Genetic Characterization of a *Sinorhizobium meliloti* Chromosomal Region Involved in Lipopolysaccharide Biosynthesis

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The genetic characterization of a 5.5-kb chromosomal region of *Sinorhizobium meliloti* 2011 that contains *lpsB*, a gene required for the normal development of symbiosis with *Medicago* spp., is presented. The nucleotide sequence of this DNA fragment revealed the presence of six genes: *greA* and *lpsB*, transcribed in the forward direction; and *lpsE*, *lpsD*, *lpsC*, and *lrp*, transcribed in the reverse direction. Except for *lpsB*, none of the *lps* genes were relevant for nodulation and nitrogen fixation. Analysis of the transcriptional organization of *lpsB* showed that *greA* and *lpsB* are part of separate transcriptional units, which is in agreement with the finding of a DNA stretch homologous to a "nonnitrogen" promoter consensus sequence between *greA* and *lpsB*. The opposite orientation of *lpsB* with respect to its first downstream coding sequence, *lpsE*, indicated that the altered LPS and the defective symbiosis of *lpsB* mutants are both consequences of a primary nonpolar defect in a single gene. Global sequence comparisons revealed that the *greA-lpsB* and *lrp* genes of *S. meliloti* have a genetic organization similar to that of their homologous loci in *R. leguminosarum* bv. viciae. In particular, high sequence similarity was found between the translation product of *lpsB* and a core-related biosynthetic mannosyltransferase of *R. leguminosarum* bv. viciae encoded by the *lpcC* gene. The functional relationship between these two genes was demonstrated in genetic complementation experiments in which the *S. meliloti lpsB* gene restored the wild-type LPS phenotype when introduced into *lpcC* mutants of *R. leguminosarum*. These results support the view that *S. meliloti lpsB* also encodes a mannosyltransferase that participates in the biosynthesis of the LPS core. Evidence is provided for the presence of other *lpsB*-homologous sequences in several members of the family *Rhizobiaceae*.

The infection of legume roots by soil bacteria of the genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* results in the development of specialized root organs, the nitrogen-fixing nodules (53). The establishment of functional root nodules is the result of a complex process that involves an active signal exchange between the host roots and the infecting rhizobia (7). One of the earliest events in the symbiotic dialog involves the secretion of flavonoid compounds by the host plant and the subsequent production of nodulation (Nod) factors by the rhizobia (38). Although the Nod factors are the best-characterized signal molecules of rhizobia, independent evidence supports the view that some of the bacterial surface polysaccharides are also active in signaling the plant (2, 16, 19, 22, 39, 40, 57, 60). It has thus been shown that the pretreatment of alfalfa roots with specific fractions of *Sinorhizobium meliloti* exopolysaccharides (EPS) conferred on symbiosis-deficient EPS mutants the ability to develop nitrogen-fixing nodules at a significant rate (2, 22, 57, 60). This and similar results for other plant-rhizobium associations (19) indicate that the EPSs induce durable changes in the plant root that are essential for symbiosis. That

the active *S. meliloti* EPS can be partially replaced by certain molecular forms of a capsular polysaccharide (KPS) suggested that these two *S. meliloti* polysaccharides have similar modes of action in symbiosis (45, 50). It was recently reported, however, that there are differences in the efficiencies of nodule invasion mediated by the succinoglycan (EPSI), the galactoglucan (EPSII), and the KPS (43). Interestingly, KPS has genetic loci in common with the biosynthetic pathway of another surface polysaccharide, the outer membrane lipopolysaccharide (LPS) (11, 31). Nevertheless, no symbiotic relationships between the *S. meliloti* KPS or EPS and the LPS have been demonstrated. It is well known that in those *S. meliloti* strains with no symbiotically active KPS, the remaining unaltered LPS does not support wild-type nodulation in EPS mutants (30, 40). Further analysis will thus be required to elucidate the ultimate genetic and functional relationships among the bacterial surface polysaccharides involved in symbiosis.

The genetics and biochemistry of *S. meliloti* LPS have been little investigated (15) in comparison with the LPS of other rhizobia (42, 44, 55, 59), possibly in part because several *S. meliloti* LPS mutants were reported to have normal symbiosis with the host plant alfalfa (15). *lpsB* mutants, however, were found to be compromised in their competitiveness for nodulation in alfalfa (34) and displayed a Fix⁻ phenotype in *Medicago truncatula* (41). At the moment, there is no simple model to explain the role of the *S. meliloti* LPS in symbiosis. The most

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TABLE 1. Strains used and constructed for this work

Strain	Relevant characteristics	Source and/or reference
Agrobacterim tumefaciens C58	Wild type	A. G. Matthysse
Bradyrhizobium japonicum USDA 110	Wild type, symbiont of *Glycine max*	P. van Berkum
Escherichia coli DH5α	*recA*1, Δ*lac*U169 80d*lac*ZΔM15; host strain used in cloning experiments	Bethesda Research Laboratories
E. coli XL1-Blue	Host strain of the nested-deletion clones generated by exonuclease III for DNA sequencing	Bullock et al. (10)
E. coli S17-1	MM294, RP4-2-Tc::Mu-Km::Tn7 chromosomally integrated	Simon et al. (54)
Mesorhizobium ciceri USDA 3383	Wild type, symbiont of *Cicer arietinum* L.	P. van Berkum
M. tianshanense USDA 3592	Wild type, symbiont of *Glycirrhiza pallidiflora*	P. van Berkum
Rhizobium etli CFN42, USDA 9032	Wild type, symbiont of *Phaseolus vulgaris*	E. Martínez
R. galegae USDA 4128	Wild type, symbiont of *Galega orientalis*	P. van Berkum
R. hainanensis USDA 3588	Wild type, symbiont of *Desmodium sinuatum*	P. van Berkum
R. huatlense USDA 4900	Wild type, symbiont of *Sesbania herbaceae*	P. van Berkum
R. leguminosarum bv. viciae VF39	Wild type, symbiont of *Pisum sativum*	U. Priefer
R. leguminosarum bv. viciae 3855	Wild type, symbiont of *P. sativum*, Sm^r derivative of wild-type strain 128C53	C. Ronson (28)
R. leguminosarum bv. viciae RSKnH	*R. leguminosarum* bv. viciae 3855 *lpcC*::*nptII*	C. Ronson (28)
R. leguminosarum bv. trifolii ANU843	Wild type, symbiont of *Trifolium* spp.	P. van Berkum
R. mongolense USDA 1844	Wild type, symbiont of *Medicago ruthenica*	P. van Berkum
Rhizobium sp. strain GRH2	Wild type, isolated from *Acacia cyanophylla*, nodulates herbaceous legumes such as *Phaseolus* and *Trifolium* spp.	N. Toro
Rhizobium sp. strain LPU83	Wild type, nodulates *M. sativa*, *P. vulgaris*, and *Leucaena leucocephala*, closely related to *Rhizobium* sp. strain Or191	Del Papa et al. (17)
Rhizobium sp. strain NGR234	Wild type, isolated from root nodules of *Lablab purpureus*, nodulates several legumes	W. J. Broughton
Rhizobium sp. strain Or191	Wild type, nodulates *M. sativa*, *P. vulgaris*, and *L. leucocephala*	P. van Berkum
R. tropici CIAT 299 (type 2A)	Wild type, symbiont of *P. vulgaris*	M. Aguilar
R. tropici CIAT 899 (type 2B), USDA 9030	Wild type, symbiont of *P. vulgaris*	P. van Berkum
Sinorhizobium sp. strain BR816	Wild type, symbiont of *P. vulgaris*	M. Aguilar
Sinorhizobium fredii HH103	Wild type, symbiont of *G. max*	M. Megias
S. fredii USDA 191	Wild type, symbiont of *G. max*	S. Pueppke
S. fredii USDA 257	Wild type, symbiont of *G. max*	S. Pueppke
S. medicae USDA 1037	Wild type, symbiont of *M. sativa*	P. van Berkum
S. meliloti 2011	Symbiont of *M. sativa*, Sm^r derivative of strain SU-47	Casse et al. (12)
S. meliloti 6963	*S. meliloti* 2011 *lpsB*::Tn5-6963 Nm^r Sm^r	Lagares et al. (34)
S. meliloti 7555	*S. meliloti* SU47 *lpsC*::Tn5 Nm^r Sm^r	Clover et al. (15)
S. meliloti 20-B+	*S. meliloti* 2011 *lpsB*::*lacZ*-Gm (sense *lacZ* fusion at *Kpn*I site in *lpsB*) Sm^r Gm^r	This work
S. meliloti 20-B	*S. meliloti* 2011 *lpsB*::*lacZ*-Gm (antisense *lacZ* fusion at *Kpn*I site in *lpsB* Sm^r Gm^r	This work
S. meliloti 20-C	*S. meliloti* 2011 *lpsC*::pK18mob, *lacZ* promoter of vector reads downstream of interrupted coding sequence, Sm^r Nm^r	This work
S. meliloti 20-D	*S. meliloti* 2011 *lpsD*::pK18mob, *lacZ* promoter of vector reads downstream of interrupted coding sequence, Sm^r Nm^r	This work
S. meliloti 20-E	*S. meliloti* 2011 *lpsE*::pK18mob, *lacZ* promoter of vector reads downstream of interrupted coding sequence, Sm^r Nm^r	This work
S. saheli USDA 4893	Wild type, symbiont of *Sesbania cannabina*	P. van Berkum
S. teranga USDA 4894	Wild type, symbiont of *Sesbania laeta*	P. van Berkum

passive role would be that of a surface molecule merely masking the presentation of naturally hidden bacterial structures that disturb plant penetration (i.e., preserving the bacterial surface charge or hydrophobicity [18]); a more active role for LPS in signaling the plant has yet to be considered (16). At this point, none of these possibilities can be excluded.

The altered symbiotic phenotype of *S. meliloti lpsB* mutants with respect to *Medicago* spp. implicates *lpsB* as an informative locus for investigating the genetics and biochemistry of the LPS structures that are required for supporting wild-type symbiosis. We present here the genetic analysis of a 5.5-kb chromosomal DNA fragment from *S. meliloti* that contains the *lpsB* gene. Based on sequence data and results from genetic complementation experiments, a putative function is proposed for the *lpsB* gene product. In addition, two new genes, *lpsE* and *lpsD*, that map immediately downstream from *lpsB* and participate in LPS biosynthesis have been identified.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively.

Culture media and bacterial growth conditions. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (35) at 37°C. *S. meliloti* strains were grown in TY (6) or LB medium at 28°C. Antibiotics were added as required at the following concentrations (micrograms per milliliter): streptomycin (400) gentamicin (50) and neomycin (120) for *S. meliloti*; ampicillin (200), gentamicin (10), and kanamycin (50) for *E. coli*.

LPS preparations and SDS-PAGE. LPS samples for electrophoretic analysis were purified by affinity chromatography as indicated by Valverde et al. (58), and

TABLE 2. Plasmids used and constructed for this work

Plasmid	Relevant characteristics	Source or reference
pA1	pK18mob carrying an *Sst*I-*Kpn*I insert containing the complete *greA* gene and the 5′ end of the *lpsB* gene of *S. meliloti* 2011; coding sequences of the insert in sense orientation relative to the p*lac* promoter from the vector	This work
pA2	pK19mob carrying the same insert as in pA1; coding sequences of the insert in antisense orientation relative to p*lac* promoter from the vector	This work
pAB2001	Plasmid containing a promoterless *lacZ-aacC1* cassette conferring Gm^r	Becker et al. (5)
pAL100	pACYC184-Gm-*mob* carrying in *Dra*I a 5.5-kb *Sst*I DNA fragment from *S. meliloti* 2011 that complements the LPS mutations in *S. meliloti* 6963 (blunt-end cloned as *Ecl*136II), Gm^r	Niechaus et al. (41)
pAL101	pSVB30/ΔB-H carrying in *Sst*I the 5.5-kb *Sst*I fragment from *S. meliloti* 2011 that complements *S. meliloti* 6963	This work
pAL103A	pSVB30 carrying in *Sal*I (Klenow) the 5.5-kb *Sst*I fragment from pAL101 blunt-end cloned as *Ecl*136II; insert orientation with its *Hind*III site close to the *lac* promoter from the vector	This work
pAL103B	pSVB30 carrying in the *Sal*I restriction site (Klenow) the 5.5-kb *Sst*I fragment from pAL101 blunt-end cloned as *Ecl*136II; insert orientation opposite that in pAL103A	This work
pB1	pK18mob carrying a *Bam*HI-*Kpn*I insert that contains the 3′ end of the *greA* gene and the 5′ portion of the *lpsB* gene of *S. meliloti* 2011; coding sequences of the insert in sense orientation relative to the *lac* promoter from the vector	This work
pB2	pK19mob carrying the same insert as in pB1; coding sequences of the insert in antisense orientation relative to the *lac* promoter from the vector	This work
pC1	pK18mob carrying an internal fragment of the *lpsB* gene that extends from a few codons downstream of the putative GTG start codon to the central *Kpn*I site; coding sequences of the insert in sense orientation relative to the *lac* promoter from the vector	This work
pC2	pK19mob carrying the same insert as in pC1; coding sequences of the insert in antisense orientation relative to the *lac* promoter from the vector	This work
pJB3Tc19	Derivative of the minimal replicon of plasmid RK2, Ap^r Tc^r	Blatny et al. (8)
pJBIpsSme	pJB3Tc19 carrying a 4.9-kb *Sst*I-*Hind*III fragment containing the *greA-lpsD* region from *S. meliloti* 2011 cloned in the polylinker of the vector as *Eco*RI-*Hind*III, Km^r	This work
pJL200	pSVB30 carrying in *Sst*I an 11-kb *Sst*I Tn5-containing fragment from *S. meliloti* 6963; insert homologous to that of pAL101 but carrying the Tn5 within *lpsB*	Niehaus et al. (41)
pJL200/ΔB-H	pSVB30/ΔB-H carrying in *Sst*I the 11-kb *Sst*I Tn5-containing fragment from *S. meliloti* 6963	This work
pJL201	pACYC184-Gm-*mob* carrying in *Dra*I an 11-kb *Sst*I Tn5-containing fragment from pJL200 blunt-end cloned as *Ecl*136II, Nm^r Gm^r; insert homologous to that of pAL100 but carrying the Tn5 within *lpsB*	Niehaus et al. (41)
pK18mob	pK18 derivative, *mob*, Km^r	Schäfer et al. (52)
pK19mob	pK19 derivative, *mob*, Km^r	Schäfer et al. (52)
pPN120	pLAFR1 with a 4.4-kb *Eco*RI fragment containing *greA, lpcC, dctA*, and the 5′ region of *dctB* from *R. leguminosarum* bv. viciae, Tc^r	C. Ronson (28)
pSVB30	pUC8 derivative, Ap^r	Arnold and Pühler (1)
pSVB30/ΔB-H	pSVB30 with a deletion in the *Bam*HI-*Hind*III region of the polylinker	This work

sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (SDS-PAGE) was performed as described elsewhere (34), using a Mini-Protean II 2-D cell (Bio-Rad). Gels were fixed and silver stained as described by Reuhs et al. (47).

Recombinant DNA techniques. Plasmid DNA preparation, restriction enzyme analysis, cloning procedures, and *E. coli* transformation were performed according to established techniques (35). Southern hybridizations were carried out using DNA probes labeled with digoxigenin. The probes were synthesized by PCR using digoxigenin-dUTP (Boehringer Mannheim) and appropriate primers to amplify the region of interest. For hybridizations, DNA extracted from bacteria was digested and transferred to nitrocellulose membranes (Hybond N; Amersham) as described by Chomczynski (14). The digoxigenin-labeled DNA probes were hybridized to the membranes at 65°C overnight after the blocking of nonspecific binding sites 1 h at 68°C, using the solutions and experimental conditions specified by Boehringer Mannheim (catalog no. 1093 657). For the visualization of positive bands, the membranes were incubated with an antibody against the digoxigenin ligand and washed, and a final color reaction was initiated at alkaline pH by the addition of X-phosphate plus nitroblue tetrazolium chloride as specified by the manufacturer.

DNA sequencing. The wild-type DNA fragment complementing *S. meliloti* 6963 was cloned into a modified pSVB30 vector in order to perform nested exonuclease III deletions as follows. First, plasmid pJL200/ΔB-H was generated by transferring the 11-kb *Sst*I DNA fragment of *S. meliloti* 6963 from pJL200 to the *Sst*I site of pSVB30/ΔB-H and then replaced the *Bam*HI-*Hind*III portion containing the Tn5 insertion from this plasmid with the homologous wild-type *Bam*HI-*Hind*III segment (devoid of the transposon) from pAL100 to produce plasmid pAL101 (Table 2). Finally, we generated plasmids pAL103A and

pAL103B by removing the *Sst*I insert from pAL101 with *Ecl*136II (isoschizomer of *Sst*I producing blunt ends) and cloning the fragment into the filled-in *Sal*I site of pSVB30 in forward and reverse orientations, respectively. Using these plasmids, appropriate subclones for sequencing were constructed by creating a set of overlapping nested deletions by the method of Henikoff (24). Sequencing reactions were carried out using universal and reverse primers and an Auto Read Sequencing kit (Pharmacia-LKB) according to a protocol devised by Zimmermann et al. (62). Sequence data were obtained for both DNA strands using an A.L.F. DNA sequencer (Pharmacia-LKB). Short gaps in the nucleotide sequence were resolved either by sequencing from the upstream region by means of specific primers or by reading from the pSVB30 vector toward different subcloned restriction fragments by means of standard M13 primers (Pharmacia). Sequence analysis and the determination of coding probabilities were done with programs from the Staden software package (56).

Vector-mediated chromosome walking. An *Stu*I-*Eco*RI restriction fragment containing the start codon and the upstream portion of *lrp* was obtained for DNA sequencing as described by Hozbor et al. (26). After excision of an internal region of the *lrp* from pAL101 with *Stu*I-*Sac*I, this segment was cloned into the suicide vector pK18mob (52) and then promoted the integration of this construction into the chromosome of *S. meliloti* 2011 by homologous recombination. After self-ligation of the *Eco*RI-digested total DNA from the resulting recombinant strain (neomycin and streptomycin resistant [Nm^r Sm^r]), the reaction mixture containing the desired circularized fragment was transformed into *E. coli* DH5α. The sequence of the 5′ end of *lrp* was obtained by reading from the vector through the *Stu*I site up to the first in-frame start codon (ATG).

Oligonucleotide primers and PCR hybridization conditions. We designed deoxyoligonucleotide primers in order to amplify a 267-bp fragment of the *S.*
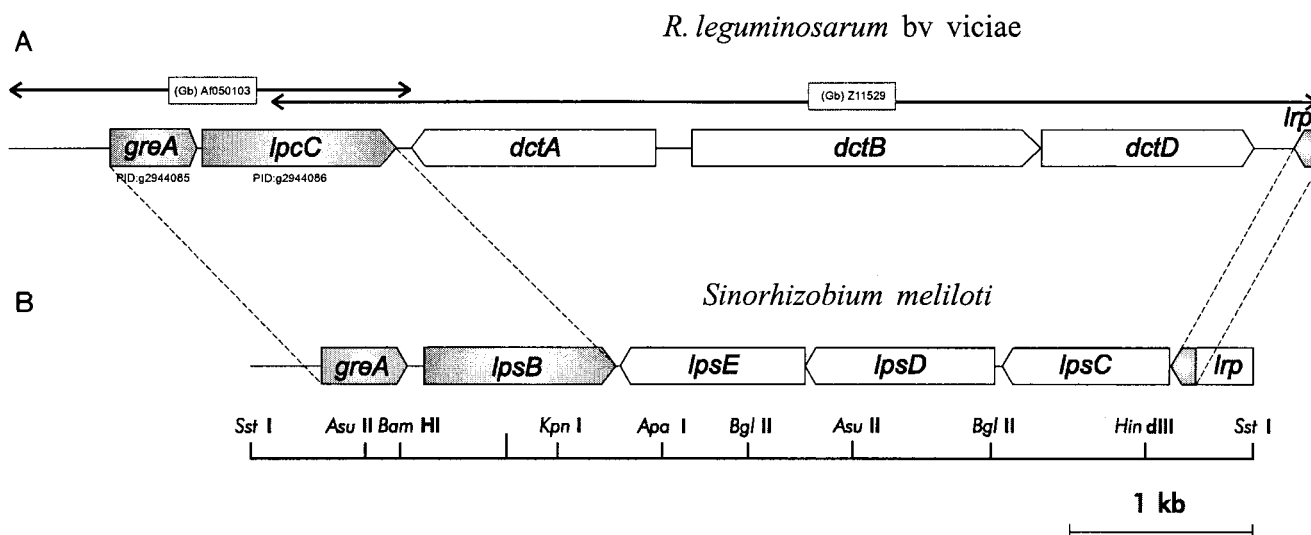
FIG. 1. Genomic arrangement of *greA, lpsB*, and *lrp* in *S. meliloti* compared to the arrangement of homologous genes in *R. leguminosarum* bv. viciae. (A) Gene arrangement in the vicinity of the *lpcC* gene in *R. leguminosarum* bv. viciae (GenBank accession no. AF050103 and Z11529). (B) Gene arrangement within the 5.5-kb *SstI* fragment that contains the *lpsB* gene in *S. meliloti* 2011 (GenBank accession no. AF193023 [this work]). Shaded regions correspond to homologous coding sequences in both rhizobia.

meliloti lpsB and the *Rhizobium leguminosarum* bv. viciae *lpcC* based on the sequence conservation of both genes (GenBank accession no. AAF06008 and AAC05215, respectively). These primers, synthesized by DNAgency (Malvern, Pa), had the sequences 5′-GTICGCCATCAGAAAGG-3′ (LPSB2F) and 5′-G AGCGGCGTCAGGCCGAAGC-3′ (LPSB2R). The PCRs were performed as described by Del Papa et al. (17). Stated in brief, PCR mixtures of 25 μl contained 50 mM Tris (pH 8.3), 500 μg of bovine serum albumin per ml, deoxynucleoside triphosphates, 3 mM MgCl$_2$, 1 U of *Taq* polymerase, each primer at 0.5 μM, and 5 to 10 μl of template DNA, obtained previously by heating a freshly isolated bacterial colony at 100°C for 15 min in 50 μl of distilled water. In some instances, appropriate dilutions of total DNA prepared from the bacteria by classical phenol-Tris extraction were used as the template. The amplification reactions were run in capillary tubes in an Idaho 1605 Air Thermo Cycler (ATC; Idaho Technology) under the following cycling conditions: 94°C for 1 min; 35 cycles at 94°C for 15 s, 53°C for 10 s, and 72°C for 10 s; and a final holding step at 72°C for 15 s. After the reaction, 10-μl volumes of the PCR products were separated on 1.5% (wt/vol) agarose gels containing 0.5 to 1.0 μg of ethidium bromide per ml, and the resulting banding pattern was photographed using a Kodak model DC120 digital camera under UV illumination. Southern blot hybridization of the PCR products from Fig. 5B were carried out at 65°C, using a 532-bp digoxigenin-labeled DNA probe corresponding to an internal portion of *lpsB* whose total sequence included that of the PCR-amplified region.

Chromosomal single-copy *lacZ* transcriptional fusions and allelic exchange at the *lps* locus. Single-copy chromosomal transcriptional fusions between a promotorless *lacZ-accC1* cassette (5) and *lpsB* were effected through the homologous recombination in vivo of the insert from either pHL-B1 (sense fusion) or pHL-B2 (antisense fusion). All plasmids for the site-specific gene replacement were constructed using pK18mob as a suicide vector (52). Plasmids were transferred from *E. coli* S17-1 to *S. meliloti* 2011 by conjugation. *S. meliloti* clones that had presumably undergone the marker exchange were identified as the result of a double-crossover event by their expected gentamicin-resistant (Gm^r; cassette *accC1* gene), Sm^r (marker of the recipient bacteria), and Nm^s (after plasmid segregation) phenotype. Genomic structures of site-specific gene replacements were confirmed by Southern transfer analysis.

Analysis of *lpsB* transcriptional organization. Different fragments of the *greA-lpsB* region were subcloned into the mobilizable suicide vectors pK18mob and pK19mob, and the hybrid plasmids were transferred to strain *S. meliloti* 20-B+ (Table 1). Integration of the hybrid plasmids into the genome of the *S. meliloti* by a single crossover was inferred on the basis of an acquisition of the vector-encoded antibiotic resistance. Transconjugants carrying different deletions upstream from the *lpsB-lacZ* transcriptional fusion were assayed for β-galactosidase activity.

Assay for β-galactosidase. β-Galactosidase activity was measured by the *o*-nitrophenyl-D-galactopyranoside method as described by Miller (36) except that the cells were grown on TY medium and permeabilized with 50 μl of chloroform. Bacterial cultures in log phase of growth were collected by centrifugation and resuspended in culture medium, and the concentration of bacteria was adjusted depending on the enzyme activity for each strain. All values were expressed as averages of at least three independent determinations.

Nodulation tests. Surface-sterilized seeds were germinated on water-agar (1.5%, wt/vol). Two-day-old seedlings were transferred to gamma-irradiated sterilized plastic growth pouches (Mega Minneapolis International, Minneapolis, Minn.) containing 10 ml of nitrogen-free Jensen mineral solution, pH 6.7 (27). Three days later, primary roots were inoculated with 10^6 rhizobia by dripping 100 μl of a bacterial suspension onto the root from the tip toward the base. The plants were cultured in a growth chamber at 22°C and a 16-h photoperiod. Four weeks after inoculation, the dry weight of the aereal part of plants inoculated with LPS mutants was compared with that of controls inoculated with the parental strain. The number of nodules per individual plant was examined before harvest.

Nucleotide sequence accession number. The sequence data reported have been deposited in GenBank and assigned accession no. AF193023.

RESULTS

Sequence analysis of the 5.5-kb DNA fragment that complemented *lpsB* mutations. We have previously shown that the *lpsB* mutant *S. meliloti* 6963 carries a Tn5 insertion within the *lpsB* region (41) according to the complementation groups previously established by Clover et al. (15). A 5.5-kb *SstI* restriction fragment recovered from the wild-type strain 2011 of *S. meliloti* and delivered via the plasmid pAL100 (Table 2) was able to complement the mutation in *S. meliloti* 6963 that affected both its LPS structure and symbiotic capacity (34, 41). To elucidate the genetic structure of the *lpsB* complementation group (15), the 5.5-kb *SstI* DNA fragment that complements the mutant *S. meliloti* 6963 was sequenced. A computer-assisted analysis of codon usage resulted in the open reading frame (ORF) structures presented in Fig. 1B. We assigned *lpsB* to an ORF of 1,056 bp by sequencing the DNA region flanking

the Tn5 insertion site within *S. meliloti* 6963. We inferred a putative GTG start codon for *lpsB* on the basis of a likely ribosome-binding site located 8 bp upstream from that triplet. The deduced gene product for LpsB corresponds to a polypeptide of 351 amino acids with a molecular mass of 38.6 kDa. We detected no hydrophobic segments that could form transmembrane helices within the protein. The complete predicted sequence for LpsB showed strong homology to the *lpcC*-encoded mannosyltransferase required for LPS core biosynthesis in *R. leguminosarum* bv. viciae (accession no. AAC05215; BLAST identities = 195/348, 56%; positives = 240/348, 69%) (28). We also found moderate homology of the central region of *lpsB* (amino acid sequence identity of between 24 and 33%) to many glycosyltransferases related to the biosynthesis of LPSs and of other surface polysaccharides (i.e., the RfbU-related protein from *Methanobacterium thermoautotrophicum* [AAB84956], EpsG from *Streptococcus thermophilus* [U40830], the RfbU protein homolog from *Methanococcus jannaschii* [F64500], the RfaK α-1,2-*N*-acetylglucosamine transferase from *Neisseria meningitidis* [AAC44648], and the IcsA LPS glycosyltransferase from *N. meningitidis* [AAC45156]).

A second putative ORF, of 477 bp and with high coding probability, was located 82 bp upstream from *lpsB*. Within the first 21 bp, this ORF contains two potential in-frame ATG start codons; between them, also in-frame, is a GTG codon. The two ATG are preceded by highly likely ribosome-binding sites. The first of these codons would give rise to a deduced amino acid sequence corresponding to a polypeptide of 158 amino acids with a calculated molecular mass of 17.5 kDa. We provisionally designated this ORF *greA* because the predicted translation product showed high homology to the GreA transcriptional factors from several organisms, including *R. leguminosarum* bv. viciae (AAC05214; here at 77% identity). A global sequence comparison among known GreA proteins indicated the presence of two consensus prokaryotic transcription elongation factor signatures, Prosite PS00829 and PS00830. Both of these sequence motifs are present in all GreA proteins, including the gene product from *S. meliloti*.

Downstream from *lpsB* and transcribed in the opposite direction, three complete ORFs were identified: *lpsE* (1,023 bp), *lpsD* (1,032 bp) (this work), and the previously defined *lpsC* (918 bp) (15) (Fig. 1A). Restriction enzyme analysis of a DNA fragment that complemented *lpsB* and *lpsC* mutants had previously shown that these two genes mapped 2 to 3 kb apart (15). Analysis of the DNA region flanking the Tn5 insertion in mutant *S. meliloti* 7555 (Table 1) allowed us to assign *lpsC* to the rightmost of the three sequential ORFs. The predicted translation product of the 918-bp *lpsC* (305 amino acids, 34.4 kDa) displays significant homology to the β-1, 4-glycosyltransferases from *N. meningitidis* (AAC44647; 25% sequence identity over 259 amino acids) and *Aquifex aeolicus* (AAC07593; 23% sequence identity over 254 amino acids). Mutations in this gene have been previously reported to lead to changes in LPS structure (15). The predicted translation products of *lpsE* (340 amino acids, 37.1 kDa) and *lpsD* (343 amino acids, 38.7 kDa) exhibit striking sequence resemblance to one another (53%). The 2-bp overlapping sequence (**TG**) between the **ATG** start codon of *lpsE* and the **TG**A termination codon of *lpsD* suggests the presence of a possible translational coupling between the two putative genes. Sequence comparison of the translation

products of *lpsE* and *lpsD* against the nonredundant protein database from GenBank revealed several partial homologies to proteins involved in the biosynthesis of different bacterial polysaccharides. The higher scores corresponded to a capsular biosynthesis-related protein from *A. aeolicus* (AAC07522; 25% identity over 192 amino acids), to the CAPM protein from *Rickettsia prowazekii* (CAA14871) and to RfbU-related LPS biosynthetic enzymes from *M. jannaschii* (F64500) and *Methanobacterium thermoautotrophicum* (AAB84679) (sequence identities of 21 and 33% over 143 and 293 amino acids, respectively).

Finally, upstream from *lpsC* and in the same coding direction, a truncated ORF homologous to the transcription factor gene *lrp* from several organisms was identified. The missing 5′ region of this *lrp*-like gene was obtained by cloning a portion of DNA lying upstream from the 5′ *Sst*I site and extending to the next chromosomal *Eco*RI site by means of a vector-mediated chromosome-walking strategy (Materials and Methods). Partial sequencing of the recovered fragment showed that 18 bp separated the *Sst*I site from a putative ATG start codon. Sequence analysis reveals the presence of a prokaryotic signature consensus characteristic of the transcription-regulatory proteins of the *asnC* subfamily (Prosite PS00519). Other *lrp* gene homologs that have been cloned in members of the family *Rhizobiaceae*, include those from *Bradyrhizobium japonicum* (AAB49303) (32), *Agrobacterium tumefaciens* (AAC43979) (13), and a more distant putative transcription factor from *Rhizobium* sp. strain NGR234 (P55658). Through sequence comparison we also found the 3′ end of an *lrp* gene homolog from *R. leguminosarum* bv. viciae, located immediately downstream from the reported *dctD* gene (GenBank accession no. Z11529) (Fig. 1) (see below). The 44 amino acids of the C-terminal region of the Lrp protein corresponding to this identified DNA stretch exhibit a 75% sequence identity with the homologous product of *S. meliloti* 2011.

Results from our laboratory have shown that the 5.5-kb *Sst*I fragment presented in Fig. 1 has a striking size conservation in all *S. meliloti* strains from different geographic origins thus far tested (not shown).

Construction and characterization of *lpsD* and *lpsE* mutants. *S. meliloti* strains 20-C, 20-D, and 20-E, carrying nonpolar disruptions within *lpsC*, *lpsD*, and *lpsE*, respectively, were constructed by chromosome integration of the suicide plasmid pK18mob within their respective coding sequences. To avoid polar effects of the mutations, the *lacZ* promoter of the integrated vector pK18mob read downstream of the interrupted coding sequences (it is well known that the *lacZ* promoter is functional in *S. meliloti* [3, 4] [see Fig. 4]). All constructed mutants showed similar LPS phenotypes with a clear shift in their smooth LPS (S-LPS) component (Fig. 2A). In addition, a new rough LPS (R-LPS) component with high SDS-PAGE mobility was observed in the *lpsC, lpsD*, and *lpsE* mutants (Fig. 2B). The observed changes in the R-LPS patterns were all intermediate between the wild type and the *lpsB* mutant previously reported (Fig. 2B). The results indicate that the three genes are involved in LPS biosynthesis. Plant inoculation assays using *M. sativa* and *M. truncatula* showed that strains 20-C, 20-D, and 20-E have symbiotic phenotypes similar to that of the wild-type strain 2011, as evaluated through the number of root nodules and the plant dry weight 4 weeks postinoculation
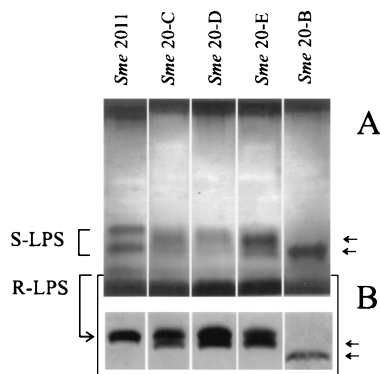
FIG. 2. SDS-PAGE of affinity-purified LPS extracted from *S. meliloti* 2011 and from mutant strains. Each well contained the total LPS extracted from about 10 mg (wet weight) of cells of the indicated bacterial strain, using EDTA and polymyxin B as described in Materials and Methods. (A) SDS-PAGE (18% polyacrylamide); (B) R-LPS components from a gel where increased resolution was achieved by extending the running time. The positions of LPS components that are present only in the mutants are indicated to the left. Genotypes of *S. meliloti* (*Sme*) strains 20-B, 20-E, 20-D, and 20-C are described in Table 1; *Sme* 2011 corresponds to the parental wild-type strain.
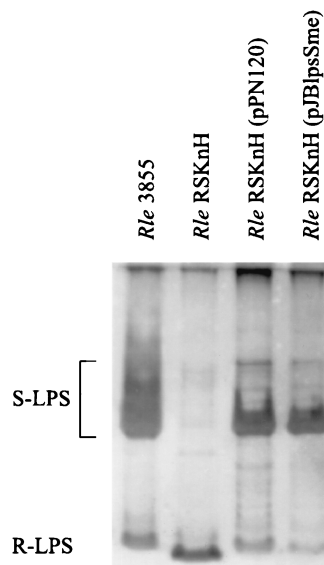

FIG. 3. SDS-PAGE (18% polyacrylamide) analysis showing the genetic complementation of an *lpcC* mutant of *R. leguminosarum* bv. viciae (Rle) by the *lpsB* gene of *S. meliloti*. Lanes: Rle 3855, wild-type strain; Rle RSKnH, *lpcC* mutant; Rle RSKnH (pPN120), *lpcC* mutant carrying a plasmid copy of the wild-type *lpcC* gene; Rle RSKnH (pJBlpsSme), *lpcC* mutant carrying a plasmid copy of the wild-type *lpsB* gene of *S. meliloti* (Table 1). Each well contained the total LPS extracted from about 10 mg (wet weight) of cells of the indicated bacterial strain, using EDTA and polymyxin B as described in Materials and Methods. The positions of LPS components in the gel are indicated to the left.

(not shown). Results presented here and previously (34, 41) implicate *lpsB* as the only *S. meliloti* LPS mutant affected in symbiosis.

Similarities between the gene arrangement of *greA-lpsB* and *lrp* in *S. meliloti* and their homologous loci in *R. leguminosarum* bv. viciae. Comparison of the nucleotide sequence corresponding to the 5.5-kb region presented in this work against the GenBank database revealed that three genes homologous to *S. meliloti lpsB, greA,* and *lrp* are present in *R. leguminosarum* bv. viciae (GenBank accession no. AF050103 and Z11529). These genes are close to each other in both *S. meliloti* and *R. leguminosarum* (Fig. 1). The genes *greA-lpsB* are contiguous and transcribed in the same direction in *S. meliloti*, as is also the case with the homologous genes *greA-lpcC* in *R. leguminosarum*. Moreover, both species of rhizobia have the transcription factor gene *lrp* located comparably in orientation and distance with respect to *greA* within the bacterial chromosome. In *S. meliloti, lrp* maps 3 kb downstream from *lpsB*, on the opposite DNA strand. Likewise, in *R. leguminosarum, lrp* maps 5 kb downstream from *lpcC*, the *lpsB* homolog. Different genes, however, separate *lpsB* from *lrp* in *S. meliloti* and *lpcC* from *lrp* in *R. leguminosarum*. Whereas in *S. meliloti* there are three genes homologous to glycosyltransferase genes (see above) downstream from *lpsB*, in *R. leguminosarum* the *dctA-dctBD* cluster (*dct*, dicarboxylic acid transport) lies downstream from *lpcC*. By contrast, the homologous *dct* cluster in *S. meliloti* has been previously mapped outside the bacterial chromosome within the second symbiotic megaplasmid (61).

Genetic complementation of *lpcC* mutants by *S. meliloti lpsB*. The striking nucleotide sequence similarity between *lpsB* and *lpcC* suggested that these genes could also have comparable sugar transferase activities. To evaluate this possibility, we carried out genetic complementation experiments in which the wild-type *lpsB* and *lpcC* genes were introduced into *R. leguminosarum lpcC* and *S. meliloti lpsB* mutants. Plasmids were introduced into the rhizobial strains by conjugation. Result

presented in Fig. 3 show that when the plasmid-borne *S. meliloti lpsB* gene is introduced into the *lpcC* mutant of *R. leguminosarum* bv. viciae strain RSKnH, the ability to synthesize complete LPS molecules is restored, resulting in an LPS pattern indistinguishable from that of the wild-type strain 3855 (positive complementation). In a reverse experiment, however, introduction of the *R. leguminosarum lpcC* gene into the *S. meliloti lpsB* mutant via the shuttle vector pPN120 did not restore the wild-type *S. meliloti* LPS (not shown). The presence of pPN120 and plasmid pJBlpsSme in the *R. leguminosarum* and *S. meliloti* transconjugants was confirmed by agarose gel electrophoresis.

Transcriptional organization of *lpsB*. To investigate the transcriptional organization of *lpsB*, we integrated nonreplicative plasmids containing progressively 5′-deleted fragments from the *greA-lpsB* region into the genome of *S. meliloti* 20-B+ that carried a *lpsB::lacZ* transcriptional fusion (Fig. 4A and B). β-Galactosidase activities resulting from the *lacZ* transcriptional fusions occurring downstream from the integrated vector were analyzed (Fig. 4C). When the integrated plasmid contained at the 5′ end the 939 bp lying upstream from the putative *lpsB* start codon (plasmid pA2), the β-galactosidase activity of the resulting bacterial derivatives (39.6 Miller units) was similar to that exhibited by the recipient strain *S. meliloti* 20-B+ (32.1 Miller units). With the deleted fragments retaining at the 5′ end the 122 bp upstream from the start codon (plasmid pB2), a similar pattern of enzyme activity was observed (Fig. 4C). Higher activities were obtained with the recombinants carrying integrated plasmids having the *lac* pro-
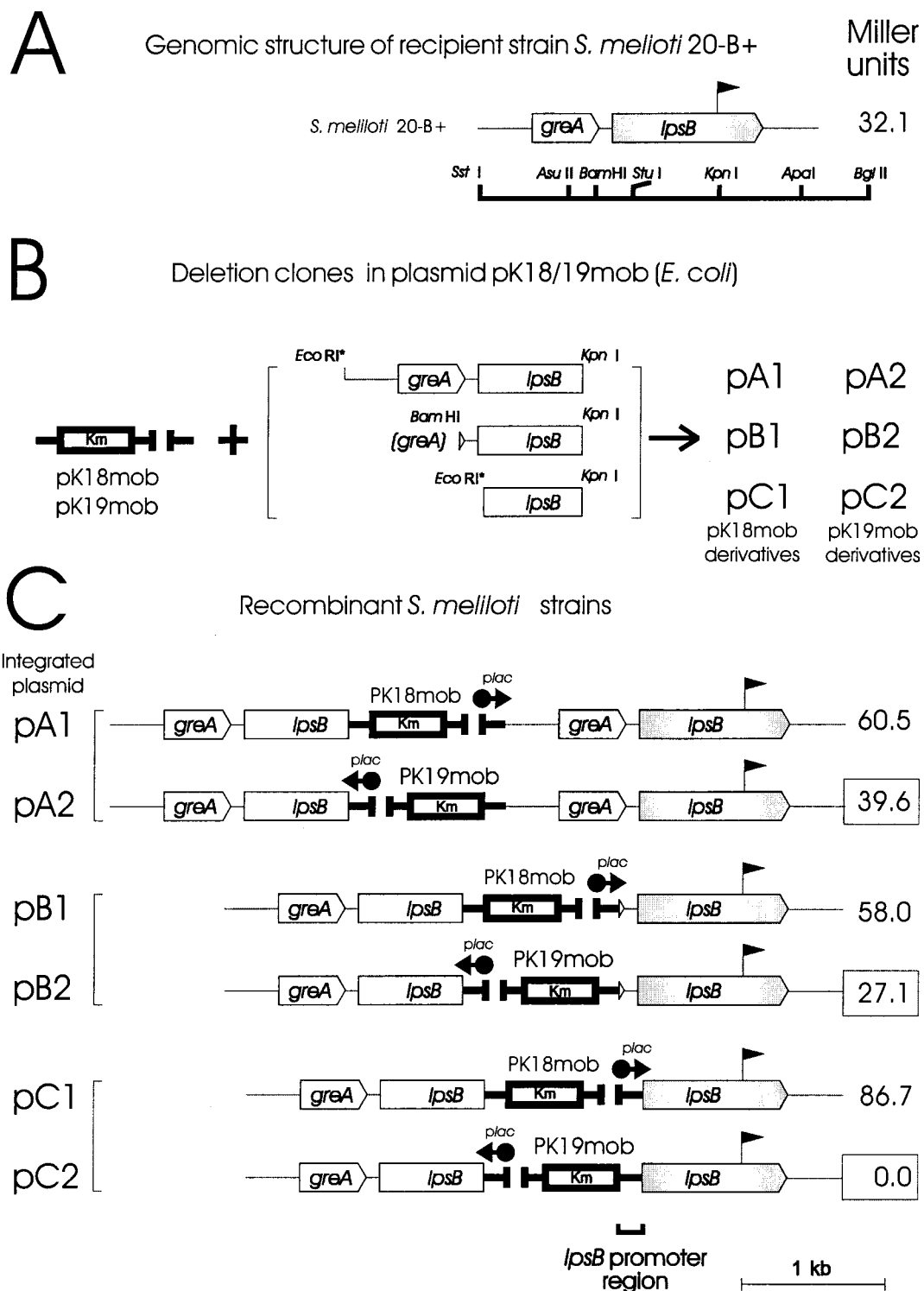
FIG. 4. Schematic representation of constructs used for transcriptional analysis of *lpsB*. (A) The promoterless *lacZ-accC1* cassette (pennant symbol) was inserted in sense orientation in the *Kpn*I site of the *lpsB* gene of *S. meliloti* 2011 to form *S. meliloti* 20-B+ (Table 1). The restriction map of *lpsB* and the flanking regions is shown below. (B) The three *greA-lpsB* deletion clones indicated on the right were subcloned between either the *Eco*RI (subclones A and C) or the *Bam*HI (subclone B) site and the *Kpn*I site within the polylinkers of the suicide plasmids pK18mob and pK19mob, shown on the left. Recombinant plasmids carrying the *lacZ* promoter in sense and antisense orientations relative to the inserts *greA-lpsB* were designated pA1-pB1-pC1 and pA2-pB2-pC2, respectively. (C) The plasmid constructs were integrated into the genome of *S. meliloti* 20-B+ via a single cross-over as described in Materials and Methods to produce the corresponding derivative strains indicated. The β-galactosidase activity (in Miller units) measured in each recombinant is shown to the right. The series 2 strains, having the *lac* promoter (p*lac*) oriented in the reverse direction, are the experimentally informative ones in that the associated enzymatic activity reflects the functioning of any *S. meliloti* endogenous promoters upstream from the cassette. By contrast, those in series 1 constitute positive controls since significant β-galactosidase production would be expected under the direction of the *lac* promoter. Control strain *S. meliloti* 20-B, having the cassette in the reverse (antisense) direction, exhibited only negligible β-galactosidase activity (not shown).

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moter in sense orientation with respect to the *lacZ* cassette (plasmids pA1 and pB1). By contrast, the integration of plasmid pC2, carrying a deletion that included up to a small 5′ portion of the *lpsB* coding region, abolished the linked transcription of *lpsB-lacZ*. Nevertheless, plasmid pC1, containing the vector in the opposite orientation (the positive control), showed the high level of β-galactosidase expression expected for transcription initiated from the vector's upstream *lac* promoter. We thus conclude that a span of 122 bp upstream from the *lpsB* start codon is sufficient to direct transcription of the gene and very likely contains the *lpsB* promoter. Consistent with this possibility is the occurrence of a "nonnitrogen" promoter consensus sequence [TTP$_u$ANN 16–17 bases P$_u$A(P$_u$)$_4$ 3–5 bases CA] (51) between *greA* and *lpsB* immediately upstream from the putative GTG start codon. The location of this consensus sequence gave us a preliminary indication that *greA* and *lpsB* might not be part of the same transcriptional unit. That there is moreover extremely low transcriptional activity of *lpsB* as a result of readthrough from the *greA* promoter is indicated by the small decrement in activity in the recombinant strain carrying plasmid pB2 compared to the control strain *S. meliloti* 20-B+. The use of *S. meliloti* 20-B+ to analyze the expression of *lpsB* in symbiosis showed that the gene is expressed at the root hair curling site, within the infection thread, and within the nodules in the central nitrogen-fixing tissue (not shown).

Presence of DNA sequences homologous to *lpsB* in several bacterial species of the family *Rhizobiaceae*. We have previously suggested that alterations in LPS arising from *lpsB* mutations are likely to be associated with changes in the core region of the molecule (34). Core sugars and their linkages are frequently conserved among related bacteria, in contrast to the highly variable sugar structure of the O antigen. Having established that *lpcC* and *lpsB* are both core-related biosynthetic genes, we used a PCR-hybridization assay to test for the presence of similar sequences in several bacterial species of the family *Rhizobiaceae* (Fig. 5). The PCR primers were designed based on the sequence conservation between *lpcC* and *lpsB* (Materials and Methods). Positive amplification-hybridization corresponding to a PCR product of ca. 267 bp in length was obtained for *Mezorhizobrum ciceri* USDA 3383, *Rhizobium etli* CE3, *R. galegae* USDA 4128, *R. hainensis* USDA 3588, *R. huatlense* USDA 4900, *R. leguminosarum* bv. trifolii ANU843, *R. leguminosarum* bv. viciae VF39, *Rhizobium* sp. strains GRH2 (*Acacia*), LPU83, Or191, and NGR 234, *R. mongolense* USDA 1844, *R. tianshanense* USDA 3592, *R. tropici* CIAT 299, *R. tropici* CIAT 899, *S. fredii* 191, *S. fredii* 257, *S. fredii* HH103, *S. medicae* USDA 1037, *S. meliloti* 2011, *S. saheli* USDA 4893, *S. teranga* USDA 4894, *A. tumefaciens* C58, and *B. japonicum* USDA 110. The results suggest that *lpsB*-homologous genetic loci are most likely present in several species in the family *Rhizobiaceae*.

DISCUSSION

Previous results from our laboratories showed that *lpsB* mutants of *S. meliloti* 2011 exhibited an altered symbiotic capability when inoculated into different species of *Medicago* (34, 41). In this work we have characterized a 5.5-kb *Sst*I DNA fragment which contains the *S. meliloti lpsB* chromosomal gene

(15, 23, 33). In addition to *lpsB*, five other ORFs were identified, including one corresponding to the previously reported *lpsC* gene (15) and two others with sequence similarities to the regions coding for the transcription factors GreA and Lrp, respectively, found in several other gram-negative bacteria (9, 37). Immediately downstream of *lpsB* we have identified two new genes involved in LPS biosynthesis, *lpsE* and *lpsD*; these genes showed partial sequence similarities to bacterial sugar transferase genes, and their nonpolar interruption resulted in LPS modifications as visualized by SDS-PAGE. A previous report by Clover et al. (15) had shown that a Tn5 *lpsC* mutant was not altered in its symbiosis with alfalfa. We showed here that the disruption of neither *lpsD* nor *lpsE* affected symbiosis with *M. sativa* or *M. truncatula*. Current evidence indicates that *lpsB* is the only LPS-associated mutation leading to changes in symbiosis (34, 41). In addition, results presented in this work show that the transcription of *lpsB* appears to be under its own promoter, with little readthrough from the *greA* gene, and that the altered LPS and the defective symbiosis of *lpsB* mutants are both consequences of a primary nonpolar defect in a single gene.

A global sequence comparison of the 5.5-kb DNA region with the GenBank database revealed remarkable similarities between the gene arrangements of *greA, lpsB,* and *lrp* in *S. meliloti* and their homologous loci in *R. leguminosarum* bv. viciae (chromosomal synteny). However, whereas in *R. leguminosarum* the *dctA* and *dctBD* genes were downstream from *lrp*, the unrelated genes *lpsC, lpsD,* and *lpsE* were found in *S. meliloti*. In the latter rhizobium, the *dctA* and *dctBD* genes had been previously mapped on the second symbiotic megaplasmid (61). Sequence analysis also showed that the predicted protein LpsB has a strong homology to the *R. leguminosarum lpcC*-encoded mannosyltransferase required for the LPS core biosynthesis (28, 29). That the two proteins exhibit striking amino acid sequence similarity suggested that they could also have comparable sugar transferase activities. Kadrmas et al. (28) previously reported, however, that membranes from *S. meliloti* when tested in glycosyltransferase assays in vitro possessed very little mannose transfer activity from the donor GDP-mannose to the acceptor Kdo2-lipid IV$_A$ (28). It is likely that the experimental conditions required to detect the core-associated mannosyltransferase activity in cell extracts of *S. meliloti* may not coincide with the optima established for *R. leguminosarum*. In fact, the genetic complementation experiment presented in this work revealed that *S. meliloti lpsB* restored the biosynthesis of complete LPS molecules when introduced into an (otherwise rough) *lpcC* mutant of *R. leguminosarum* bv. viciae. This result strongly supports that the *lpsB* gene product is a core biosynthetic mannosyltransferase. It is worth noting that in a reverse-complementation experiment, the *R. leguminosarum lpcC* gene did not restore a wild-type LPS when introduced into an *lpsB* mutant of *S. meliloti*. One possible explanation for this observation is that *lpcC* is not properly expressed in the genetic background of *S. meliloti*. Alternatively, LpcC may have a stricter substrate specificity than LpsB. To gain further insight into this question, the enzyme activities of LpcC and LpsB should be analyzed in vitro as previously described (28), using homologous and heterologous core biosynthetic substrates.

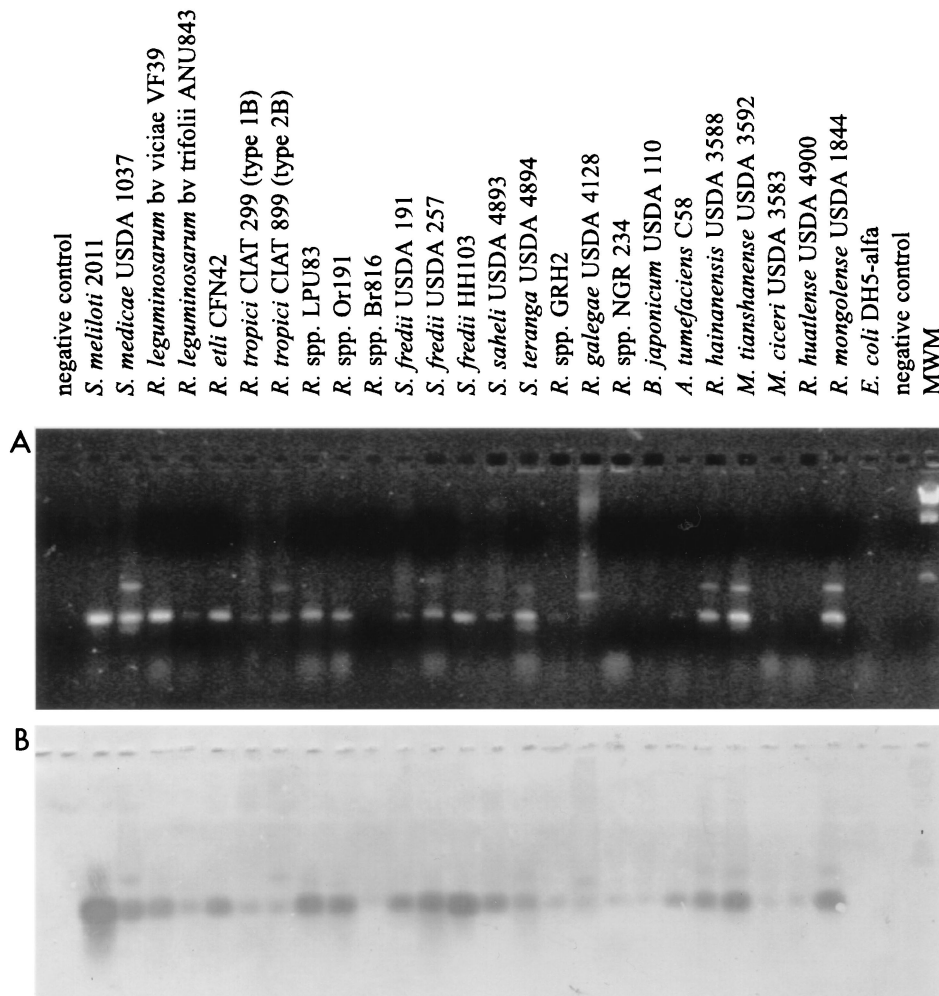Unfortunately, only one LPS structure has been elucidated

FIG. 5. PCR-hybridization analysis of *lpsB*-homologous sequences in various bacteria. (A) A PCR assay was carried out with primers LPSB2F and LPSB2R, designed according to the sequence conservation between *S. meliloti lpsB* and the homologous *lpcC* of *R. leguminosarum* bv. viciae. PCR products were separated on 2% (wt/vol) agarose gels containing 0.5 to 1 μg of ethidium bromide per ml and photographed with a Kodak DC120 digital camera under UV illumination. Strains are indicated above the lanes. MWM, molecular weight marker (lambda phage DNA digested with *Hin*dIII). (B) The PCR products from panel A were analyzed by Southern blot hybridization using a 532-bp digoxigenin-labeled DNA probe which is internal to *lpsB* and contains the amplified region.

in rhizobia (20, 21), and little information is available on conserved core sugars and their linkages among different *Rhizobium* species. The striking conservation of *lpsB* in all *S. meliloti* strains that we have tested suggests that mannose could be a ubiquitous component of the LPS core of these rhizobia. In addition, the detection of *lpsB*-related sequences in several members of the family *Rhizobiaceae* gives rise to the possibility that such sequences could also code for *lpsB*- or *lpcC*-related mannosyltransferases.

With respect to the *lpsB* mutants, there is the possibility that other surface polysaccharides could be affected. Other authors have shown that LPS and KPS have some biosynthetic reactions in common (11, 31). Nevertheless, several observations make it unlikely that KPS is affected in *lpsB* mutants. First, mannose is not present in the KPSs of the *S. meliloti* strains thus far analyzed (48) (the same is true for the EPSs [25, 46]). Second, results from our laboratory showed that *lpsB* mutants derived from *S. meliloti* 41 are sensitive to the KPS-dependent

phage φ16-3. Both observations indicate that *lpsB* mutants have wild-type versions of this polysaccharide.

The principal questions remaining concern the mechanisms underlying the inability of the core-affected *S. meliloti lpsB* mutants to establish fully compatible associations with *Medicago* hosts. In most species of rhizobia, changes in symbiosis associated with LPS alterations occur after the loss or modification of the O antigen (18, 42, 44), topologically the outermost region of the molecule. However, this seems not to be the case for *S. meliloti lpsB* mutants that, being affected in symbiosis, still retain an O antigen linked to the altered core polysaccharide. Previous evidence has also shown that the LPS of *S. meliloti* has several unusual characteristics compared with LPSs from other bacteria. It has been observed, for example, that the R-LPS of *S. meliloti* is the major molecular form of the LPS (48). In addition, a strong immunodominance was associated with the R-LPS components (34, 49), a characteristic that in most other bacteria is associated with the O antigen. It

is worth noting that *lpsB* mutants have lost most of the R-LPS immunoreactivity (34) that is present in the parental strain. However, no relationships have been established between specific epitopes and symbiosis. Studies in this direction are expected to be facilitated by the use of currently available anti-*S. meliloti* LPS core monoclonal antibodies (49).

Current data taken together implicate *lpsB* as the key locus for investigating the participation of *S. meliloti* LPS in symbioses with *Medicago* spp. Further experiments should be aimed at investigating whether the impaired symbiosis of *lpsB* mutants results from the loss of specific chemical groups that have to be sensed by the plant, or whether the plant phenotype is the consequence of secondary, as yet unidentified bacterial modifications that disturb plant penetration.

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