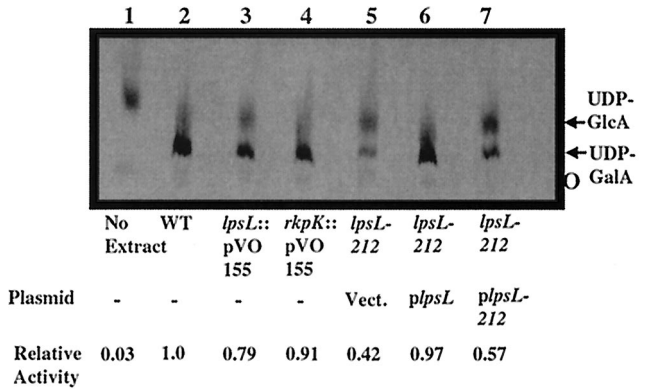

FIG. 4. Assay of UDP-glucuronic acid epimerase activity in the wild type and *lpsL* mutants. Wild-type and mutant extracts were assayed for UDP-glucuronic acid epimerase as described in Materials and Methods. Lane 1, no extract; lane 2, strain Rm1021 (wild type [WT]); lane 3, strain DKR62 (*lpsL*::pVO155); lane 4, DKR63 (*rkpK*::pVO155); lane 5, DKR58 (*lps-212* pVector); lane 6, DKR59 (*lps-212* p*lpsL*); lane 7, DKR60 (*lps-212* p*lpsL212*). UDP-GlcA, UDP-glucuronic acid; UDP-GalA, UDP-galacturonic acid; O, origin. Radioactive species were identified by migration in relation to unlabeled standards. Similar results were seen in four assays performed with independently prepared extracts.

distinct but overlapping insert. Comparison with the GenBank database showed that the nucleotide sequence of the inserts had >90% nucleotide identity to *lpsL* and *rkpK* of *S. meliloti* strain Rm41 (Fig. 3). The *S. meliloti* strain Rm41 *rkpK* gene was previously reported to encode UDP-glucose dehydrogenase, which converts UDP-glucose to UDP-glucuronic acid (19). The upstream gene, *lpsL,* had significant sequence similarity to nucleotide-sugar epimerases (19). Sequencing of PCR-amplified *lpsL* from the wild-type and DKR1 strains demonstrated a single nucleotide difference between the genes isolated from the two different sources, a deletion of a guanosine nucleotide near the predicted C terminus (nucleotide 847), which resulted in a shift of the reading frame and premature truncation of the predicted protein (Fig. 3).

The primary sequence information therefore suggested that *lps-212* was an allele of *lpsL.* To confirm these data, minimal clones containing either *lpsL* or *rkpK* were constructed and inserted into plasmid pMS03 downstream of the *Salmonella enterica* serovar Typhimurium *trp* promoter, which allows constitutive expression in *S. meliloti*. The plasmids were conjugated into DKR1, and complementation of the LPS defect was scored by assaying deoxycholate sensitivity, in vitro polysaccharide sulfation of plasmid-bearing extracts, and LPS structures.

A minimal clone containing the *lpsL* gene derived from the wild-type strain complemented DKR1, as judged by recovery of wild-type polysaccharide structure (Fig. 1A), in vivo LPS sulfation (Fig. 1B), and in vitro sulfation activity (Fig. 2A), suggesting that the mutated gene in strain *lps-212* was an allele of *lpsL.* A minimal clone containing *lpsL* isolated from strain DKR1 was unable to complement the alteration of polysaccharide structure (Fig. 1A), LPS sulfation in vivo (Fig. 1B), or in vitro sulfation (Fig. 2A), implying that the mutation in the *lpsL* gene of strain DKR1 eliminated its ability to restore wild-type phenotypes to strains containing the original *lps-212* mutation. Minimal *rkpK* clones from wild-type *S. meliloti* were unable to complement any of the phenotypes of strain DKR1 (D. H. Keating, M. G. Willits, and S. R. Long, unpublished results).

To demonstrate that *lps-212* mutant phenotypes arose from an altered allele of *lpsL*, we constructed null mutations in either *lpsL* or *rkpK* with the insertional mutagenic plasmid pVO155 (28). Strain DKR62, containing an insertion in *lpsL,* and DKR63, which contains an insertion in *rkpK,* were both found to be affected in LPS biosynthesis, as judged by deoxycholate sensitivity (D. H. Keating, M. G. Willits, and S. R. Long, unpublished results) and in vitro sulfation (Fig. 2B). This suggested that both *lpsL* and *rkpK* are required for both LPS synthesis and its modification with sulfate.

***lpsL* is required for UDP-glucuronic acid epimerase activity.** As mentioned above, *lpsL* isolated from strain Rm41 has been reported previously to bear a high degree of similarity to sugar epimerases (19). The gene *lpsL* from *S. meliloti* strain Rm1021 is also highly similar to genes encoding sugar epimerases (51% amino acid identity to *Escherichia coli galE*). Previous studies of *lpsL* from strain Rm41 predicted that UDP-glucose is converted to UDP-glucuronic acid via RkpK, which is then epimerized to UDP-galacturonic acid by LpsL (19).

To determine if *lpsL* encodes an epimerase activity capable of converting UDP-glucuronic acid to UDP-galacturonic acid, we used a thin-layer chromatography system which separates UDP-galacturonic acid from UDP-glucuronic acid. Radiochemically pure UDP-glucuronic acid was incubated with clarified *S. meliloti* cell extracts and then analyzed by thin-layer chromatography (Fig. 4). Wild-type Rm1021 extract was able to convert a detectable amount of UDP-glucuronic acid to UDP-galacturonic acid (as judged by migration with respect to unlabeled standards). However, the extract from strain DKR1 showed a reduced ability to carry out this reaction in vitro. The extract from strain DKR62, which carries an insertionally inactivated *lpsL*, also had a reduced amount of epimerase activity (Fig. 4), although greater than that of the strain DKR1 extract. The activity in strain DKR1 could be restored by addition of wild-type *lpsL* on a multicopy plasmid but not by a multicopy plasmid carrying the *lpsL212* allele, indicating that the product of the wild-type *lpsL* gene is required for nucleotide sugar epimerase activity in vitro. Strain DKR63, containing an insertionally inactivated *rkpK,* was found to have nucleotide sugar epimerase activity similar to that of the wild type (Fig. 4).

Strains Rm1021 and DKR1 and strains carrying insertions that inactivated *rkpK* or *lpsL* were also assayed for UDP-glucose dehydrogenase activity. Extracts prepared from the

TABLE 2. UDP-glucose dehydrogenase activity of wild-type and mutant strains

Strain	Genotype	UDP-glucuronic acid dehydrogenase activity (nmol/min/mg of protein)	Relative activity[a]
None		<0.23	<0.003
Rm1021	Wild type	74	1.0
DKR1	*lpsL212*	66	0.89
DKR62	*lpsL*::pDKR62	23	0.31
DKR63	*rkpK*::pDKR63	<0.23	<0.003

[a] The activity of strain Rm1021 was set at 1.

wild-type and DKR1 strains retained wild-type UDP-glucose dehydrogenase activity (Table 2), whereas extracts prepared from strain DKR63 (*rkpK*::pVO155) lacked detectable UDP-glucose dehydrogenase activity (Table 2). Interestingly, extracts prepared from strain DKR62 (*lpsL*::pVO155) were found to have a ca. 3-fold reduction in dehydrogenase activity. Because extracts from strain DKR1 had wild-type activity, the reduction in UDP-glucose dehydrogenase activity seen in DKR62 may result from a polar effect of the *lpsL*::pVO155 insertion on the transcription of *rkpK* (which is downstream of *lpsL*).

***lps-212* mutant is deficient in nodulation.** We assayed the ability of strain DKR1 to enter into a nitrogen-fixing symbiosis with alfalfa and found that DKR1 was deficient in several symbiotic phenotypes. First, the number of nodules produced by DKR1 was consistently lower than that formed by the wild type (Fig. 5A). Second, the DKR1-induced nodules had undetectable levels of nitrogenase activity (Fig. 5B). In fact, the rates of nitrogen fixation were the same as those of plants inoculated with an *S. meliloti nodH* mutant, which is incapable of producing nodules on alfalfa (Fig. 5A). Strain DKR1 harboring a wild-type copy of *lpsL* on a multicopy plasmid showed a significant but not wild-type level of nitrogen fixation. Strain DKR1 containing a plasmid with the *lpsL212* allele showed no detectable nitrogen fixation.

We believe that the inability of plasmid-borne wild-type *lpsL* to restore normal levels of nitrogen fixation is due to loss of the plasmid under the nonselective conditions present in planta. The pTB93G plasmid, which is based on the same vector as pMS03, has been reported previously to be unstable in planta (7). Strain DKR62 containing the *lpsL*::pVO155 mutation also produced nodules with a reduced amount of nitrogen fixation, but this activity was significantly higher than the level seen in plants inoculated with strain DKR1 (Fig. 5B).

Strain DKR1 is altered in nodule invasion. To further characterize the nature of the symbiotic deficiency associated with strain DKR1, we compared infection with the wild-type and DKR1 strains. using a chromogenic reporter to monitor the bacteria in planta. The wild-type and DKR1 strains were transformed with plasmid pXLGD4, which contains *lacZ* under the control of the *hemA* promoter. The *hemA* promoter allows strong constitutive expression of β-galactosidase in bacterial cells inhabiting the infection thread and plant cytoplasm (1, 23). The β-galactosidase activity encoded by *lacZ* could be visualized by staining the fixed nodule tissue with X-Gal, allowing observation of bacteria within the infection thread and nodule.

Analysis of the infection by wild-type *S. meliloti* demonstrated typical infection threads that rapidly penetrated the epidermal and cortical layers of the root (Fig. 6). Strain DKR1 showed a very different infection phenotype. Strain DKR1 was able to enter the plant (as judged by the ability of the X-Gal-staining cells to survive treatment with bleach). However, the infection threads differed from those of the wild type in that they had a thicker appearance and were unable to efficiently penetrate the developing nodule (Fig. 6). Instead, the bacteria formed darkly staining clusters, suggesting that the mutant bacteria were able to replicate extensively and were viable but were unable to enter the developing nodule.

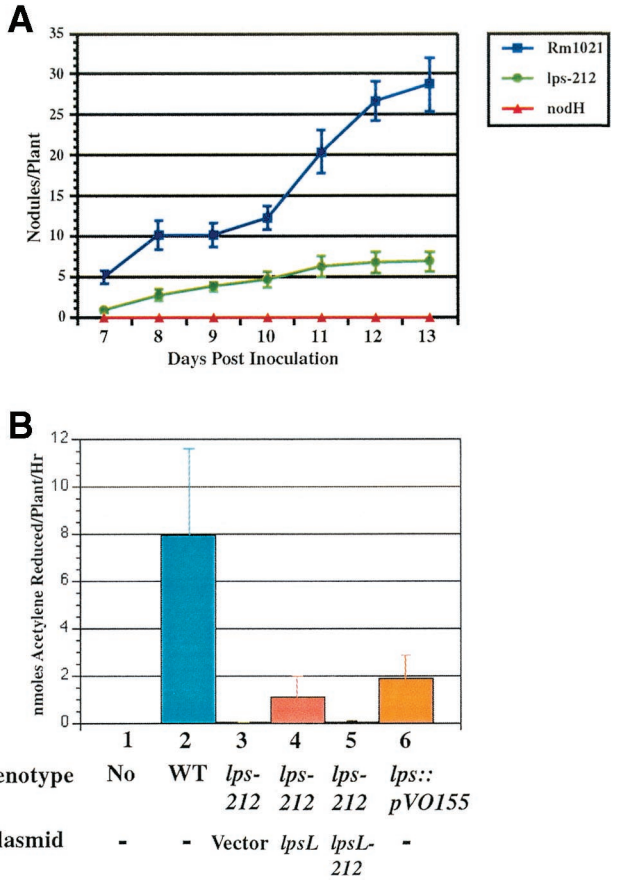

FIG. 5. Symbiotic assay of Rm1021 (wild type) and DKR1 mutant on alfalfa hosts. (A) Nodulation kinetics of Rm1021 (wild type), DKR1 (*lps-212)*, and JT210 *(nodH*::Tn*5*). Plants were grown on agar slants in the absence of added nitrogen and inoculated with bacterial strains, and the number of nodules was quantitated and plotted as a function of time as described in Materials and Methods. Error bars denote standard error in nodule number. (B) Nitrogen fixation assay of alfalfa nodules produced by wild-type and mutant *S. meliloti*. Plants were inoculated with *S. meliloti* and assayed for nitrogen fixation 21 days after inoculation as described in Materials and Methods. Lane 1, mock inoculation; lane 2, Rm1021 (wild-type [WT]); lane 3, DKR58 (*lps-212* pVector); lane 4, DKR59 (*lps-212* p*lpsL*); lane 5, DKR60 (*lps-212* p*lpsL212*); lane 6, DKR62 (*lpsL*::pVO155). Error bars denote standard error.

DISCUSSION

A hallmark of the legume nitrogen-fixing symbiosis is the limited number of plant symbiotic partners that are responsive

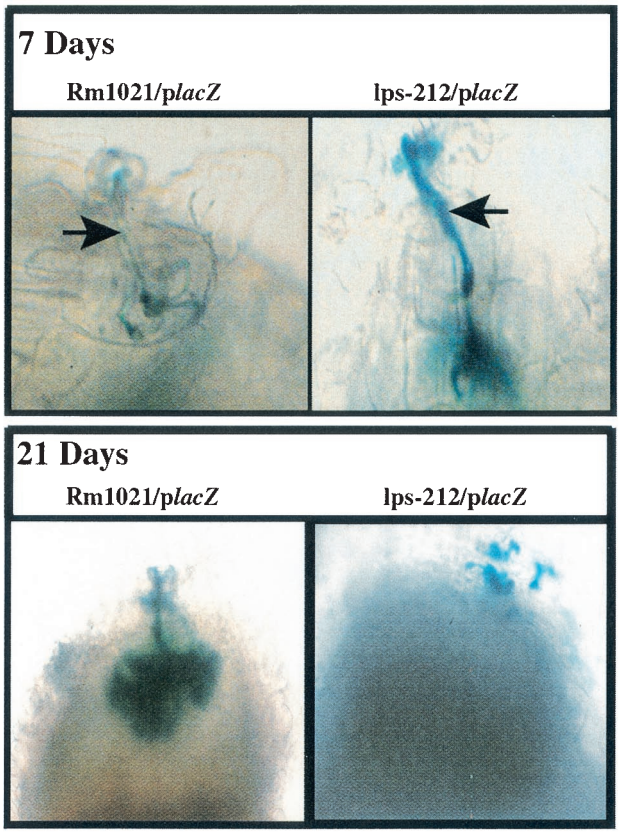

FIG. 6. Visualization of infection threads elicited by wild-type and *lps-212* mutant strains. Plasmid pXLGD4 containing a *hemA*::*lacZ* transcriptional fusion was mobilized into strain Rm1021 (wild type) and DKR1 (*lps-212*) by conjugation. The transconjugants were inoculated onto plants, and the progress of infection was examined at either 7 days or 28 days postinoculation by fixing and staining the plants for β-galactosidase activity as described in Materials and Methods. The arrows point to infection threads.

to a given species of bacteria. Chemical modifications of the Nod factors produced by the bacteria are essential for this host specificity (9–11, 15, 17, 38). However, several lines of evidence suggest that additional determinants of host specificity must exist. This was implied by experiments with heterologous rhizobia engineered to produce the *S. meliloti* Nod factor, which could elicit nodule formation on alfalfa but could not establish a nitrogen-fixing symbiosis (14).

Consistent with the requirement for additional types of host-specific signals, three classes of mutants which are able to elicit the formation of nodules but cannot establish an effective symbiosis have been isolated. Mutants of *S. meliloti* strain 1021 which lack a specific class of exopolysaccharide (known as succinoglycan), mutants blocked in synthesis of capsular polysaccharides (K-antigens) in *S. meliloti* strain Rm41, and mutations that disrupt the synthesis of LPS in *Rhizobium leguminosarum* all elicit the formation of nodules that are unable to fix nitrogen (7, 22, 29). Each of these mutants have in common defects in the synthesis of carbohydrates associated with the cell surface. The findings presented here with the *lpsL212* mutant further implicate cell surface carbohydrate species in the establishment of the *S. meliloti*-alfalfa symbiosis.

The *lpsL212* mutation present in strain DKR1 arose spontaneously in strain JSS12, a $nodP_1Q_1$ mutant with a reduced ability to produce 3′-phosphoadenosine-5′-phosphosulfate, the form of sulfate used as a substrate in all carbohydrate sulfotransfer reactions. Despite the intriguing connection between $nodP_1Q_1$ and *lpsL*, we have been unable to assign a role for the *lpsL212* mutation in this genetic context. The growth rates of JSS12 ($nodP_1Q_1$ *lpsL212*) and JSS12Tr ($nodP_1Q_1$ in an otherwise wild-type background) were similar, and no difference in viability was seen under a variety of conditions, including stationary phase and sulfate deprivation (D. H. Keating, M. G. Willits, and S. R. Long, unpublished results).

The mutation in strain DKR1 is an allele of *lpsL*, previously predicted to encode a UDP-sugar epimerase, based on substantial sequence identity to epimerases which convert UDP-glucose and UDP-galactose (19). Based on the phenotypes of *lpsL* and *rkpK* insertion mutations and their genomic proximity, *lpsL* was predicted to encode an epimerase that converts UDP-glucuronic acid (produced by RkpK) to UDP-galacturonic acid (19). In this report we demonstrated that *lpsL* is clearly required for UDP-glucuronic acid epimerase activity in vitro. Our studies with Rm1021 demonstrated that both *rkpK* and *lpsL* mutants are altered in cell surface, as judged by deoxycholate sensitivity and LPS analysis. *rkpK* mutants of strain Rm41were also found to have defects in the synthesis of K-antigen; this has not been tested in strain Rm1021, which has a K-antigen of distinct but as yet unknown polysaccharide composition (B. Reuhs, personal communication). Mutations in *lpsL* did not affect the synthesis of K-antigen in strain Rm41 (19), but this has not been tested in strain Rm1021.

Although the *lpsL212* mutation present in strain DKR1 shows phenotypic similarities with the *lpsL*::pVO155 insertion in strain DKR62, several differences also exist between the two different alleles. The *lpsL*::pVO155 insertion mutant was better able to fix nitrogen than the *lpsL212* mutant. This increased ability to fix nitrogen may in part result from loss of the *lpsL*::pVO155 insertion in planta, which would restore a wild-type gene and was shown to occur in ca. 1% of the bacteria isolated from the nodules of *lpsL*::pVO155-infected plants. However, the *lpsL*::Tn*5* insertion in strain Rm41 was also found to be capable of nitrogen fixation (19). The *lpsL* studies in strain Rm41 lacked a quantitative measure of nitrogen fixation and likely would have missed a subtle phenotype, such as was seen with the *lpsL*::pVO155 insertion. Thus, although the differences between the Rm41 and Rm1021 alleles of *lpsL* could simply be the result of differences between the two strain backgrounds, it is also possible that they reflect differences between the insertionally inactivated allele of *lpsL* and the *lpsL212* allele.

The differences seen between the *lpsL212* and *lpsL*::pVO155 alleles in symbiotic assays reflect those seen in biochemical assays of UDP-glucuronic epimerase activity. Extracts prepared from the *lpsL212* mutant consistently showed a reduced ability to produce UDP-galacturonic acid in vitro compared to extracts from the *lpsL*::pVO155 mutant. Although we do not yet understand the basis for the discrepancy in biochemical behavior between the *lpsL212* and *lpsL*::pVO155 alleles in this assay, *S. meliloti* contains five open reading frames that show substantial sequence identity to genes encoding UDP-sugar epimerases. In the absence of LpsL activity, proteins encoded

by these additional open reading frames may contribute to the low level of UDP-galacturonic acid synthesis seen in the *lpsL*::pVO155 mutant. The *lpsL212* allele, on the other hand, may prevent conversion of UDP-glucuronic acid to UDP-galacturonic acid by these alternative epimerases. The *lpsL212* allele is a frameshift mutation that results in a polypeptide which is truncated by only 31 amino acids (ca. 9% of the open reading frame). This truncated open reading frame may retain an ability to bind UDP-glucuronic acid or otherwise interfere with UDP-sugar metabolism.

Although we currently do not understand the biochemical mechanism by which *lpsL212* differs from *lpsL*::pVO155, it is clear that that *lpsL212* renders strain DKR1 incapable of entering into a nitrogen-fixing symbiosis. There are two potential explanations for the inability of strain DKR1 to establish an effective symbiosis. First, the abnormal infection thread structures seen in the chromatically tagged DKR1 strains could arise from a simple lack of viability within the infection thread microenvironment. While this remains a formal possibility, two lines of evidence are inconsistent with such a model. First, DKR1 can be recovered and efficiently cultured from infected nodules, indicating that viable bacteria are present. However, this assay is not quantitative, and subtle variations in viability would not have been detectable.

Second, although the infection thread structures in DKR1-infected nodules were clearly abnormal, they appeared to be filled with bacteria, as judged by expression of reporter fusions. It should be pointed out that the chromatic visualization technique used in these experiments relies on a protein molecule that is relatively stable within bacterial cells. Therefore, it is possible that the majority of cells within the infection thread are no longer viable and that the β-galactosidase activity seen in nodules arose from accumulation prior to the loss of bacterial viability. Future studies with fluorescent and chromatic tags with shorter half-lives are required to eliminate this possibility.

Despite the lack of quantitative measures for infection thread viability, qualitative assessment clearly showed that bacteria were present within the infection threads elicited by strain DKR1. This argues against a model postulating simple lack of viability of strain DKR1 in planta. In this respect, the *lpsL212* mutant is similar to *exo* mutants, which are unable to produce the acidic exopolysaccharide succinoglycan. Such *exo* mutants have been reported to elicit the formation of altered infection thread structures containing clusters of bacteria (7, 29), leading to the hypothesis that exopolysaccharides act as chemical signals between the bacteria and plant.

Based on the similarity of the phenotypes of DKR1 and *exo* mutants, it is possible that the inability of DKR1 to invade nodules stems from a similar lack of a signal required for infection thread growth. Strain DKR1 produces wild-type quantities of succinoglycan (D. H. Keating, M. G. Willits, and S. R. Long, unpublished results), and thus we propose that either the structure of the LPS on the *S. meliloti* cell surface or possibly its modification by sulfate is a likely candidate for such a signal. Studies of additional *S. meliloti* mutations affecting the synthesis of LPS and K-antigen as well as the identification of mutants specifically blocked in the sulfate modification of cell surface are required to test this hypothesis.

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