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The *rkp-1* Cluster Is Required for Secretion of Kdo Homopolymeric Capsular Polysaccharide in *Sinorhizobium meliloti* Strain Rm1021▽†

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Under conditions of nitrogen stress, leguminous plants form symbioses with soil bacteria called rhizobia. This partnership results in the development of structures called root nodules, in which differentiated endosymbiotic bacteria reduce molecular dinitrogen for the host. The establishment of rhizobium-legume symbioses requires the bacterial synthesis of oligosaccharides, exopolysaccharides, and capsular polysaccharides. Previous studies suggested that the 3-deoxy-D-manno-oct-2-ulopyranosonic acid (Kdo) homopolymeric capsular polysaccharide produced by strain *Sinorhizobium meliloti* Rm1021 contributes to symbiosis with *Medicago sativa* under some conditions. However, a conclusive symbiotic role for this polysaccharide could not be determined due to a lack of mutants affecting its synthesis. In this study, we have further characterized the synthesis, secretion, and symbiotic function of the Kdo homopolymeric capsule. We showed that mutants lacking the enigmatic *rkp-1* gene cluster fail to display the Kdo capsule on the cell surface but accumulate an intracellular polysaccharide of unusually high M_r. In addition, we have demonstrated that mutations in *kdsB2*, smb20804, and smb20805 affect the polymerization of the Kdo homopolymeric capsule. Our studies also suggest a role for the capsular polysaccharide in symbiosis. Previous reports have shown that the overexpression of *rkpZ* from strain Rm41 allows for the symbiosis of *exoY* mutants of Rm1021 that are unable to produce the exopolysaccharide succinoglycan. Our results demonstrate that mutations in the *rkp-1* cluster prevent this phenotypic suppression of *exoY* mutants, although mutations in *kdsB2*, smb20804, and smb20805 have no effect.

When nitrogen is limited, many leguminous plants enter into symbiotic relationships with members of the rhizobia, a group of gram-negative soil bacteria. Rhizobia are able to elicit the formation of specialized symbiotic structures located on the plant root, referred to as nodules. The bacteria colonize the developing nodules via a complex series of developmental steps. Initially, the rhizobia adhere to root hairs, specialized epidermal cells located on the surface of the plant root. The adhered bacteria then alter the morphology of the root hairs, such that a curled structure called a shepherd's crook is formed, which entraps a microcolony of the bacteria. Bacteria entrapped within the shepherd's crook subsequently elicit the formation of a tubular invagination of the root hair, referred to as an infection thread. The rhizobia occupy the infection thread, which extends to the base of the root hair and penetrates into the developing nodule. Finally, the bacteria are released from the infection thread into the cytoplasm of the nodule cells, where they differentiate into bacteroids and reduce molecular dinitrogen for the plant (6, 7, 32, 60, 75).

The establishment of a successful rhizobium-legume symbiosis requires an exchange of molecular signals between the symbiotic partners. Plants secrete small molecules into the surrounding soil, such as flavonoids, isoflavonoids, and betaines. These compounds induce the transcription of *nod* genes within the bacteria (50, 51, 53, 56, 64, 66), which are responsible for the biosynthesis of Nod factor (16, 17, 43, 46, 63). Nod factor, in turn, elicits symbiotic responses within the plant that lead to cortical cell dedifferentiation and division, resulting in the formation of a nodule (15, 16, 19, 21, 46, 49, 69, 72).

Besides Nod factor, three additional classes of polysaccharides have been shown to be required for symbiosis: lipopolysaccharides (LPS), exopolysaccharides, and capsular polysaccharides (K antigens) (57). Mutations that affect the structure of LPS, or its modification by small molecules, result in defects in the ability of the bacteria to colonize root nodules or prevent the differentiation of intracytoplasmic bacteria into bacteroids (4, 14, 39, 68). Mutations in *exo* genes of *Sinorhizobium meliloti* strain Rm1021 prevent the biosynthesis of the acidic exopolysaccharide succinoglycan and result in a lack of infection thread extension into the developing nodule (10, 18, 40, 48, 70, 80). The requirement for succinoglycan can be bypassed by mutations that activate the production of a normally cryptic galactoglucan (also known as exopolysaccharide II), which can functionally replace succinoglycan during symbiosis (28–30, 48, 81).

Although *exo* mutants unable to produce succinoglycan display strong symbiotic phenotypes in strain Rm1021, the production of succinoglycan is not required for symbiosis in other *S. meliloti* strains. In *S. meliloti* strain Rm41, the lack of a symbiotic requirement for succinoglycan results from the synthesis of a capsular polysaccharide, which can functionally replace succinoglycan during symbiosis (59, 79). This capsular polysaccharide is a copolymer of glucuronic acid and pseudaminic acid (57–59), and its biosynthesis is dependent on the *rkp-1*, *rkp-2*, and *rkp-3* gene clusters (36, 54). The *rkp-1* and *rkp-2* clusters appear to be present among other *Sinorhizobium*

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species, whereas the third gene cluster seems to be specific to *S. meliloti* strain Rm41 (37). The *rkp-1* cluster includes the genes *rkpA-F*, which display significant sequence similarity to type I fatty acid synthases (27, 52) or polyketide synthases (4). Consistently with the proposed function of the *rkpA-F* cluster in the biosynthesis of fatty acids, the *rkpF* gene has been shown to undergo a posttranslational modification by 4′-phosphopantetheine, which is required for the biological activity of all fatty acid synthases studied to date (23). Additionally, the *rkp-1* cluster includes *rkpG*, predicted to encode an acyl-transferase; *rkpH*, predicted to encode an oxidoreductase; and the *rkpIJ* genes, predicted to encode part of an export apparatus (38). Collectively, the genes of the *rkp-1* cluster have been proposed to function in generating a lipid or polyketide necessary for the production of capsule (27, 38, 52). The *rkp-2* gene cluster is necessary for the synthesis of the glucuronic acid component of the capsule and is composed of two genes: *lpsL*, which encodes a UDP-glucuronic acid epimerase, and *rkpK*, a gene encoding UDP-glucose dehydrogenase (36). Finally, the genes in the *rkp-3* cluster encode proteins involved in the production of precursors of the capsule polymer (*rkpL-Q*) as well as proteins responsible for the export of the capsular polysaccharide to the cell surface (*rkpR-T*) (37).

Strain Rm41 normally produces a capsule of 20 to 25 kDa (59). Mutants of Rm41 lacking the *rkpZ* gene produce a capsule of approximately 25 to 35 kDa, which cannot replace succinoglycan during symbiosis (59). Consequently, the *rkpZ* gene has been suggested to encode a chain length determinator (59). The expression of *rkpZ* also can affect polysaccharide formation in other *S. meliloti* strains. The introduction of *rkpZ* into strain Rm1021 partially corrects the symbiotic defect of *exo* mutants (48, 59, 67, 79). These data suggested that strain Rm1021 produces the same capsular polysaccharide as Rm41, but the degree of polymerization of the capsule is inappropriate to promote symbiosis.

A recent study reported the characterization of a capsular polysaccharide derived from *S. meliloti* strain Rm1021 (25). Unlike the capsular polysaccharide detected in strain Rm41, this polysaccharide was reported to be a homopolymer of 3-deoxy-D-manno-oct-2-ulopyranosonic acid (Kdo) (25). The Kdo homopolymer produced by *S. meliloti* Rm1021 was reported to be of low M_r (3 to 6 kDa). However, the introduction of the *rkpZ* gene from strain Rm41 resulted in an increase in the M_r of this polysaccharide (3 to 30 kDa) as well as an increase in its abundance (67).

Because the introduction of *rkpZ* from Rm41 altered the M_r of the Kdo homopolymer and suppressed the symbiotic phenotype of Rm1021 *exo* mutants, the previous study concluded that this Kdo homopolymeric capsular polysaccharide could functionally replace succinoglycan during symbiosis (67). However, because of a lack of mutants that disrupt the synthesis of the Kdo homopolymer, the authors were unable to eliminate the possibility that the expression of *rkpZ* influences the biosynthesis of an alternative polysaccharide, which resulted in the observed symbiotic phenotypes.

In this communication, we describe a genetic characterization of capsular polysaccharide biosynthesis in *S. meliloti* strain Rm1021. We identified a gene cluster involved in the optimum polymerization of the Kdo homopolymeric capsule. Furthermore, we show that the *rkp-1* cluster is required for the detection of the Kdo homopolymer on the cell surface. Finally, we demonstrate that the *rkp-1* cluster is required for the suppression of the *exo* mutant symbiotic phenotype by *rkpZ*.

MATERIALS AND METHODS

Bacterial strains and media. All *Sinorhizobium meliloti* strains used in this study are derivatives of either Rm1021 or Rm41 and are listed in Table 1. *S. meliloti* strains were cultured in Luria-Bertani (LB) (13) or tryptone yeast extract (TY) medium (5) with antibiotics as previously described (45).

Strain construction. Plasmid DNA was introduced into all *S. meliloti* strains via triparental mating utilizing the *Escherichia coli* helper strain MT616, as previously described (20).

Strain MGM024 was constructed by introducing plasmid pMG006, a derivative of plasmid pJQ200 (55), containing a neomycin-resistant (Nm^r) marked deletion fragment of *rkpA*, into strain Rm1021, followed by selection for gentamicin resistance. Plasmid pMG006 cannot replicate in *S. meliloti* and can be stably maintained only through a recombination event between the *rkpA* gene fragment on the plasmid and the *rkpA* gene on the chromosome. The gentamicin-resistant colonies then were streaked onto LB medium containing 5% sucrose. Plasmid pMG006 contains the *sacB* gene, which confers sensitivity to sucrose. Sucrose-resistant colonies that were sensitive to gentamicin but resistant to neomycin were presumed to have undergone an allelic exchange event that resulted in the replacement of the wild-type *rkpA* fragment on the chromosome with the Nm^r-marked *rkpA* deletion. The mutant construct then was verified by PCR amplification and sequencing. Strain MGM164 was constructed in strain Rm41 by the same method.

Strain MGM260 was constructed by the introduction of plasmid pMG043 (a derivative of plasmid pVO155 [45], harboring an internal fragment of *rkpA*) into Rm1021 and selection for neomycin-resistant colonies. Plasmid pMG043 cannot replicate within Rm1021, therefore colonies resistant to neomycin can arise only following recombination between the *rkpA* gene fragment on plasmid pMG043 and the genomic copy of *rkpA*. Colonies that were neomycin resistant were presumed to have undergone recombination events that integrated the plasmid into the genome, thereby disrupting the *rkpA* gene. The insertion events then were confirmed via PCR. Strain DKR530 was constructed by the introduction of plasmid pDKR530 (a derivative of plasmid pDW33 [45], which contains an internal fragment of the *exoY* gene) into Rm1021 and selection for hygromycin-resistant colonies. Colonies that were hygromycin resistant were presumed to have undergone recombination events that integrated plasmid pDKR530 into the genome, disrupting the *exoY* gene, which was confirmed by PCR analysis and the examination of succinoglycan production. Strains MGM003, MGM445, MGM418, MGM419, DKR520, MGM420, and MGM365 were constructed in an equivalent manner via the introduction of the plasmids pMG042, pMG510, pMG511, pMG512, pDK520, pMG502, and pMG503, respectively, selection for hygromycin resistance, and confirmation by PCR.

Strain MGM349 was constructed via the introduction of *rkpA*::Nm (from strain MGM024) into strain DKR530 using bacteriophage N3. Similarly, strains MGM395, MGM464, MGM427, MGM428, MGM435, and MGM436 were constructed by the bacteriophage N3-mediated introduction of *rkpA*::Nm into strains MGM003, MGM445, MGM418, MGM419, MGM420, and MGM365, respectively.

The mutant strains MGM340 and MGM417 were constructed via the introduction of *kdsB2*::pDW33 with bacteriophage N3 into strains DKR145 and MGM024, respectively.

Plasmid construction. All plasmids used in this study are listed in Table 1. Plasmid pMTO001 was constructed by amplifying a 2,156-bp internal fragment of *rkpA* from *S. meliloti* Rm1021 genomic DNA via PCR using the primers 5′-GGATCCGGGCACCCCCACGTCACCT-3′ and 5′-GGGCCCGGCTCCGGTACGCTCGCCT, followed by being cloned into plasmid pCR2.1 (Invitrogen). Using two SmaI restriction sites located within the cloned *rkpA* fragment, 1,258 bp of DNA was excised to yield plasmid pMTO002. The *rkpA* gene fragment containing the 1,258-bp deletion then was liberated from plasmid pMTO002 using restriction enzymes ApaI and BamHI and ligated into vector pJQ200 (digested with the same enzymes) to generate plasmid pMG003. A 1,497-bp fragment containing the neomycin resistance gene (*nptII*) was liberated from plasmid pVO155 through digestion with NruI. This fragment subsequently was ligated into plasmid pMG003 digested with SmaI to yield plasmid pMG006. The presence of the fragment was verified via PCR, restriction digestion, and the demonstration of neomycin resistance.

Plasmid pMG043 was constructed by excising a 2,156-bp internal *rkpA* fragment from plasmid pMTO001 using the restriction enzymes BamHI and XbaI

TABLE 1. Strains and plasmids

Strain and plasmid	Genotype	Reference or source
S. meliloti		
Rm1021	SU47 Sm^r	42
MGM044	Rm1021/pTE3	This study
MGM312	Rm1021/pMW23	This study
MGM522	Rm1021/pTE3, pMS04	This study
MGM523	Rm1021/pMW23, pMS04	This study
MGM490	Rm1021/pTE3, pMB393	This study
MGM491	Rm1021/pMW23, pMB393	This study
MGM024	*rkpA*::Nm	This study
MGM050	*rkpA*::Nm/pTE3	This study
MGM314	*rkpA*::Nm/pMW23	This study
MGM003	*rkpG*::pDW33	This study
MGM048	*rkpG*::pDW33/pTE3	This study
MGM388	*rkpG*::pDW33/pMW23	This study
MGM445	*rkpH*::pDW33	This study
MGM454	*rkpH*::pDW33/pTE3	This study
MGM455	*rkpH*::pDW33/pMW23	This study
MGM418	*rkpI*::pDW33	This study
MGM423	*rkpI*::pDW33/pTE3	This study
MGM424	*rkpI*::pDW33/pMW23	This study
MGM419	*rkpJ*::pDW33	This study
MGM425	*rkpJ*::pDW33/pTE3	This study
MGM426	*rkpJ*::pDW33/pMW23	This study
MGM532	*rkpA*::Nm/pTE3, pMS04	This study
MGM508	*rkpA*::Nm/pTE3, pMS04::*rkpGH*	This study
MGM533	*rkpA*::Nm/pMW23, pMS04	This study
MGM509	*rkpA*::Nm/pMW23, pMS04::*rkpGH*	This study
MGM528	*rkpG*::pDW33/pTE3, pMS04	This study
MGM510	*rkpG*::pDW33/pTE3, pMS04::*rkpGH*	This study
MGM529	*rkpG*::pDW33/pMW23, pMS04	This study
MGM511	*rkpG*::pDW33/pMW23, pMS04::*rkpGH*	This study
MGM530	*rkpH*::pDW33/pTE3, pMS04	This study
MGM512	*rkpH*::pDW33/pTE3, pMS04::*rkpGH*	This study
MGM531	*rkpH*::pDW33/pMW23, pMS04	This study
MGM513	*rkpH*::pDW33/pMW23, pMS04::*rkpGH*	This study
MGM395	*rkpG*::pDW33 *rkpA*::Nm	This study
MGM400	*rkpG*::pDW33 *rkpA*::Nm/pTE3	This study
MGM402	*rkpG*::pDW33 *rkpA*::Nm/pMW23	This study
MGM464	*rkpH*::pDW33 *rkpA*::Nm	This study
MGM465	*rkpH*::pDW33 *rkpA*::Nm/pTE3	This study
MGM466	*rkpH*::pDW33 *rkpA*::Nm/pMW23	This study
MGM427	*rkpI*::pDW33 *rkpA*::Nm	This study
MGM429	*rkpI*::pDW33 *rkpA*::Nm/pTE3	This study
MGM430	*rkpI*::pDW33 *rkpA*::Nm/pMW23	This study
MGM428	*rkpJ*::pDW33 *rkpA*::Nm	This study
MGM431	*rkpJ*::pDW33 *rkpA*::Nm/pTE3	This study
MGM432	*rkpJ*::pDW33 *rkpA*::Nm/pMW23	This study
MGM403	*kdsB2*::pDW33	This study
MGM404	*kdsB2*::pDW33/pTE3	This study
MGM405	*kdsB2*::pDW33/pMW23	This study
MGM420	smb20804::pDW33	This study
MGM433	smb20804::pDW33/pTE3	This study
MGM434	smb20804::pDW33/pMW23	This study
MGM365	smb20805::pDW33	This study
MGM383	smb20805::pDW33/pTE3	This study
MGM385	smb20805::pDW33/pMW23	This study
MGM494	*kdsB2*::pDW33/pMW23, pMB393	This study
MGM486	*kdsB2*::pDW33/pMW23, pMB393::smb20805	This study
MGM547	smb20804::pDW33/pMW23, pMB393	This study
MGM526	smb20804::pDW33/pMW23, pMB393::smb20805	This study
MGM492	smb20805::pDW33/pMW23, pMB393	This study
MGM488	smb20805::pDW33/pMW23, pMB393::smb20805	This study
DKR145	*exoY*::pVO155	78
MGM081	*exoY*::pVO155/pTE3	This study
MGM358	*exoY*::pVO155/pMW23	This study
MGM340	*exoY*::pVO155 *kdsB2*::pDW33	This study
MGM352	*exoY*::pVO155 *kdsB2*::pDW33/pTE3	This study

Continued on following page

TABLE 1—*Continued*

Strain and plasmid	Genotype	Reference or source
MGM354	*exoY*::pVO155 *kdsB2*::pDW33/pMW23	This study
DKR530	*exoY*::pDW33	This study
MGM359	*exoY*::pDW33/pTE3	This study
MGM361	*exoY*::pDW33/pMW23	This study
MGM349	*exoY*::pDW33 *rkpA*::Nm	This study
MGM355	*exoY*::pDW33 *rkpA*::Nm/pTE3	This study
MGM357	*exoY*::pDW33 *rkpA*::Nm/pMW23	This study
Escherichia coli		
DH5α	F$^-$ ϕ*80lacZΔM15* Δ(*lacZYA-argF*)*U169 deoR recA endA1 hsdR17*(r_K^- m_K^+) *phoA supE44 thi-1 gyrA96 relA1*	65
MT616	*E. coli* pRK600 (conjugation helper strain)	40
Plasmids		
pCR2.1	TOPO cloning vector	Invitrogen
pVO155	Insertional inactivation plasmid Nm^r	45
pDW33	Insertional inactivation plasmid Hyg^r	12
pJQ200	Suicide plasmid Gm^r *sacB*	55
pTE3	pLAFR containing the *trp* promoter from *Salmonella typhi* serovar Typhimurium	3
pMW23	pRK404, harbors 4.0-kb PstI fragment containing *rkpZ* from Rm41	79
pMB393	Broad-host-range cloning vector; Sp^r	2
pMS04	Broad-host-range vector, high-copy-number derivative of pMB393 containing the *trp* promoter from *Salmonella typhi* serovar Typhimurium	35
pMTO001	pCR2.1::*rkpA* (2,156-bp internal fragment)	This study
pMTO002	pCR2.1::*rkpA* (2,156-bp internal fragment, carrying a 1,258-bp deletion)	This study
pMG003	pJQ200::*rkpA* (2,156-bp internal fragment, carrying a 1,258-bp deletion)	This study
pMG006	pJQ200::*rkpA*::Nm (2,156-bp internal fragment, a 1,258-bp deletion with insertion of Nm^r cassette)	This study
pMG043	pVO155::*rkpA* (internal fragment)	This study
pMG042	pDW33::*rkpG* (internal fragment)	This study
pMG510	pDW33::*rkpH* (internal fragment)	This study
pMG511	pDW33::*rkpI* (internal fragment)	This study
pMG512	pDW33::*rkpJ* (internal fragment)	This study
pDKR520	pDW33::*kdsB2* (internal fragment)	This study
pMG502	pDW33::smb20804 (internal fragment)	This study
pMG503	pDW33::smb20805 (internal fragment)	This study
pDKR530	pDW33::*exoY* (internal fragment)	This study
pMG060	pMS04::*rkpGH* (full length)	This study
pMG052	pMB393::smb20805 (full length)	This study

and ligating it into vector pVO155, which had been digested with the same restriction enzymes. The presence of the fragment was verified by PCR and restriction digestion.

The plasmid pMG042 was constructed by amplifying an internal fragment of *rkpG* from Rm1021 genomic DNA using the primers 5′-GGATCCCCGCGTC GGCGAGCCGCCT-3′ and 5′-CTCGAGATCCGCGGTGTCGTTGTGC-3′. The resulting PCR product was cloned into plasmid pCR2.1, yielding plasmid pMTO509. The *rkpG*-containing fragment then was excised from plasmid pMTO509 via restriction digestion with the enzymes BamHI and XhoI and ligated into plasmid pDW33, which had been digested with the same enzymes. The presence of the fragment was verified via PCR and restriction digestion.

Plasmid pMG510 was constructed using the PCR primers 5′-CCGGATCCA TTCGAGGCGATGCAGG-3′ and 5′-ACCTCGAGGCGCAGTAGGCGGTA TA-3′ to amplify an internal fragment of the *rkpH* gene from Rm1021 genomic DNA, which subsequently was cloned into plasmid pCR2.1 to yield plasmid pMTO510. The resulting plasmid then was digested with the restriction enzymes BamHI and XhoI and ligated into plasmid pDW33, which had been digested with the same enzymes.

Plasmid pMG511 was constructed by the PCR amplification of an internal fragment of *rkpI* from Rm1021 genomic DNA using primers 5′-TCGGATCCG TCGTCTTCCATTTCAT-3′ and 5′-CCCTCGAGGACATTGCTCAAGCGC C-3′ and was cloned into plasmid pCR2.1 to yield plasmid pMTO511. The *rkpI*-containing fragment then was liberated from pMTO511 by digestion with restriction enzymes BamHI and XhoI and ligated into plasmid pDW33, which had been treated with the same enzymes.

The plasmid pMG512 was constructed by amplifying an internal fragment of *rkpJ* from Rm1021 genomic DNA using the PCR primers 5′-CCGGTCGACG AATATGCCGGGTGGA-3′ and 5′-TTCTCGAGCGGGTGCACCTTGACG G-3′. The resulting PCR product was cloned into plasmid pCR2.1 to yield plasmid pMTO512. The *rkpJ* fragment then was excised with the restriction enzymes SalI and XhoI and ligated into plasmid pDW33, which had been digested with the same enzymes.

Plasmid pDKR520 was constructed by PCR amplification of a *kdsB2* internal fragment from Rm1021 genomic DNA utilizing the primers 5′-ACGTCGACA AGCTGTTCGCGCCGCA-3′ and 5′-TCTGGGATCCTCGAAGAGATTTCC GCAGCG-3′. The *kdsB2*-containing PCR fragment was subsequently digested with the enzymes SalI and BamHI and ligated into plasmid pDW33, which had been treated with the same enzymes.

The plasmid pMG502 was constructed by amplifying an internal fragment of ORF smb20804 from Rm1021 genomic DNA using the primers 5′-AGGTCGA CGGAACCACTGCGAACGC-3′ and 5′-TTGGATCCGGCGTCCATCTCGA GGG-3′. The PCR product then was cloned into plasmid pCR2.1 to yield plasmid pMTO502. The smb20804-containing fragment then was excised from plasmid pMTO502 with the restriction enzymes SalI and BamHI and ligated into plasmid pDW33, which had been digested with the same enzymes.

The plasmid pMG503 was constructed by amplifying an internal fragment of open reading frame (ORF) smb20805 from Rm1021 genomic DNA using the primers 5′-TCCCAGCGTAATCGCAAAGCCTCTG-3′ and 5′-GGTGTCTAG ACGTGCCACCCCGTAC-3′. The PCR product then was cloned into plasmid pCR2.1 to yield plasmid pMTO503. The smb20805-containing fragment then was excised with the restriction enzymes BamHI and XhoI and ligated into plasmid pDW33, which had been digested with the same enzymes.

Plasmid pDKR530 was constructed by the PCR amplification of an *exoY* internal fragment from Rm1021 genomic DNA utilizing the primers 5′-AGGT

CGACGAAGAGTTTTTCAGGATCAACC-3′ and 5′-TGCCGGATCCCCGTCAGGCCGGGACGCGAT-3′. The *exoY*-containing PCR fragment subsequently was digested with the enzymes SalI and BamHI and ligated into plasmid pDW33, which had been treated with the same enzymes.

The plasmid pMG060 was constructed by amplifying ORFs *rkpG* and *rkpH* from Rm1021 genomic DNA using the primers 5′-ATTGTCTAGAACAAGCATGGCCGCGGGCGC-3′ and 5′-TGTCGGATCCGACAAGGAAAAGCA-3′. The PCR product then was cloned into plasmid pCR2.1 to yield plasmid pMTO060. The *rkpGH*-containing fragment then was excised with the restriction enzymes BamHI and XbaI and ligated into plasmid pMS04, which had been digested with the same enzymes.

The plasmid pMG052 was constructed by amplifying ORF smb20805 from Rm1021 genomic DNA using the primers 5′-TTGCTCGCTCAACGCCTGCGTTAGA-3′ and 5′-CTTTAACGTCAGCTAACGCAATCCC-3′. The PCR product then was cloned into plasmid pCR2.1 to yield plasmid pMTO052. The smb20805-containing fragment then was excised with the restriction enzymes XbaI and BamHI and ligated into plasmid pMB393, which had been digested with the same enzymes.

Extraction and analysis of cellular polysaccharides. Cellular polysaccharides were obtained by phenol-water extraction as described previously (71) with the following modifications: cells were grown to stationary phase in 1 ml LB liquid medium (in the presence of antibiotics), pelleted, and washed in 1 ml of LB medium. The resulting cell pellet was resuspended in 180 μl of solution A (0.05 M Na_2HPO_4, 0.005 M EDTA, pH 7), and 180 μl of 90% phenol subsequently was added.

Alternatively, cellular polysaccharides were extracted from *S. meliloti* cells harvested either from 1-ml LB liquid cultures or directly from LB medium plates using EDTA-triethylamine (EDTA-TEA) buffer as described previously (74), with the following modifications. Briefly, cells were washed once in LB liquid medium and resuspended in 50 μl of 100 mM EDTA (titrated to pH 7.0 with TEA) per approximately 20 mg of wet cell mass. Samples then were incubated at room temperature for 15 min before centrifugation at 10,000 × *g* for 2 min. The supernatant was removed, 25 μl sodium dodecyl sulfate (SDS) sample buffer was added, and samples were incubated at 100°C for 5 min.

Detection of polysaccharides. After the addition of SDS sample buffer, the polysaccharide extracts were fractionated via Tris-Tricine SDS-polyacrylamide gel electrophoresis (PAGE) as described previously (44), and polysaccharides were visualized by staining with Alcian blue (11).

Fractionation of polysaccharides and glycosyl composition analysis. Polysaccharides from 1-liter cell cultures were extracted by the phenol-water procedure, and the water layers were treated sequentially with a mixture of DNase and RNase (20 μl/ml each) and Pronase (20 μl/ml). Following dialysis and lyophilization, the total yield of polysaccharide was approximately 80 mg from each extract. Portions (10 mg) of the polysaccharide extracts were fractionated by size-exclusion chromatography under dissociative conditions on a column of Sephacryl S-400 HR (1.1 by 100 cm) equilibrated in 10 mM Tris buffer (pH 9.2) containing 0.25% sodium deoxycholate, 0.2 M NaCl, and 1.0 mM EDTA. Chromatography was performed on an AKTA system (G.E. Healthcare/Amersham), and the eluant was monitored for the refractive index (RID-10A detector; Shimadzu Corp., Kyoto, Japan) and by UV absorbance at 215, 254, and 280 nm. The fractions obtained from size-exclusion chromatography were dialyzed to remove detergent (57) and then lyophilized. Aliquots (100 to 200 μg) were subjected to glycosyl composition analysis by preparing the trimethylsilyl methyl glycoside derivatives (70), and gas chromatography-mass spectrometry was performed as described previously (71).

In vivo labeling of cellular polysaccharides. Wild-type and mutant *S. meliloti* cells were inoculated into 1 ml LB medium in the presence of 2 μCi of [U-^{14}C]glucose and cultured to saturation (optical density at 600 nm of ~3). The cells were washed once in LB medium, and the radiolabeled polysaccharides then were extracted via phenol-water or EDTA-TEA as described above. The radiolabeled polysaccharides were subsequently fractionated on Tris-Tricine SDS-PAGE, and the incorporation of ^{14}C was detected by phosphorimaging.

Periplasmic fractionation. The preparation of periplasmic fractions was carried out by osmotic shock treatment as described previously (41), with the following modifications. *S. meliloti* strains were cultured to stationary phase in 1 ml LB liquid medium (in the presence of antibiotics), and the cells were harvested via centrifugation at 16,000 × *g*. After being washed once in LB medium, the cells were resuspended in 0.4 ml of 50 mM Tris-HCl (pH 8.0), 20% (wt/vol) sucrose, 2 mM EDTA containing 1 mg/ml lysozyme and were incubated for 15 min on ice. NaCl then was added to a final concentration of 100 mM, and samples were incubated for an additional 10 min on ice. Samples were centrifuged for 10 min at 6,800 × *g*. Material from both the soluble and insoluble fractions then were extracted via the phenol-water method and fractionated by Tris-Tricine PAGE, and polysaccharides detected by Alcian blue staining or by phosphorimaging.

Subcellular fractionation of very-high-molecular-weight polysaccharide. *S. meliloti* strains were cultured to stationary phase in 200 ml LB (in the presence of antibiotics). Cells were harvested at 5,500 × *g* and exposed to osmotic shock treatment (as described above, except that cells were washed in 25 ml LB medium and resuspended in 10 ml osmotic shock buffer). The insoluble pellet was frozen at −20°C, thawed, and resuspended in 15 ml buffer A. The suspensions were treated with DNase and RNase (20 μl/ml each) at 37°C for several hours, until they showed clearing. Pronase subsequently was added at 20 μl/ml, and suspensions were incubated again at 37°C for several hours. Suspensions were centrifuged at 8,000 × *g* for 15 min, and the supernatant was removed and centrifuged again to be cleared completely of debris. The supernatants then were exposed to ultracentrifugation for 30 min at 237,000 × *g* (Beckman TL-100 Ultracentrifuge, Rotor TLA-100.3). Polysaccharides were extracted from the resulting pellet fractions using phenol-water (as described above), while the supernatants were left untreated.

Nodulation assay. Nodulation assays were performed as described previously (22) on Jensen's medium agar (76) in glass tubes. Plants were grown for 80 days.

Recovery of bacteria from nodules. Nodules were removed from the root of the plant and pooled (15 plants for each treatment listed). The nodules then were treated with 20% sodium hypochlorite for 2 min, followed by three washes with sterile water. The surface-sterilized nodules were resuspended in LB liquid medium containing 0.3 M sucrose and homogenized. The resulting suspension then was serially diluted and plated on LB medium plates containing streptomycin. After 3 to 4 days the colonies were counted.

Dry weight measurements. Eighty days after inoculation, the plants were dried for 5 h at 95°C and their dry weights determined.

β-Glucuronidase assay. The β-glucuronidase activity was determined by a spectrophotometric assay, as described by Jefferson et al. (34).

RESULTS

Inactivation of *rkpA-J* in *S. meliloti* strain Rm1021. To better understand the physiological function of polysaccharides in strain *S. meliloti* Rm1021, we sought to construct mutants deficient in the synthesis of the Kdo homopolymeric capsule. In strain Rm41, the production of capsular polysaccharide has been reported to require the *rkp-1* cluster (38, 52, 54). Although the *rkp-1* cluster also is present in strain Rm1021, to our knowledge a characterization of its physiological function has not been reported. We inactivated each of the five genes in the *rkp-1* cluster and investigated the effects of the mutations on the synthesis of cell surface polysaccharides. We constructed a 1,258-bp deletion in the *rkpA* gene, which was replaced with a neomycin resistance cassette. Mutations in the remaining four genes (*rkpG*, *rkpH*, *rkpI*, and *rkpJ*) were generated via vector integration with plasmid pDW33, a derivative of plasmid pVO155 (45). Plasmid pDW33 contains a ColE1 origin (which is incapable of replication within *S. meliloti*) and harbors a promoterless copy of *uidA*, allowing for the construction of transcriptional fusions, and it encodes hygromycin resistance (12, 45).

Mutations in the *rkp-1* gene cluster prevent detection of surface-associated Kdo homopolymeric capsule. We cultured wild-type *S. meliloti* and each of the *rkp-1* cluster mutants in LB medium in the presence of [U-^{14}C]glucose, recovered cell surface polysaccharides via extraction with EDTA-TEA buffer, fractionated the extracts on Tris-Tricine SDS-PAGE, and visualized the polysaccharides by staining with Alcian blue (Fig. 1A) or by phosphorimaging (Fig. 1B). When fractionated on Tris-Tricine SDS-PAGE, LPS migrates as two species: rough LPS, which migrates at a lower M_r, and smooth LPS, which migrates at a higher M_r. Consistently with previous reports (25, 67), we observed a polysaccharide that migrated between the

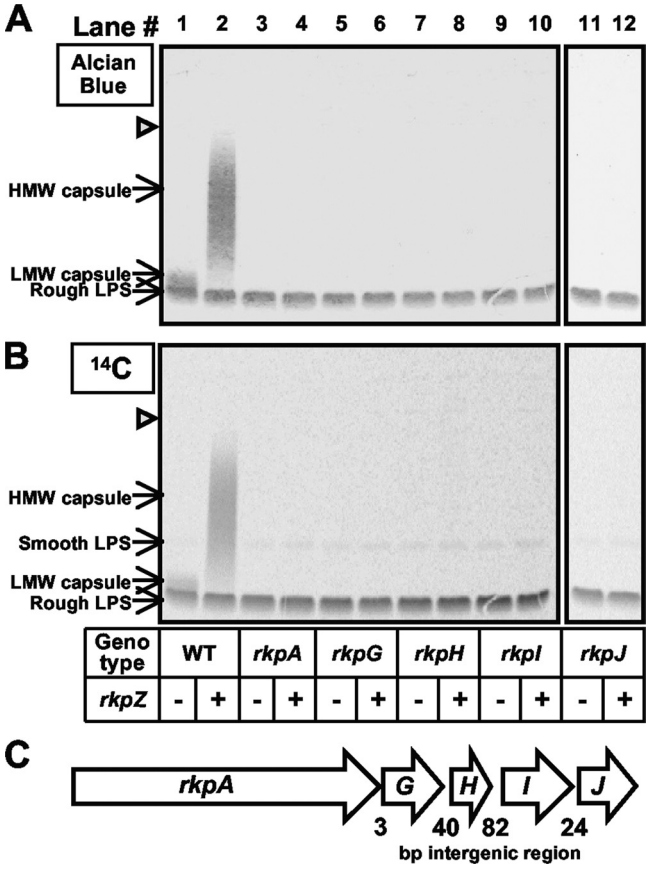


FIG. 1. Analysis of capsular polysaccharides from wild-type *S. meliloti* Rm1021 and mutants affecting the *rkp-1* gene cluster. Wild-type and mutant strains were cultured in the presence of [U-^{14}C]glucose, and cell surface polysaccharides were isolated via EDTA-TEA extraction, fractionated using Tris-Tricine SDS-PAGE, and detected by staining with Alcian blue (A) or by phosphorimaging (B) as described in Materials and Methods. Lane 1, strain MGM044 (wild-type Rm1021/pTE3 [WT/pTE3]); lane 2, MGM312 (WT/pMW23); lane 3, MGM050 (*rkpA*::Nm/pTE3); lane 4, MGM314 (*rkpA*::Nm/pMW23); lane 5, MGM048 (*rkpG*::pDW33/pTE3); lane 6, MGM388 (*rkpG*::pDW 33/pMW23); lane 7, MGM454 (*rkpH*::pDW33/pTE3); lane 8, MGM455 (*rkpH*::pDW33/pMW23); lane 9, MGM423 (*rkpI*::pDW33/pTE3); lane 10, MGM424 (*rkpI*::pDW33 pMW23); lane 11, MGM425 (*rkpJ*::pDW 33/pTE3); and lane 12, MGM426 (*rkpJ*::pDW33/pMW23). The presence of the plasmid containing *rkpZ* (pMW23) is indicated beneath each lane with a plus, while the presence of the control plasmid (pTE3) is indicated with a minus. The open triangle refers to the interface between the stacking and separating gel. (C) Organization of the *rkp-1* gene cluster in *S. meliloti* Rm1021. Genes are represented as arrows, with the length of the intergenic regions depicted in base pairs. HMW, high molecular weight; LMW, low molecular weight.

rough and smooth LPS, forming a light crown above the intensely stained rough LPS band (Fig. 1A and B).

Whereas wild-type strains produced detectable quantities of this polysaccharide species, it was undetectable in extracts derived from mutants affecting the *rkp-1* gene cluster (*rkpA-J*) (Fig. 1A and B). Despite the lack of detectable capsular polysaccharide, the LPS of each of the *rkp-1* mutant strains was present at levels similar to those of wild-type cells and exhibited a similar banding pattern. These results suggest that mutations in the *rkp-1* gene cluster prevent the detection of the capsule but do not result in a gross perturbation of polysaccharide synthesis.

Previous studies showed that the introduction of the *rkpZ* gene (from *S. meliloti* strain Rm41) into strain Rm1021 results in a higher degree of the polymerization of the Kdo homopolymeric capsule as well as an increase in overall capsule production (67). To better understand the role of the *rkp-1* cluster in the production of capsular polysaccharide, we introduced plasmid pMW23 (which contains the *rkpZ* gene from Rm41 [79]) into wild-type Rm1021 and the *rkp-1* mutants. Similarly to what had been observed previously (67), when examined by Tris-Tricine SDS-PAGE and Alcian blue staining, extracts prepared from wild-type Rm1021 harboring plasmid pMW23 displayed a ladder of bands, which extended from the region corresponding to the rough LPS band to the top of the separating portion of the gel (Fig. 1A and B). In contrast, we did not observe the production of this ladder of bands in the *rkp-1* mutants. We thus conclude that the *rkp-1* gene cluster is required for the detection of the Kdo homopolymeric polysaccharide in strain Rm1021.

Function of individual *rkp-1* cluster genes in expression of the Kdo homopolymer. Mutations in each of the genes comprising the *rkp-1* cluster resulted in a lack of detectable Kdo homopolymer. Although little is known about the structure of rhizobial promoters, the intergenic regions between the *rkpA-J* genes are short in length (Fig. 1C). Therefore, it was possible that the genes within the *rkp-1* cluster are cotranscribed, and that mutations in the upstream genes of this cluster exert a polar effect on the transcription of the downstream genes. The *rkpG*::pDW33, *rkpH*::pDW33, *rkpI*::pDW33, and *rkpJ*::pDW33 insertions result in a transcriptional fusion of the respective gene to *uidA*. We measured the β-glucuronidase activity of the *rkpG*::*uidA*, *rkpH*::*uidA*, *rkpI*::*uidA*, and *rkpJ*::*uidA* transcriptional fusions in a wild-type background or in the presence of the *rkpA*::Nm mutation. In the presence of the *rkpA*::Nm mutation, the expression of *rkpG*::*uidA* and *rkpH*::*uidA* fusions was reduced ca. 83% with respect to the expression in a wild-type background (Fig. 2). These data suggest that the *rkpA*::Nm mutation is polar on the expression of the *rkpG* and *rkpH* genes. In contrast, the expression of the *rkpI*::*uidA* and *rkpJ*::*uidA* fusions was unaffected by the presence of the *rkpA*::Nm mutation (Fig. 2), suggesting that the *rkpA* and *rkpIJ* genes are transcribed independently. Complementation experiments with plasmid-borne *rkpG* and *rkpGH* were consistent with these findings (see Fig. S1 in the supplemental material).

Identification of a very-high-molecular-weight polysaccharide species in *rkp-1* mutant strains harboring *rkpZ*. While mutations in the *rkp-1* cluster resulted in a lack of detectable capsular polysaccharide under our assay conditions, the reason behind this lack of polysaccharide production was unclear. The method we employed for polysaccharide preparation involved the treatment of the cells with EDTA-TEA, which is expected primarily to extract macromolecules present in the outer membrane (39, 61, 73). We therefore utilized an alternative method, phenol-water extraction (71), to isolate total radiolabeled cellular polysaccharides, which then were fractionated on Tris-Tricine SDS-PAGE and visualized using phosphorimaging (Fig. 3A). Consistently with the results observed with the EDTA-TEA protocol, we did not detect the capsular polysaccharide in the *rkpA*::Nm mutant harboring vector alone when

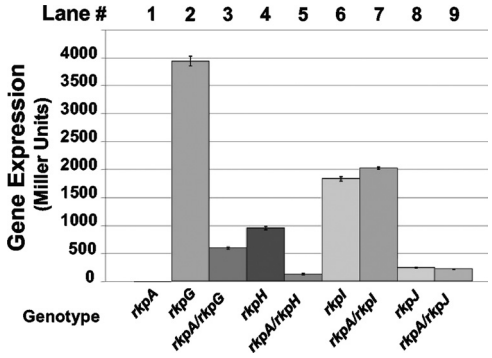


FIG. 2. Expression of *rkp-1* cluster genes. Transcriptional fusions between the *rkpG*, *rkpH*, *rkpI*, and *rkpJ* genes and *uidA* (encoding β-glucuronidase) were constructed using plasmid pDW33. The strains were grown to stationary phase (optical density at 600 nm of ~2.5) and assayed for activity as described in Materials and Methods. Enzyme activity is indicated in Miller units. Error bars represent standard deviations from experiments carried out in triplicate. Lane 1, MGM050 (*rkpA*::Nm/pTE3); lane 2, MGM048 (*rkpG*::pDW33/pTE3); lane 3, MGM400 (*rkpG*::pDW33 *rkpA*::Nm/pTE3); lane 4, MGM454 (*rkpH*::pDW33/pTE3); lane 5, MGM465 (*rkpH*::pDW33 *rkpA*::Nm/pTE3); lane 6, MGM423 (*rkpI*::pDW33/pTE3); lane 7, MGM429 (*rkpI*::pDW33 *rkpA*::Nm/pTE3); lane 8, MGM425 (*rkpJ*::pDW33/pTE3); and lane 9, MGM431 (*rkpJ*::pDW33 *rkpA*::Nm/pTE3). Plasmid pTE3 had no effect on the expression of any of the *rkp*::*uidA* fusions (M. G. Müller et al., unpublished).

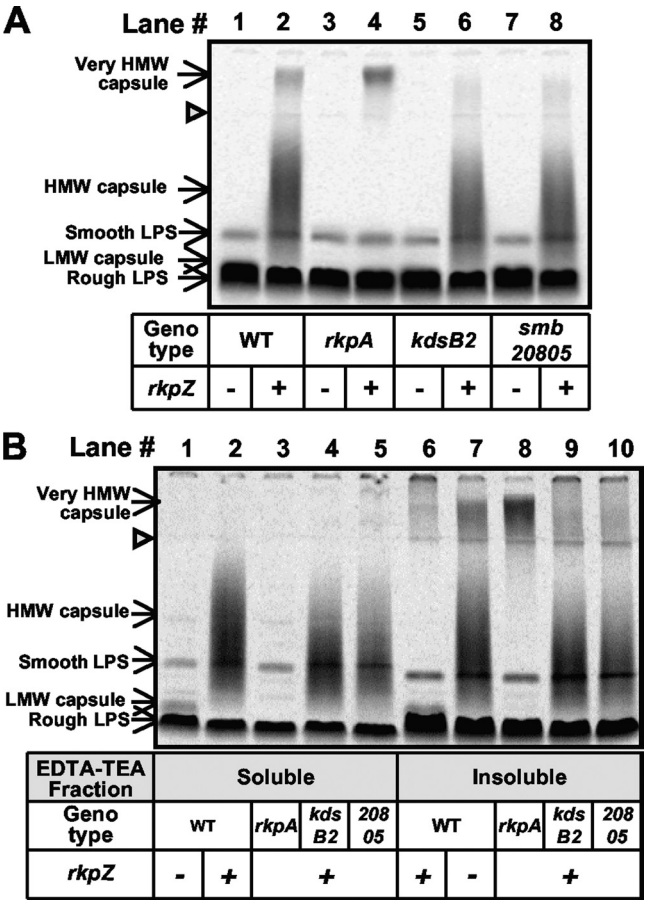


FIG. 3. Mutants deficient in *rkpA* but harboring pMW23 produce a very-high-molecular-weight polysaccharide. (A) Wild-type and mutant strains, harboring the vector control or pMW23, were cultured in the presence of [U-^{14}C]glucose. Polysaccharides then were extracted via phenol-water, fractionated by Tris-Tricine PAGE, and visualized by phosphorimaging. Lane 1, MGM044 (wild-type Rm1021/pTE3 [WT/pTE3]); lane 2, MGM312 (WT/pMW23); lane 3, MGM050 (*rkpA*::Nm/pTE3); lane 4, MGM314 (*rkpA*::Nm/pMW23); lane 5, MGM404 (*kdsB2*::pDW33/pTE3); lane 6, MGM405 (*kdsB2*::pDW33/pMW23); lane 7, MGM383 (smb20805::pDW33/pTE3); and lane 8, MGM385 (smb20805::pDW33 ::pDW33/pMW23). In panels A and B the presence of the plasmid containing *rkpZ* (pMW23) is indicated beneath each lane with a plus, and the presence of the control plasmid (pTE3) is indicated with minus. The open triangle refers to the interface between the stacking and separating gel. (B) Cells were treated with EDTA-TEA and polysaccharides from both the EDTA-TEA-soluble fractions (lanes 1 to 5) and EDTA-TEA-insoluble fractions (lanes 6 to 10) and then were extracted via phenol-water, fractionated by Tris-Tricine PAGE, and visualized by phosphorimaging. Lanes 1 and 6, MGM044; lanes 2 and 7, MGM312; lanes 3 and 8, MGM314; lanes 4 and 9, MGM405; and lanes 5 and 10, MGM385. HMW, high molecular weight; LMW, low molecular weight.

extracting with phenol-water. However, the phenol-water extraction of the *rkpA*::Nm mutant harboring pMW23 resulted in the detection of a very-high-molecular-weight species that migrated in the stacking portion of the Tris-Tricine gel. A similar phenotype was observed with the *rkpA*::pVO155, *rkpG*::pDW33, *rkpH*::pDW33, *rkpI*::pDW33, and *rkpJ*::pDW33 mutants harboring plasmid pMW23 (M. G. Müller, L. S. Forsberg, and D. H. Keating, unpublished data). Thus, *rkp-1* mutant strains harboring plasmid pMW23 produce a very-high-molecular-weight polysaccharide that cannot be liberated via extraction with EDTA-TEA, suggesting that it is not present on the cell surface. Interestingly, we also observed the presence of the very-high-molecular-weight species in phenol-water extracts derived from wild-type Rm1021 containing pMW23 (Fig. 3A), although the species was present in reduced quantity compared to those of the *rkp-1* mutants.

To determine if the very-high-molecular-weight species observed in *rkp-1* mutants containing plasmid pMW23 represents an intracellular polysaccharide, we examined the subcellular localization of the polysaccharides produced by both wild-type and *rkpA* mutant strains harboring pMW23. Treatment with EDTA-TEA divides cellular material into two fractions: a soluble fraction, which is expected to consist of outer membrane components (and likely components of the periplasm), and an EDTA-TEA-insoluble fraction, expected to contain intact cells, components of the periplasm, and the cytoplasm and cytoplasmic membrane (39, 61, 73). We examined the polysaccharide components of the EDTA-TEA-insoluble fraction by extraction via phenol-water and detection by phosphorimaging (Fig. 3B). Using this approach, we were able to demonstrate that the EDTA-TEA-insoluble fraction of both wild-type and *rkpA*::Nm mutant strains harboring pMW23 contained the very-high-molecular-weight polysaccharide species. Our ability to extract the very-high-molecular-weight polysaccharide exclusively from this fraction suggested that it was located in the periplasm, the cytoplasmic membrane, or within the cytoplasm. To determine if the very-high-molecular-weight polysaccharide was present in the periplasm, we subjected wild-type cells and the *rkpA*::Nm mutant harboring pMW23 to osmotic shock. This method has been used previously with *S. meliloti* strain Rm1021 for the recovery of periplasmic contents (41). We then

employed phenol-water extraction to prepare polysaccharides from the periplasmic fraction and detected the polysaccharides by staining with Alcian blue. We did not detect the very-high-molecular-weight polysaccharide in the osmotic shock fraction of either the wild type or the *rkpA* mutant (Fig. 4A). Instead, we detected the very-high-molecular-weight polysaccharide in the insoluble material remaining after osmotic shock extraction (Fig. 4B).

Our inability to observe the very-high-molecular-weight polysaccharide in either the EDTA-TEA-soluble fraction or the osmotic shock fraction suggested that the polysaccharide was located in the cytoplasm or associated with the cytoplasmic membrane. Thus, we subjected the insoluble fraction after osmotic shock treatment to freeze-thaw, low-speed centrifugation to remove cell debris and unbroken cells, and then ultracentrifugation. The resulting pelleted material then was extracted using phenol-water. Utilizing this approach, we found that the very-high-molecular-weight polysaccharide was present in the supernatants resulting from the ultracentrifugation of both the wild type and the *rkpA*::Nm mutant harboring pMW23 (although more was observed in the supernatants derived from *rkpA::*Nm), and only a small portion was recovered from the ultracentrifuge-pelleted fractions (Fig. 4B). The ultracentrifuge pellets contained significant amounts of what appeared to be LPS, indicating the presence of outer membrane material in this fraction. However, these results suggest that the majority of the very-high-molecular-weight polysaccharide is found in the soluble fraction, consistently with a cytoplasmic location.

Compositional analysis of very-high-molecular-weight polysaccharide. To determine if the very-high-molecular-weight polysaccharide observed in *rkp-1* mutants harboring pMW23 was an intracellular form of the Kdo homopolymeric capsular polysaccharide normally on the cell surface, we prepared total cellular polysaccharides from the wild type and *rkp-1* mutants harboring pMW23 and subjected them to additional analysis. The chromatography of the polysaccharide extracts derived from the wild-type Rm1021 and the *rkpA* mutant harboring plasmid pMW23 indicated that both strains produced very-high-molecular-weight material that migrated as a distinct peak at the exclusion volume of an S-400 column when chromatographed in the presence of detergent (dissociative conditions) (Fig. 5A). This indicates an apparent molecular mass of approximately 2×10^6 Da or greater, in agreement with the reported exclusion limit of this matrix as measured with polysaccharides. This fraction was isolated and subjected to glycosyl composition analysis (Fig. 5B). The predominant carbohydrate in both strains was Kdo, suggesting that the primary high-molecular-weight polysaccharide component was a Kdo-rich polysaccharide (K antigen). Small amounts of other glycosyl components also were present in this fraction; however, the structural relationships of these sugars with Kdo were not investigated further.

Identification of genes required for optimum polymerization of the very-high-molecular-weight polysaccharide. Our data suggested that *rkp-1* mutants fail to express the capsular polysaccharide on their cell surface. Instead, the mutants produce a very-high-molecular-weight intracellular polysaccharide species. Compositional analysis demonstrated that the very-high-molecular-weight polysaccharide is enriched in Kdo, suggesting that it is an altered form of the Kdo homopolymer normally found on the cell surface. To determine if the two polysaccharides are related, we sought to construct mutants that affect the polymerization of the Kdo homopolymer. The identification of

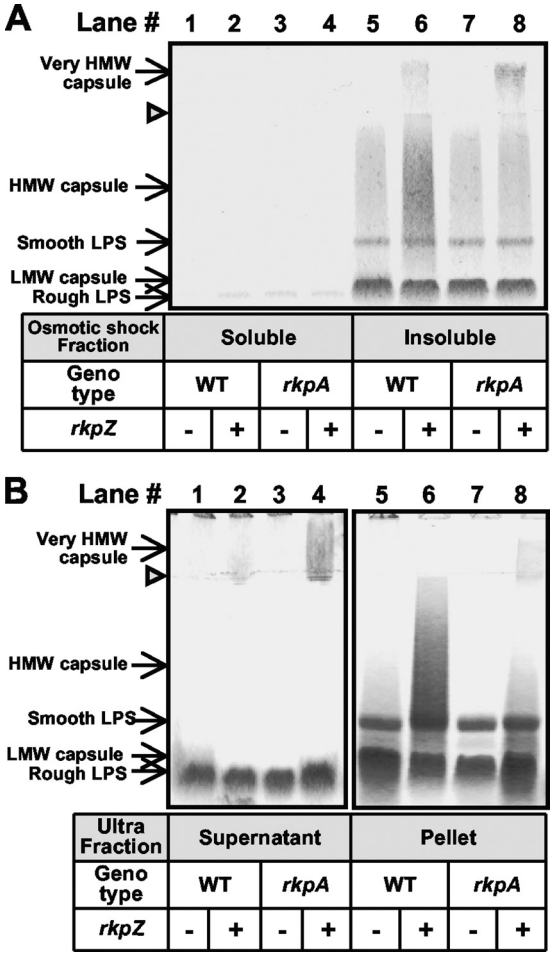
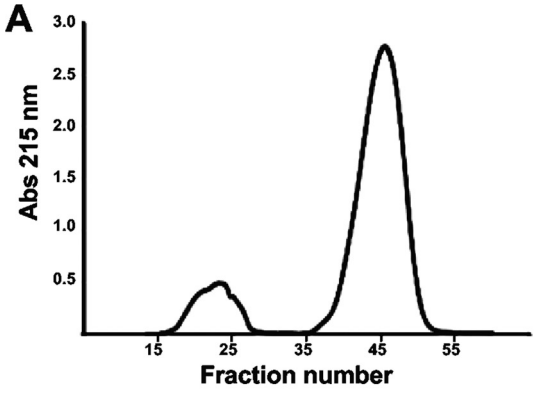


FIG. 4. Cellular location of very-high-molecular-weight polysaccharides. (A) Analysis of periplasmic contents of wild-type and *rkpA*::Nm cells. Periplasmic polysaccharides were liberated from wild-type and mutant strains using osmotic shock as described in Materials and Methods. Polysaccharides recovered from both the soluble (lanes 1 to 4) and insoluble osmotic shock fractions (lanes 5 to 8) then were subjected to Tris-Tricine SDS-PAGE, and the polysaccharides were visualized by staining with Alcian blue. Lanes 1 and 5, strain MGM044 (wild-type Rm1021/pTE3 [WT/pTE3]); lanes 2 and 6, MGM312 (WT/pMW23); lanes 3 and 7, MGM050 (*rkpA*::Nm/pTE3); and lanes 4 and 8, MGM314 (*rkpA*::Nm/pMW23). The presence of the plasmid containing *rkpZ* (pMW23) is indicated beneath each lane with a plus, and the presence of the control plasmid (pTE3) is indicated with a minus. The open triangle refers to the interface between the stacking and separating gel. (B) Subcellular fractionation of very-high-molecular-weight capsular polysaccharide extracted from insoluble osmotic shock fractions. Wild-type and mutant cells were treated with osmotic shock. The insoluble pellets then were exposed to freeze-thaw followed by ultracentrifugation. Polysaccharides were extracted from the resulting pellet fraction via phenol-water extraction (lanes 5 to 8) or supernatants were left untreated (lanes 1 to 4). Finally, polysaccharides from both ultracentrifugation fractions were fractionated using Tris-Tricine SDS-PAGE and stained with Alcian blue. Lanes 1 and 5, strain MGM044; lanes 2 and 6, MGM312; lanes 3 and 7, MGM050; and lanes 4 and 8, MGM314. HMW, high molecular weight; LMW, low molecular weight.



B

Mole ratios of glycosyl residues		
glycosyl residue	Vo fraction (HMW)	
	Rm1021/pMW23	*rkpA*::Nm/pMW23
Arabinose	0.09	0.43
Ribose	0.05	0.14
Xylose	1.28	2.86
Mannose	2.00	2.00
Galactose	1.61	0.39
Glucose	2.91	3.13
Kdo	51.93	18.80

FIG. 5. Compositional analysis of very high-molecular-weight (HMW) polysaccharide. Polysaccharide was prepared from the wild type and *rkpA* mutants harboring plasmid pMW23. (A) Fractionation of polysaccharide extract derived from Rm1021 containing pMW23 by size-exclusion chromatography. Chromatography was performed in the presence of detergent (dissociative conditions) on a column of Sephacryl S-400 HR. The high-molecular-weight polysaccharide (fractions 15 to 26) migrates at the column void volume, indicating an approximate molecular weight of $\geq 1 \times 10^6$ to 2×10^6. LPS migrated in fractions 37 to 53 as revealed by glycosyl composition analysis (M. G. Müller et al., unpublished). Abs, absorbance. (B) Compositional analysis of polysaccharide isolated from void volume (Vo). The molar ratios of glycosyl residues were normalized such that the amount of mannose was equivalent in both the Rm1021 and *rkpA*::Nm samples.

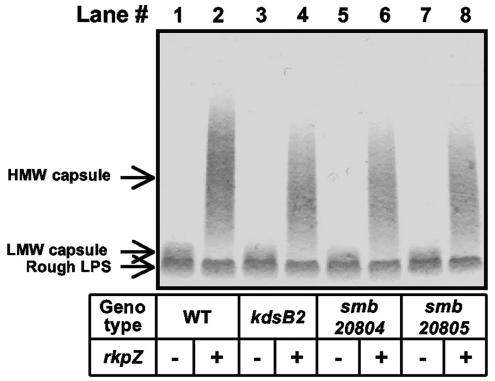


FIG. 6. Analysis of capsular polysaccharides from wild-type *S. meliloti* and *kdsB2*, smb20804, and smb20805 mutant strains. Wild-type and mutant strains were cultured in liquid medium, cell surface polysaccharides then were isolated via EDTA-TEA extraction and fractionated using Tris-Tricine SDS-PAGE, and polysaccharides were visualized by staining with Alcian blue, as described in Materials and Methods. Lane 1, strain MGM044 (wild-type Rm1021/pTE3 [WT/pTE3]); lane 2, MGM312 (WT/pMW23); lane 3, MGM404 (*kdsB2*::pDW33/pTE3); lane 4, MGM405 (*kdsB2*::pDW33/pMW23); lane 5, MGM433 (smb20804::pDW33/pTE3); lane 6, MGM434 (smb20804::pDW33/pMW23); lane 7, MGM383 (smb20805::pDW33/pTE3); and lane 8, MGM385 (smb20805::pDW33 ::pDW33/pMW23). The presence of the plasmid containing *rkpZ* (pMW23) is indicated beneath each lane with a plus, and the presence of the control plasmid (pTE3) is indicated with a minus. HMW, high molecular weight; LMW, low molecular weight.

mutations that affect the Kdo homopolymeric capsule and the very-high-molecular-weight polysaccharide would provide evidence of a precursor-product relationship between the two. The *S. meliloti* gene *kdsB2* was annotated as a CMP-Kdo cytidylyltransferase (1, 24, 26) based on sequence identity to known enzymes. We hypothesized that *kdsB2* is necessary for the synthesis of the homopolymeric capsule. We generated a disruption of *kdsB2* via vector integration with pDW33 and assayed the strain for the production of Kdo homopolymer in the presence of either plasmid pMW23 or vector control. The polysaccharide profile for *kdsB2*::pDW33 harboring the vector control was indistinguishable from that seen for the wild type (Fig. 6). However, in the presence of plasmid pMW23 the capsular polysaccharide isolated from the *kdsB2*::pDW33 mutant exhibited a small but reproducible decrease in M_r compared to that of wild-type strains harboring pMW23 (Fig. 6, compare lanes 2 and 4). Thus, we concluded that *kdsB2* is required for the optimum polymerization of the Kdo homopolymer.

Interestingly, the *kdsB2* gene is located directly upstream of two genes: smb20804 (predicted to encode a membrane protein of unknown function) and smb20805 (predicted to encode a glycosyltransferase). We constructed mutations in both smb20804 and smb20805 and visualized the cell surface polysaccharides by Tris-Tricine SDS-PAGE and staining with Alcian blue. Both the smb20804::pDW33 and smb20805::pDW33 mutations resulted in a phenotype similar to that observed in the *kdsB2*::pDW33 mutant in the presence of pMW23 (Fig. 6). Complementation experiments clearly demonstrated that smb20805 is required for the optimum synthesis of the Kdo homopolymer; results with *kdsB2* and smb20804 were inconclusive (see Fig. S2 in the supplemental material).

KdsB2 and Smb20805 affect the synthesis of the very-high-molecular-weight polysaccharide. To determine if the *kdsB2* gene cluster mutants affected synthesis of the very-high-molecular-weight polysaccharide, we used the phenol-water extraction method to recover cellular polysaccharides from wild-type Rm1021, the *rkpA*::Nm mutant, and the *kdsB2*::pDW33 and smb20805::pDW33 mutant strains, each harboring the pMW23 plasmid. We did not observe the presence of the very-high-molecular-weight polysaccharide species in the *kdsB2*::pDW33 and smb20805::pDW33 mutants harboring the pMW23 plasmid (Fig. 3A, compare lanes 2, 4, 6, and 8). The ability of the *kdsB2*::pDW33 and smb20805::pDW33 mutants to affect the polymerization of both polysaccharides suggests that they are variants of the same capsular species.

Symbiotic effects of mutations affecting Kdo homopolymer synthesis. Our studies identified mutations that affect the

TABLE 2. Symbiosis of mutants affected in synthesis or display of the Kdo homopolymer

S. meliloti strain	*rkpZ*[a]	No. of green plants	No. of plants tested
Rm1021	−	15	15
	+	14	15
Rm1021 *rkpA*::Nm	−	15	15
	+	15	15
Rm1021 *kdsB2*::pDW33	−	15	15
	+	15	15
Rm1021 *exoY*::pDW33	−	1	15
	+	7	15
RM1021 *exoY*::pDW33 *rkpA*::Nm	−	0	15
	+	1	15
Rm1021 *exoY*::VO155 *kdsB2*::pDW33	−	1	15
	+	6	15
Mock inoculation	NA	0	15

[a] The presence of the plasmid containing *rkpZ* (pMW23) is indicated with a plus, while the presence of the control plasmid (pTE3) is indicated with a minus. NA, not applicable. Similar results were observed in two independent experiments.

polymerization (*kdsB2*, smb20804, and smb20805) or surface display (*rkp-1*) of the Kdo homopolymer. In addition, none of these mutants displayed obvious differences in growth in laboratory medium (M. G. Müller et al., unpublished). Thus, these mutants provided molecular tools to alter the production of the Kdo homopolymer and examine its symbiotic function. We inoculated *Medicago sativa* (alfalfa) seedlings with wild-type *S. meliloti* Rm1021, mutants affecting the production of the Kdo homopolymer (the *rkpA*::Nm and *kdsB2*::pDW33 mutants), and mutants affecting the production of succinoglycan (the *exoY*::pDW33 and *exoY*::pVO155 mutants), each harboring either the vector control or plasmid pMW23. Additionally, we inoculated plants with double mutants that affect both the Kdo homopolymer and succinoglycan production (the *rkpA*::Nm *exoY*::pDW33 double mutant and the *exoY*::pVO155 *kdsB2*:: pDW33 double mutant), each carrying pMW23 or the vector control. At various time points postinoculation, we measured the rate of nodule formation. Consistently with what had been reported previously (67), all of the strains assayed were able to elicit the formation of root nodules.

Although all strains could elicit nodules on *M. sativa*, they differed in their ability to fix nitrogen for the host. Wild-type Rm1021, as well as the *rkpA*::Nm and *kdsB2*::pDW33 mutant strains, elicited the formation of pink nodules on at least 14/15 plants, a result that was unaffected by the presence or absence of pMW23 (Table 2). Furthermore, all of the plants displayed robust growth on the nitrogen-free Jensen medium, generating healthy green shoots. Similar results were observed in plant dry weight measurements (Fig. 7), which have been used in several prior studies as an estimate of nitrogen fixation (31, 33, 76, 77). Collectively, these data suggest that the Kdo homopolymeric capsule is not essential for either nodule formation or nitrogen fixation in otherwise wild-type Rm1021.

In contrast, mutants unable to produce succinoglycan, both in a wild-type background (*exoY*::pDW33) or when combined with a mutation that alters Kdo capsule polymerization (*exoY*::pVO155 *kdsB2*::pDW33) or with a mutation preventing capsule surface display (*exoY*::pDW33 *rkpA*::Nm), triggered the formation of white nodules. The corresponding plants displayed stunted growth and were yellow in color. We observed only a single plant under these conditions that was green and carried a few pink nodules (Table 2). Consistently with these results, we observed a decrease in measurements of the dry weights of plants inoculated with these strains (Fig. 7). In the presence of pMW23, however, the number of green plants carrying pink nodules increased to 7/15 plants when inoculated with the *exoY*::pDW33 mutant and 6/15 plants when inoculated with the *exoY*::pVO155 *kdsB2*::pDW33 double mutant. We also observed an increase in mean plant dry weight, although it was reduced with respect to the wild type (Fig. 7). This result is similar to those of previous studies, which also reported only a partial restoration of nitrogen-fixing symbiosis in *exo* mutants carrying pMW23 (48, 59, 67, 79).

In contrast, we observed only a single green plant with pink nodules when inoculated with the *exoY*::pDW33 *rkpA*::Nm double mutant harboring pMW23 (Table 2). Plants inoculated with this strain displayed a mean dry weight similar to that of the same mutant harboring the vector control, which was reduced compared to the dry weight observed for the wild type or for the *kdsB2*::pDW33 and *rkpA*::Nm single-mutant strains harboring pMW23 (Fig. 7). We concluded that the rescue of the symbiotic phenotype of Rm1021 *exoY* mutants by *rkpZ* is dependent on the *rkp-1* cluster. Interestingly, the *kdsB2*::pDW33 mutation did not affect the rescue of *exoY*::pVO155 mutants by

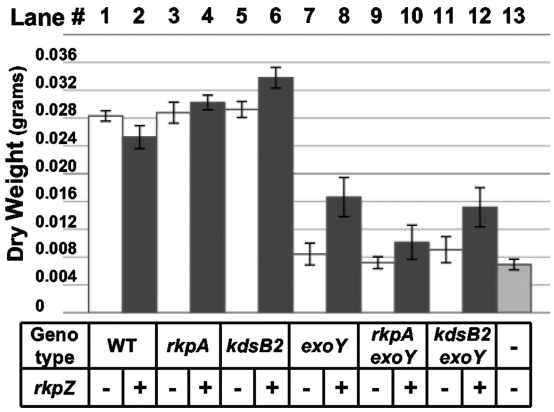


FIG. 7. Nitrogen-fixing ability of wild-type and mutant strains affected in capsule biosynthesis. Sterile *M. sativa* seedlings were placed onto Jensen agar slants. Plants were inoculated 48 h after planting with wild-type *S. meliloti* or mutant strains harboring either vector control (pTE3) or pMW23. Plants were harvested 80 days postinoculation, and their dry weight was measured. Lane 1, strain MGM044 (wild-type Rm1021/pTE3 [WT/pTE3]); lane 2, MGM312 (WT/pMW23); lane 3, MGM050 (*rkpA*::Nm/pTE3); lane 4, MGM314 (*rkpA*::Nm/pMW23); lane 5, MGM404 (*kdsB2*::pDW33/pTE3); lane 6, MGM405 (*kdsB2*::pDW 33/pMW23); lane 7, MGM359 (*exoY*::pDW33/pTE3); lane 8, MGM361 (*exoY*::pDW33/pMW23); lane 9, MGM355 (*exoY*::pDW33 *rkpA*::Nm/ pTE3); lane 10, MGM357 (*exoY*::pDW33 *rkpA*::Nm/pMW23); lane 11, MGM352 (*exoY*::pVO155 *kdsB2*::pDW33/pTE3), lane 12, MGM354 (*exoY*::pVO155 *kdsB2*::pDW33/pMW23); and lane 13, sample mock inoculated with sterile 10 mM $MgSO_4$. The presence of the plasmid containing *rkpZ* (pMW23) is indicated beneath each lane with a plus, and the presence of the control plasmid (pTE3) is indicated with a minus. The *exoY*::pDW33 mutant and *exoY*::pVO155 mutant behave identically under all conditions tested (M. G. Müller et al., unpublished). Similar results were observed in an additional, independent experiment (M. G. Müller et al., unpublished).

rkpZ, suggesting that changes in the degree of polymerization do not affect symbiotic efficiency. In addition, we observed that plants displaying a reduced fixation of nitrogen contained nodules with fewer viable bacteria than those of plants inoculated with wild-type Rm1021 (see Table S1 in the supplemental material).

DISCUSSION

Cell surface polysaccharides, such as LPS and capsule polysaccharide, contribute to diverse microbe-host interactions, including the nitrogen-fixing symbioses between rhizobia and leguminous plants. Studies of *S. meliloti* strain Rm41 have demonstrated a clear role for capsule polysaccharidein symbiosis. In other *S. meliloti* strains, such as the well-studied model Rm1021, the symbiotic function of capsule polysaccharide is less clear. To address this gap in knowledge, we carried out a molecular analysis of the Kdo homopolymeric capsule produced by strain Rm1021. We identified three new genes that, when disrupted, affect the degree of the polymerization of the Kdo homopolymeric capsule. We also demonstrated that the *rkp-1* cluster is required for the display of the Kdo homopolymeric capsule on the cell surface. The results of this study, combined with data from other laboratories, provide strong evidence that the Kdo homopolymeric capsule produced by strain Rm1021 can contribute to symbiosis with alfalfa.

Based on the reported capsule composition, we focused our attention on *kdsB2*, which is annotated as a CMP-Kdo cytidylyltransferase. In the presence of plasmid-borne *rkpZ* from strain Rm41, mutants disrupted for *kdsB2* or the downstream genes smb20804 and smb20805, produced capsule with a reduction in degree of polymerization compared to that of the wild type. In addition to its role as a substituent of the *S. meliloti* capsule, the sugar Kdo is a component of *S. meliloti* LPS (9). We speculate that enzymes involved in LPS polymerization are able to functionally replace KdsB2, Smb20804, and Smb20805, which would explain the modest effects of the mutations on capsule polymerization. For example, the *S. meliloti* genome contains a predicted CMP-Kdo synthetase encoded by the *kdsB* gene. We were not able to generate a mutant deficient for *kdsB*, suggesting that it is an essential gene, which prevented any insight into its potential role in either LPS or capsule biosynthesis. Future studies will examine the effect of LPS mutants on the polymerization of the Kdo homopolymeric capsule.

A much stronger effect on the Kdo homopolymeric capsule was observed in mutants of the *rkp-1* gene cluster. In the presence of the plasmid-borne *rkpZ* gene, *rkp-1* mutants of strain Rm1021 accumulated a very-high-molecular-weight polysaccharide. Interestingly, this polysaccharide species also could be observed, albeit to a lesser extent, in wild-type Rm1021 cells harboring plasmid-borne *rkpZ*. Several lines of evidence suggest that the very-high-molecular-weight polysaccharide represents an intracellular form of the Kdo homopolymeric capsule normally present on the cell surface. First, a combination of EDTA-TEA extraction, osmotic shock treatment, and ultracentrifugation suggested that this very-high-molecular-weight polysaccharide was located in the cytoplasm, with a very small fraction being present in the cytoplasmic or outer membrane. Second, mutations in *kdsB2* and smb20805 (which affect the polymerization of the Kdo homopolymer) prevented the accumulation of the very-high-molecular-weight polysaccharide. Third, an analysis of the composition of the very-high-molecular-weight polysaccharide showed that it was enriched in Kdo, which is consistent with it being a high-molecular-weight form of the Kdo homopolymer (Fig. 5). Collectively, these data strongly suggest that *rkp-1* mutants accumulate an intracellular high-molecular-weight form of the Kdo homopolymeric capsule. Interestingly, the *rkpA* gene of strain Rm1021 consists of one large ORF, whereas the equivalent region of strain Rm41 has been reported to encode six individual ORFs (*rkpA-F*) (52). To further examine this surprising difference between Rm1021 and Rm41, we partially resequenced the *rkpA* region in Rm41. Although incomplete, our initial DNA analysis suggests that the *rkpA* region of Rm41 also consists of one large ORF that is similar to that of Rm1021 (M. G. Müller et al., unpublished).

The sequence similarity between *rkpA* and genes involved in fatty acid synthesis led to the proposal that the *rkp-1* gene cluster was responsible for the synthesis, modification, or transfer of a lipid anchor or lipid carrier (38, 52). In this view, nascent cytoplasmic chains of polysaccharide (4 to 7 kDa) are transferred to an *rkp-1*-dependent lipid anchor/carrier prior to further polymerization, followed by export to the cell surface (59). A key prediction of this model is that only the 4- to 7-kDa nascent chains of polysaccharide accumulate in mutants of the *rkp-1* cluster. Consistently with this model, in the absence of plasmid-borne *rkpZ* we failed to detect capsular polysaccharide in *rkp-1* mutants (the 4- to 7-kDa polysaccharides likely are undetectable via our PAGE assay). However, in the presence of plasmid-borne *rkpZ*, we observed an accumulation of polysaccharides of very high molecular weight in *rkp-1* mutants, which appeared to be cytoplasmically located. This result stands in contrast to the prediction of the model. Mutations affecting *rkp-1* in *S. meliloti* strain Rm41 have been reported to result in a lack of detectable capsular polysaccharide (38, 52). However, our studies using [U-^{14}C]glucose labeling and phenol-water extraction also resulted in the observation of a similar very-high-molecular-weight form of capsule in *rkpA* mutants of strain Rm41 (M. G. Müller et al., unpublished). Although preliminary in nature, these data suggest that *rkp-1* mutants display similar phenotypes in both Rm1021 and Rm41.

In the presence of plasmid-borne *rkpZ*, *S. meliloti* strain Rm1021 produces Kdo homopolymeric capsule with an increased degree of polymerization (67). Within strain Rm41, however, mutants lacking *rkpZ* display an increased degree of the polymerization of capsular polysaccharide (59), suggesting that the protein functions to reduce the degree of polymerization. In either case, the results have led to the suggestion that RkpZ functions as a chain length regulator (37, 59). Interestingly, the expression of *rkpZ* in strain Rm1021 also resulted in an overall increase in the abundance of the Kdo homopolymer (67). These unexpected effects of *rkpZ* on capsule abundance led the authors of a previous study to suggest that RkpZ instead acts as a regulator of polysaccharide synthesis (67), with the altered degree of polymerization being a consequence of capsule overproduction. The results of our study are consistent with this idea. We propose that the heterologous expression of *rkpZ* leads to greatly increased capsule biosynthesis

in wild-type strain Rm1021, which exceeds the ability of the cell to transport the polysaccharide from the cytoplasm. In the absence of efficient transport from the cytoplasm, this cytoplasmic Kdo homopolymeric polysaccharide continues to undergo polymerization, resulting in the production of the very-high-molecular-weight species observed in wild-type strains harboring *rkpZ*. Mutations in *kdsB2*, smb20804, and smb20805 decrease the rate of polymerization, which allows transport to remain balanced with synthesis, thereby preventing the accumulation of the very-high-molecular-weight polysaccharide. In *rkp-1* mutants transport is blocked, leading to the production of exclusively very-high-molecular-weight polysaccharide.

While evidence from our group, as well as that from other groups (67), suggests that RkpZ acts as a regulator of Kdo synthesis, the mechanism of this regulation is unclear. The introduction of plasmid-borne *rkpZ* had no effect on the expression of *kdsB2*::*uidA*, smb20804::*uidA*, and smb20805::*uidA* transcriptional fusions, nor did it affect the expression of *rkpA*::*uidA*, *rkpG*::*uidA*, *rkpH*::*uidA*, *rkpI*::*uidA*, or *rkpJ*::*uidA* transcriptional fusions (M. G. Müller et al., unpublished data). Therefore, RkpZ does not appear to act as a regulator of transcription. Interestingly, RkpZ exhibits 42% amino acid identity to KpsC from *E. coli* (8, 47). When *kpsC* was mutated, it rendered the *E. coli* cell surface devoid of K5 antigen. Instead, the polysaccharide was present solely in the cell cytoplasm and lacked a phospholipid anchor, although its size was similar to that of wild-type *E. coli* K5 antigen (8). In addition, recent localization studies have suggested that KpsC functions as part of a complex connecting polysaccharide synthesis to transport (62), which could mean that KpsC and RkpZ carry out similar functions. Future studies will examine the role of RkpZ in polysaccharide synthesis and export.

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REFERENCES

1. **Barnett, M. J., R. F. Fisher, T. Jones, C. Komp, A. P. Abola, F. Barloy-Hubler, L. Bowser, D. Capela, F. Galibert, J. Gouzy, M. Gurjal, A. Hong, L. Huizar, R. W. Hyman, D. Kahn, M. L. Kahn, S. Kalman, D. H. Keating, C. Palm, M. C. Peck, R. Surzycki, D. H. Wells, K. C. Yeh, R. W. Davis, N. A. Federspiel, and S. R. Long.** 2001. Nucleotide sequence and predicted functions of the entire *Sinorhizobium meliloti* pSymA megaplasmid. Proc. Natl. Acad. Sci. USA **98:**9883–9888.
2. **Barnett, M. J., V. Oke, and S. R. Long.** 2000. New genetic tools for use in the Rhizobiaceae and other bacteria. BioTechniques **29:**240–245.
3. **Barnett, M. J., J. A. Swanson, and S. R. Long.** 1998. Multiple genetic controls on *Rhizobium meliloti syrA*, a regulator of exopolysaccharide abundance. Genetics **148:**19–32.
4. **Becker, A., N. Fraysse, and L. Sharypova.** 2005. Recent advances in studies on structure and symbiosis-related function of rhizobial K-antigens and lipopolysaccharides. Mol. Plant-Microbe Interact. **18:**899–905.
5. **Beringer, J. E.** 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84:**188–198.
6. **Brewin, N. J.** 1991. Development of the legume root nodule. Annu. Rev. Cell Biol. **7:**191–226.
7. **Brewin, N. J.** 1992. Nodule formation in legumes, p. 229–248. *In* J. Lederberg (ed.), Encyclopedia of microbiology, vol. 3. Academic Press, San Diego, CA.
8. **Bronner, D., V. Sieberth, C. Pazzani, I. S. Roberts, G. J. Boulnois, B. Jann, and K. Jann.** 1993. Expression of the capsular K5 polysaccharide of *Escherichia coli*: biochemical and electron microscopic analyses of mutants with defects in region 1 of the K5 gene cluster. J. Bacteriol. **175:**5984–5992.
9. **Carlson, R. W., B. Reuhs, T. B. Chen, U. R. Bhat, and K. D. Noel.** 1995. Lipopolysaccharide core structures in *Rhizobium etli* and mutants deficient in O-antigen. J. Biol. Chem. **270:**11783–11788.
10. **Cheng, H. P., and G. C. Walker.** 1998. Succinoglycan is required for initiation and elongation of infection threads during nodulation of alfalfa by *Rhizobium meliloti*. J. Bacteriol. **180:**5183–5191.
11. **Corzo, J., R. Perez-Galdona, M. Leon-Barrios, and A. M. Gutierrez-Navarro.** 1991. Alcian blue fixation allows silver staining of the isolated polysaccharide component of bacterial lipopolysaccharides in polyacrylamide gels. Electrophoresis **12:**439–441.
12. **Cronan, G. E., and D. H. Keating.** 2004. *Sinorhizobium meliloti* sulfotransferase that modifies lipopolysaccharide. J. Bacteriol. **186:**4168–4176.
13. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
14. **Dazzo, F. B., G. L. Truchet, R. I. Hollingsworth, E. M. Hrabak, H. S. Pankratz, S. Philip-Hollingsworth, J. L. Salzwedel, K. Chapman, L. Appenzeller, A. Squartini, et al.** 1991. *Rhizobium* lipopolysaccharide modulates infection thread development in white clover root hairs. J. Bacteriol. **173:**5371–5384.
15. **de Bruijn, F.** 1991. Biochemical and molecular studies: symbiotic nitrogen fixation. Curr. Opin. Biotechnol. **2:**184–192.
16. **Dénarié, J., and J. Cullimore.** 1993. Lipo-oligosaccharide nodulation factors: a new class of signaling molecules mediating recognition and morphogenesis. Cell **74:**951–954.
17. **Dénarié, J., F. Debelle, and C. Rosenberg.** 1992. Signaling and host range variation in nodulation. Annu. Rev. Microbiol. **46:**497–531.
18. **D'Haeze, W., J. Glushka, R. De Rycke, M. Holsters, and R. W. Carlson.** 2004. Structural characterization of extracellular polysaccharides of *Azorhizobium caulinodans* and importance for nodule initiation on *Sesbania rostrata*. Mol. Microbiol. **52:**485–500.
19. **D'Haeze, W., and M. Holsters.** 2002. Nod factor structures, responses, and perception during initiation of nodule development. Glycobiology **12:**79R–105R.
20. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
21. **Downie, J. A.** 1994. Signalling strategies for nodulation of legumes by rhizobia. Trends Microbiol. **2:**318–324.
22. **Ehrhardt, D. W., E. M. Atkinson, and S. R. Long.** 1992. Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. Science **256:**998–1000.
23. **Epple, G., K. M. van der Drift, J. E. Thomas-Oates, and O. Geiger.** 1998. Characterization of a novel acyl carrier protein, RkpF, encoded by an operon involved in capsular polysaccharide biosynthesis in *Sinorhizobium meliloti*. J. Bacteriol. **180:**4950–4954.
24. **Finan, T. M., S. Weidner, K. Wong, J. Buhrmester, P. Chain, F. J. Vorholter, I. Hernandez-Lucas, A. Becker, A. Cowie, J. Gouzy, B. Golding, and A. Puhler.** 2001. The complete sequence of the 1,683-kb pSymB megaplasmid from the N2-fixing endosymbiont *Sinorhizobium meliloti*. Proc. Natl. Acad. Sci. USA **98:**9889–9894.
25. **Fraysse, N., B. Lindner, Z. Kaczynski, L. Sharypova, O. Holst, K. Niehaus, and V. Poinsot.** 2005. *Sinorhizobium meliloti* strain 1021 produces a low-molecular-mass capsular polysaccharide that is a homopolymer of 3-deoxy-D-manno-oct-2-ulosonic acid harboring a phospholipid anchor. Glycobiology **15:**101–108.
26. **Galibert, F., T. M. Finan, S. R. Long, A. Puhler, P. Abola, F. Ampe, F. Barloy-Hubler, M. J. Barnett, A. Becker, P. Boistard, G. Bothe, M. Boutry, L. Bowser, J. Buhrmester, E. Cadieu, D. Capela, P. Chain, A. Cowie, R. W. Davis, S. Dreano, N. A. Federspiel, R. F. Fisher, S. Gloux, T. Godrie, A. Goffeau, B. Golding, J. Gouzy, M. Gurjal, I. Hernandez-Lucas, A. Hong, L. Huizar, R. W. Hyman, T. Jones, D. Kahn, M. L. Kahn, S. Kalman, D. H. Keating, E. Kiss, C. Komp, V. Lelaure, D. Masuy, C. Palm, M. C. Peck, T. M. Pohl, D. Portetelle, B. Purnelle, U. Ramsperger, R. Surzycki, P. Thebault, M. Vandenbol, F. J. Vorholter, S. Weidner, D. H. Wells, K. Wong, K. C. Yeh, and J. Batut.** 2001. The composite genome of the legume symbiont *Sinorhizobium meliloti*. Science **293:**668–672.
27. **Geiger, O., and I. M. Lopez-Lara.** 2002. Rhizobial acyl carrier proteins and their roles in the formation of bacterial cell-surface components that are required for the development of nitrogen-fixing root nodules on legume hosts. FEMS Microbiol. Lett. **208:**153–162.
28. **Glazebrook, J., and G. C. Walker.** 1989. A novel exopolysaccharide can function in place of the calcofluor-binding exopolysaccharide in nodulation of alfalfa by *Rhizobium meliloti*. Cell **56:**661–672.
29. **González, J. E., B. L. Reuhs, and G. C. Walker.** 1996. Low molecular weight EPS II of *Rhizobium meliloti* allows nodule invasion in *Medicago sativa*. Proc. Natl. Acad. Sci. USA **93:**8636–8641.
30. **González, J. E., G. M. York, and G. C. Walker.** 1996. *Rhizobium meliloti* exopolysaccharides: synthesis and symbiotic function. Gene **179:**141–146.
31. **Haydock, K. P., D. O. Norris, and L. T. Mannetje.** 1980. The relation between nitrogen percent and dry weight of inoculated legumes. Plant Soil **57:**353–362.
32. **Hirsch, A. M.** 1992. Developmental biology of legume nodulation. New Phytol. **122:**211–237.

33. **Hozbor, D. F., A. J. Pich Otero, A. R. Lodeiro, M. F. Del Papa, M. Pistorio, and A. Lagares.** 2004. The symbiotic defect in a *Sinorhizobium meliloti* lipopolysaccharide mutant can be overcome by expression of other surface polysaccharides. Res. Microbiol. **155:**855–860.
34. **Jefferson, R. A., S. M. Burgress, and D. Hirsch.** 1986. β-Glucuronidase from *Escherichia coli* as a gene-fusion marker. Proc. Natl. Acad. Sci. USA **83:** 8447–8451.
35. **Keating, D. H., M. G. Willits, and S. R. Long.** 2002. A *Sinorhizobium meliloti* lipopolysaccharide mutant altered in cell surface sulfation. J. Bacteriol. **184:** 6681–6689.
36. **Kereszt, A., E. Kiss, B. L. Reuhs, R. W. Carlson, A. Kondorosi, and P. Putnoky.** 1998. Novel *rkp* gene clusters of *Sinorhizobium meliloti* involved in capsular polysaccharide production and invasion of the symbiotic nodule: the *rkpK* gene encodes a UDP-glucose dehydrogenase. J. Bacteriol. **180:**5426–5431.
37. **Kiss, E., A. Kereszt, F. Barta, S. Stephens, B. L. Reuhs, A. Kondorosi, and P. Putnoky.** 2001. The *rkp-3* gene region of *Sinorhizobium meliloti* Rm41 contains strain-specific genes that determine K antigen structure. Mol. Plant-Microbe Interact. **14:**1395–1403.
38. **Kiss, E., B. L. Reuhs, J. S. Kim, A. Kereszt, G. Petrovics, P. Putnoky, I. Dusha, R. W. Carlson, and A. Kondorosi.** 1997. The *rkpGHI* and *-J* genes are involved in capsular polysaccharide production by *Rhizobium meliloti*. J. Bacteriol. **179:**2132–2140.
39. **Lagares, A., G. Caetano-Anolles, K. Niehaus, J. Lorenzen, H. D. Ljunggren, A. Puhler, and G. Favelukes.** 1992. A *Rhizobium meliloti* lipopolysaccharide mutant altered in competitiveness for nodulation of alfalfa. J. Bacteriol. **174:**5941–5952.
40. **Leigh, J. A., E. R. Signer, and G. C. Walker.** 1985. Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. Proc. Natl. Acad. Sci. USA **82:**6231–6235.
41. **Long, S., S. McCune, and G. C. Walker.** 1988. Symbiotic loci of *Rhizobium meliloti* identified by random Tn*phoA* mutagenesis. J. Bacteriol. **170:**4257–4265.
42. **Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown, and F. M. Ausubel.** 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. **149:**114–122.
43. **Mergaert, P., W. D'Haeze, D. Geelen, D. Prome, M. Van Montagu, R. Geremia, J. C. Prome, and M. Holsters.** 1995. Biosynthesis of *Azorhizobium caulinodans* Nod factors. Study of the activity of the NodABC proteins by expression of the genes in *Escherichia coli*. J. Biol. Chem. **270:**29217–29223.
44. **Niehaus, K., A. Largares, and A. Puhler.** 1998. A *Sinorhizobium meliloti* Lipopolysaccharide mutant induces effective nodules on the host plant *Medicago sativa* (Alfalfa) but fails to establish a symbiosis with *Medicago truncatula*. Mol. Plant-Microbe Interact. **11:**906–914.
45. **Oke, V., and S. R. Long.** 1999. Bacterial genes induced within the nodule during the Rhizobium-legume symbiosis. Mol. Microbiol. **32:**837–849.
46. **Oldroyd, G. E., and J. A. Downie.** 2004. Calcium, kinases and nodulation signalling in legumes. Nat. Rev. Mol. Cell Biol. **5:**566–576.
47. **Pazzani, C., C. Rosenow, G. J. Boulnois, D. Bronner, K. Jann, and I. S. Roberts.** 1993. Molecular analysis of region 1 of the *Escherichia coli* K5 antigen gene cluster: a region encoding proteins involved in cell surface expression of capsular polysaccharide. J. Bacteriol. **175:**5978–5983.
48. **Pellock, B. J., H. P. Cheng, and G. C. Walker.** 2000. Alfalfa root nodule invasion efficiency is dependent on *Sinorhizobium meliloti* polysaccharides. J. Bacteriol. **182:**4310–4318.
49. **Perret, X., C. Staehelin, and W. J. Broughton.** 2000. Molecular basis of symbiotic promiscuity. Microbiol. Mol. Biol. Rev. **64:**180–201.
50. **Peters, N. K., J. W. Frost, and S. R. Long.** 1986. A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science **233:**977–980.
51. **Peters, N. K., and D. P. Verma.** 1990. Phenolic compounds as regulators of gene expression in plant-microbe relations. Mol. Plant-Microbe Interact. **3:**4–8.
52. **Petrovics, G., P. Putnoky, B. Reuhs, J. Kim, T. A. Thorp, K. D. Noel, R. W. Carlson, and A. Kondorosi.** 1993. The presence of a novel type of surface polysaccharide in *Rhizobium meliloti* requires a new fatty acid synthase-like gene cluster involved in symbiotic nodule development. Mol. Microbiol. **8:**1083–1094.
53. **Phillips, D. A., C. M. Joseph, and C. A. Maxwell.** 1992. Trigonelline and stachydrine released from alfalfa seeds activate NodD2 protein in *Rhizobium meliloti*. Plant Physiol. **99:**1526–1531.
54. **Putnoky, P., G. Petrovics, A. Kereszt, E. Grosskopf, D. T. Ha, Z. Banfalvi, and A. Kondorosi.** 1990. *Rhizobium meliloti* lipopolysaccharide and exopolysaccharide can have the same function in the plant-bacterium interaction. J. Bacteriol. **172:**5450–5458.
55. **Quandt, J., and M. F. Hynes.** 1993. Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. Gene **127:** 15–21.
56. **Redmond, J. W., M. Batley, M. A. Djordjevic, R. W. Innes, P. L. Kuempel, and B. G. Rolfe.** 1986. Flavones induce expression of nodulation genes in Rhizobium. Nature **323:**632–635.
57. **Reuhs, B. L., R. W. Carlson, and J. S. Kim.** 1993. *Rhizobium fredii* and *Rhizobium meliloti* produce 3-deoxy-D-manno-2-octulosonic acid-containing polysaccharides that are structurally analogous to group II K antigens (capsular polysaccharides) found in *Escherichia coli*. J. Bacteriol. **175:**3570–3580.
58. **Reuhs, B. L., D. P. Geller, J. S. Kim, J. E. Fox, V. S. Kolli, and S. G. Pueppke.** 1998. *Sinorhizobium fredii* and *Sinorhizobium meliloti* produce structurally conserved lipopolysaccharides and strain-specific K antigens. Appl. Environ. Microbiol. **64:**4930–4938.
59. **Reuhs, B. L., M. N. Williams, J. S. Kim, R. W. Carlson, and F. Cote.** 1995. Suppression of the Fix$^-$ phenotype of *Rhizobium meliloti exoB* mutants by *lpsZ* is correlated to a modified expression of the K polysaccharide. J. Bacteriol. **177:**4289–4296.
60. **Ridge, R.** 1992. A model for legume root hair growth and Rhizobium infection. Symbiosis **14:**359–373.
61. **Ridley, B. L., B. S. Jeyaretnam, and R. W. Carlson.** 2000. The type and yield of lipopolysaccharide from symbiotically deficient rhizobium lipopolysaccharide mutants vary depending on the extraction method. Glycobiology **10:** 1013–1023.
62. **Rigg, G. P., B. Barrett, and I. S. Roberts.** 1998. The localization of KpsC, S and T, and KfiA, C and D proteins involved in the biosynthesis of the *Escherichia coli* K5 capsular polysaccharide: evidence for a membrane-bound complex. Microbiology **144:**2905–2914.
63. **Roche, P., F. Maillet, C. Plazanet, F. Debelle, M. Ferro, G. Truchet, J. C. Prome, and J. Denarie.** 1996. The common *nodABC* genes of *Rhizobium meliloti* are host-range determinants. Proc. Natl. Acad. Sci. USA **93:**15305–15310.
64. **Rolfe, B. G.** 1988. Flavones and isoflavones as inducing substances of legume nodulation. Biofactors **1:**3–10.
65. **Sambrook, J., E. F. Fritsch, and T. E. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., vol. 3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
66. **Schlaman, H. R., R. J. Okker, and B. J. Lugtenberg.** 1992. Regulation of nodulation gene expression by NodD in rhizobia. J. Bacteriol. **174:**5177–5182.
67. **Sharypova, L. A., G. Chataigne, N. Fraysse, A. Becker, and V. Poinsot.** 2006. Overproduction and increased molecular weight account for the symbiotic activity of the *rkpZ*-modified K polysaccharide from *Sinorhizobium meliloti* Rm1021. Glycobiology **16:**1181–1193.
68. **Spaink, H. P., and B. J. Lugtenberg.** 1994. Role of rhizobial lipo-chitin oligosaccharide signal molecules in root nodule organogenesis. Plant Mol. Biol. **26:**1413–1422.
69. **Spaink, H. P., D. M. Sheeley, A. A. van Brussel, J. Glushka, W. S. York, T. Tak, O. Geiger, E. P. Kennedy, V. N. Reinhold, and B. J. Lugtenberg.** 1991. A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of Rhizobium. Nature **354:**125–130.
70. **Staehelin, C., L. S. Forsberg, W. D'Haeze, M. Y. Gao, R. W. Carlson, Z. P. Xie, B. J. Pellock, K. M. Jones, G. C. Walker, W. R. Streit, and W. J. Broughton.** 2006. Exo-oligosaccharides of *Rhizobium* sp. strain NGR234 are required for symbiosis with various legumes. J. Bacteriol. **188:**6168–6178.
71. **Townsend, G. E., Jr., L. S. Forsberg, and D. H. Keating.** 2006. *Mesorhizobium loti* produces *nodPQ*-dependent sulfated cell surface polysaccharides. J. Bacteriol. **188:**8560–8572.
72. **Truchet, G., P. Roche, P. Lerouge, J. Vasse, S. Camut, F. de Billy, J.-C. Prome, and J. Denarie.** 1991. Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. Nature **351:**670–673.
73. **Vaara, M.** 1992. Agents that increase the permeability of the outer membrane. Microbiol. Rev. **56:**395–411.
74. **Valverde, C., D. F. Hozbor, and A. Lagares.** 1997. Rapid preparation of affinity-purified lipopolysaccharide samples for electrophoretic analysis. BioTechniques **22:**230–236.
75. **Vijn, I., L. das Nevas, A. van Kammen, H. Franssen, and T. Bisseling.** 1993. Nod factors and nodulation in plants. Science **260:**1764–1765.
76. **Vincent, J. M.** 1970. A manual for the practical study of the root-nodule bacteria. Blackwell Scientific, Oxford, United Kingdom.
77. **Wang, C., M. Saldanha, X. Sheng, K. J. Shelswell, K. T. Walsh, B. W. Sobral, and T. C. Charles.** 2007. Roles of poly-3-hydroxybutyrate (PHB) and glycogen in symbiosis of *Sinorhizobium meliloti* with *Medicago* sp. Microbiology **153:**388–398.
78. **Wells, D. H., and S. R. Long.** 2002. The *Sinorhizobium meliloti* stringent response affects multiple aspects of symbiosis. Mol. Microbiol. **43:**1115–1127.
79. **Williams, M. N., R. I. Hollingsworth, S. Klein, and E. R. Signer.** 1990. The symbiotic defect of *Rhizobium meliloti* exopolysaccharide mutants is suppressed by *lpsZ*$^+$, a gene involved in lipopolysaccharide biosynthesis. J. Bacteriol. **172:**2622–2632.
80. **Yang, C., E. R. Signer, and A. M. Hirsch.** 1992. Nodules initiated by *Rhizobium meliloti* exopolysaccharide mutants lack a discrete, persistent nodule meristem. Plant Physiol. **98:**143–151.
81. **Zhan, H. J., S. B. Levery, C. C. Lee, and J. A. Leigh.** 1989. A second exopolysaccharide of *Rhizobium meliloti* strain SU47 that can function in root nodule invasion. Proc. Natl. Acad. Sci. USA **86:**3055–3059.