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Transcriptional Regulator LsrB of *Sinorhizobium meliloti* Positively Regulates the Expression of Genes Involved in Lipopolysaccharide Biosynthesis

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Rhizobia induce nitrogen-fixing nodules on host legumes, which is important in agriculture and ecology. Lipopolysaccharide (LPS) produced by rhizobia is required for infection or bacteroid survival in host cells. Genes required for LPS biosynthesis have been identified in several *Rhizobium* species. However, the regulation of their expression is not well understood. Here, *Sinorhizobium meliloti* LsrB, a member of the LysR family of transcriptional regulators, was found to be involved in LPS biosynthesis by positively regulating the expression of the *lrp3-lpsCDE* operon. An *lsrB* in-frame deletion mutant displayed growth deficiency, sensitivity to the detergent sodium dodecyl sulfate, and acidic pH compared to the parent strain. This mutant produced slightly less LPS due to lower expression of the *lrp3* operon. Analysis of the transcriptional start sites of the *lrp3* and *lpsCDE* gene suggested that they constitute one operon. The expression of *lsrB* was positively autoregulated. The promoter region of *lrp3* was specifically precipitated by anti-LsrB antibodies *in vivo*. The promoter DNA fragment containing TN11A motifs was bound by the purified LsrB protein *in vitro*. These new findings suggest that *S. meliloti* LsrB is associated with LPS biosynthesis, which is required for symbiotic nitrogen fixation on some ecotypes of alfalfa plants.

Lipopolysaccharide (LPS) is required for *Rhizobium* infection or survival in host cells. It is composed of O-antigen, core oligosaccharide, and lipid A. LPS contributes to infection thread formation in symbioses between legumes, *Rhizobium etli*, and *R. leguminosarum* bv. trifolii and viciae (1–3). As demonstrated by *Sinorhizobium fredii*, *S. meliloti*, and *Bradyrhizobium japonicum*, bacteroid differentiation in root nodule cells requires appropriate LPS (4–7). LPS suppresses host defenses, enabling rhizobia to infect their hosts successfully and survive long term in host cells (8, 9).

In *S. meliloti*, several LPS biosynthesis genes have been identified, including *lpsB*, *lpsCDE*, *lpsL*, *rkpK*, and *ddhB* (10–14). The *lpsB*, *lpsC*, and *lpsDE* genes encode a type I glycosyltransferase, β-1,4-glycosyltransferase, and RfaG glycosyltransferases, respectively, which are involved in the biosynthesis of the core oligosaccharides. The *lpsL* gene encodes a UDP-glucuronate 5′-epimerase, *rkpK* encodes a UDP-glucose 6-dehydrogenase, and *ddhB* encodes a CDP-glucose 4,6-dehydratase to catalyze the synthesis of CDP-4-keto-6-deoxy–D-glucose from CDP–D-glucose, which is associated with O-antigen biosynthesis. Genetic evidence demonstrates that null mutants of *lpsB*, *lpsC*, *rkpK*, and *lpsL* in the *S. meliloti* 1021 background induce defective nitrogen fixation nodules on some ecotypes of *Medicago sativa* or *Medicago truncatula* A17 (11, 13). Root nodules hosting *lpsB*::Tn*phoA* mutants from *M. sativa* cv. GT-13R$^+$ or Iroquois show premature senescence, in which bacteroid differentiation is blocked at different stages and bacteroids appear to activate host innate immunity (4).

A few regulators involved in LPS biosynthesis have been identified in rhizobial strains. A small periplasmic regulator, SyrA, is coupled with the ExoS/ChvI two-component system to regulate expression of the LPS sulfotransferase gene, *lpsS*, in *S. meliloti* 1021 (12, 15). In *S. fredii* NGR234, the type III secretion system regulator TtsI controls the biosynthesis of rhamnose and a rhamnose-rich component (rhamnan) of LPS (16). In *S. fredii* HH103, the transcription elongation factor GreA could be involved in LPS production, because its null mutant synthesized defective LPS (7). In fact, some *S. meliloti* mutants also affect LPS biosynthesis (11). However, regulation of LPS core biosynthesis genes is not well understood.

LysR regulators constitute a large family of transcriptional factors in prokaryotes and play important regulatory roles in amino acid metabolism (LysR, ArgP, GltC, IlvR/Y, MetR, MtaR, GcvA, and CysB), carbon dioxide fixation (CbbR and CfxR), catechol catabolism (CatR/M), oxidative stress adaptation (OxyR), and bacterium-plant interactions (NodD, SyrM, OccR, NocR, GbpR, and PhcA) (17). As many of the rhizobial genomes have been sequenced, an increasing number of genes encoding LysR family regulators have been annotated. The sequence of the *S. meliloti* 1021 genome was published in 2001, and more than 90 genes encoding LysR regulatory proteins have been predicted (18). In our previous work, 83 genes of strain 1021 were mutated by generating plasmid insertions (19). Two mutants, *lsrA1* and *lsrB1*, induced ineffective nodules on alfalfa (19). Here, an in-frame deletion of *lsrB* (*lsrB1*$^{-2}$) was constructed in *S. meliloti* 1021 to investigate the role of LsrB in symbiosis. We found that LsrB positively regulates the expression of the *lrp3-lpsCDE* operon involved in LPS biosynthesis.

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TABLE 1 Strains and plasmids used in this study

Strain/plasmid	Relevant property(ies)	Reference/source
Strains		
E. coli DH5α	F$^-$ φ80*lacZ*ΔM15Δ(*lacZYA-argF*)*U169 deoR recA1 endA1hsdR17* (r_K^-, m_K^+) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	Laboratory stock
E. coli MT616	MM294 pRK600, Cm^r	20
E. coli BL21(DE3)	F$^-$ *dcm ompT hsdS*(r_B^- m_B^-) *gal* λ(DE3), Km^r	Laboratory stock
S. meliloti 1021 or Rm1021	SU47 but also *str-2*, Sm^r	21
S. meliloti lsrB1^{-2}	Rm1021 Δ*lsrB*, Sm^r	This study
Plasmid		
pK18mobsacB	Suicide vector, Km^r	22
pLMG1	pK18mobsacB carrying a 1,600-bp joint DNA fragment from *S. meliloti trxB* and SMc01226, Km^r	This study
pSRK-Tc	Expression vector under the control of *lac* promoter, Tc^r	23
pLMG2	pSRK-Tc carrying an *S. meliloti lsrB* gene, Tc^r	This study
pRG960	pRG930 containing the promoterless *gusA* with the start codon, Sm^r/Sp^r	24
pLMG3	pRG960 carrying the 300-bp DNA fragment upstream of *lsrB*, Sp^r	This study
pLMG4	pRG960 carrying the 300-bp DNA fragment upstream of *trxB*, Sp^r	This study
pLMG5	pRG960 carrying the 300-bp DNA fragment upstream of *lrp3*, Sp^r	This study
pLMG6	pRG960 carrying the 300-bp DNA fragment upstream of *lpsC*, Sp^r	This study
pMD-18T	Cloning vector, Ap^r	TaKaRa
pLMG7	pMD-18T carrying an *S. meliloti lsrB* gene, Ap^r	This study
pET28b	Expression vector carrying His tags, Km^r	Novagen
pLMG8	pET28b carrying an *S. meliloti lsrB* gene, Km^r	This study

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* DH5α, MT616 (20), and BL21(DE3) were grown in Luria-Bertani (LB) medium at 37°C (Table 1). *S. meliloti* 1021 (21) and the *lsrB1*$^{-2}$ mutant (Table 1) were grown in LB medium supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ (LB/MC) at 28°C (25). The following antibiotics were used: chloramphenicol, 10 μg ml^{-1}; neomycin, 200 μg ml^{-1}; kanamycin, 25 μg ml^{-1}; spectinomycin, 100 μg ml^{-1}; tetracycline, 10 μg ml^{-1}; and streptomycin, 500 μg ml^{-1}.

Construction of an *lsrB* in-frame deletion mutant. Two 800-bp DNA fragments from the proximate regions of the *lsrB* open reading frame (ORF) on the genome of *S. meliloti* 1021 were amplified using KOD plus DNA polymerase (Toyobo, Osaka, Japan). Both PCR products were purified using the gel extraction system B kit (Biodev-Tech, Beijing, China). The purified PCR products (1:1) were employed to amplify a 1.6-kb joint fragment using TransStart *Taq* DNA polymerase (Transgen, Beijing, China). All four primers (P1 to P4) are listed in Table 2. The purified PCR product was digested by EcoRI and XbaI (TaKaRa, Dalian, China) at 37°C overnight. The digested DNA then was purified using the EasyPure PCR purification kit (Transgen, Beijing, China) and cloned into pK18mobsacB (22) with T4 DNA ligase (TaKaRa, Dalian, China) overnight at 16°C. The plasmid then was transformed into *E. coli* DH5α competent cells. The plasmid, named pLMG1 (Table 1), was purified using the EasyPure plasmid miniprep kit (Transgen, Beijing, China) and identified by EcoRI and XbaI digestion. It then was transferred into *S. meliloti* 1021 by triparental mating with the helper strain *E. coli* MT616 (20). Streptomycin- and neomycin-resistant colonies were spread onto LB/MC agar plates containing 5% sucrose and streptomycin. The resulting colonies were screened for sensitivity to neomycin and identified by PCR. The PCR product was sequenced (Invitrogen, Shanghai, China). The confirmed *S. meliloti* mutant was named the *lsrB1*$^{-2}$ mutant. The *lsrB* ORF also was amplified using the P5 and P6 primers (Table 2), and the DNA was digested with NdeI and XbaI and cloned into pSRK-Tc (23). The recombinant plasmid pLMG2 (Table 1) was used to complement the *lsrB1*$^{-2}$ mutant.

LPS assay. *S. meliloti* cells were collected from LB/MC cultures (optical density at 600 nm [OD_{600}], ≈0.8). The crude LPS was extracted with both hot phenol and boiled methods and then treated by RNase, DNase I, and protease K (4, 26). The sulfate-anthrone method was performed to quantify the total sugar content of dialyzed LPS extract (27). The crude extract (with 0.5 μg of total protein) was used for DOC-PAGE (deoxycholate polyacrylamide gel electrophoresis) analysis (11).

5′-RACE. In order to determine the transcriptional start sites of the *lsrB*, *trxB*, *lrp3*, and *lpsC* genes, RNA samples were isolated from *S. meliloti* 1021 using a TransZol plant kit (Transgen, Beijing, China). Random amplification of cDNA ends was performed using a 5′ full rapid amplification of cDNA ends (RACE) kit (TaKaRa, Dalian, China). The cDNA then was amplified by PCR using a 5′-RACE anchor outer primer (OutRAP) together with the nested outer primer of one target gene (out*lsrB*). Subsequently, a nested PCR was performed using the PCR products diluted 20-fold, obtained as described above with the inner primers of one target gene (in*lsrB*) and a 5′-RACE anchor inner primer (InRAP) (Table 2). The PCR amplification cycling protocol consisted of a predenaturing step at 95°C for 5 min and 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min, followed by a final extension step at 72°C for 10 min. The corresponding P33 to P42 primers are listed in Table 2. The resulting PCR products were sequenced. The transcriptional start sites were determined by DNA sequence analysis.

RNA extraction and RT-PCR. Total RNA for reverse transcription was isolated from cells grown to log phase (OD_{600}, ~0.8) in LB/MC medium using a TransZol plant kit (Transgen, Beijing, China). RNA quality was assayed by 1% agarose gel electrophoresis, and the RNA then was treated with RNase-free DNase (TakaRa, Dalian, China) for 30 min at 37°C to digest the remaining genomic DNA, which then was analyzed by PCR using multiple pairs of primers. The RNA preparations were used to synthesize cDNAs with a Primerscript RT Master Mix Perfect real-time kit (TakaRa, Dalian, China), and the first-strand cDNA was used as a template for PCR amplification. Real-time quantitative RT-PCR (qRT-PCR) was performed using SYBR green supermix (Toyobo, Osaka, Japan). The quantitative PCR system (20 μl) consisted of the following components: 10 μl of SYBR green real-time PCR master mix, 0.5 μl of each primer, 1 μl of the diluted cDNA sample, and 9 μl of sterile water. All readings were performed in triplicate using a real-time PCR system (Bio-Rad, Hercules, CA). The program consisted of a denaturing cycle at 95°C for 3 min; 40 cycles comprising 95°C for 10 s, 62°C for 30 s, and 72°C for 30 s; and a final

TABLE 2 Primers[a]

Name	Sequence	Purpose
P1	5′-G*GAATTC*CGACCTCGTAACCGATGTCGA-3′	Construction of the *lsrB* deletion mutant
P2	5′-AAAGGCTCAGCCGGAGAAGC-3′	Construction of the *lsrB* deletion mutant
P3	5′-CTCCGGCTGAGCCTTTCGATCCGTTCAATTGCCCG-3′	Construction of the *lsrB* deletion mutant
P4	5′-GC*TCTAGA*GCACGGCGAGATGAGATGAAA-3′	Construction of the *lsrB* deletion mutant
P5	5′-GGAATTC*CATATG*GGGGGATTCTATGTCGCTGGACT-3′	Cloning of *lsrB* gene
P6	5′-GC*TCTAGA*TCAGAAGTTCCAGTTTCTCGCTTTA-3′	Cloning of *lsrB* gene
P7	5′-TGCA*CTGCAG*CCGGCGACGTGACCGAC-3′	Cloning of *lsrB* promoter
P8	5′-CG*GGATCC*GGGCGTGCCGATGAAAGA-3′	Cloning of *lsrB* promoter
P9	5′-TGCA*CTGCAG*TTGCCCAAATCCCCAATCCT-3′	Cloning of *trxB* promoter
P10	5′-CG*GGATCC*GCTTTTGTCCTTCGTCCGCC-3′	Cloning of *trxB* promoter
P11	5′-CG*GGATCC*TTGCCCAAATCCCCAATCCT-3′	Cloning of *lrp3* promoter
P12	5′-TGCA*CTGCAG*GCTTTTGTCCTTCGTCCGCC-3′	Cloning of *lrp3* promoter
P13	5′-TGCA*CTGCAG*ATTTTGTTATTGAAGAACTGACCG-3′	Cloning of *lpsC* promoter
P14	5′-CG*GGATCC*GAGACTCTCGATGCACTTGCC-3′	Cloning of *lpsC* promoter
P19	5′-TCCGGAAATCCAGGTACAGC-3′	qRT-PCR of *lsrB*
P20	5′-ATGCGATATGCGCGGAT-3′	qRT-PCR of *lsrB*
P21	5′-TCAGAAAGGGACCGACCTCTT-3′	qRT-PCR of *lpsB*
P22	5′-CCAGTCGGGAATATTCGTGTG-3′	qRT-PCR of *lpsB*
P23	5′-GCCTATCTCGGCAAGTGCAT-3′	qRT-PCR of *lpsC*
P24	5′-GATGTTGAAGCACCAGGGCT-3′	qRT-PCR of *lpsC*
P25	5′-ATCTTTGGCTGCTTGGCGA-3′	qRT-PCR of *lpsE*
P26	5′-ATGAACCATTGCGGCCCTT-3′	qRT-PCR of *lpsE*
P27	5′-ATCGTGCGCTTCTGAATGC-3′	qRT-PCR of *lrp3*
P28	5′-TCCTGAAAGCTCGCAAGGTT-3′	qRT-PCR of *lrp3*
P29	5′-AAGGTGATCTGGGACCACGA-3′	qRT-PCR of *trxB*
P30	5′-GCGAAGTTTGCCCTTGAAGA-3′	qRT-PCR of *trxB*
P31	5′-GATCGTCATGTAGCGCAGGA-3′	qRT-PCR of *rpsF*
P32	5′-CCTCGCTCGGCAGGACAT-3′	qRT-PCR of *rpsF*
P33	5′-CATGGCTACATGCTGACAGCCTA-3′	5′-RACE of outRAP
P34	5′-CGCGGATCCACAGCCTACTGATGATCAGTCGATGGAAA-3′	5′-RACE of inRAP
P35	5′-TTGTCGAGGATGAGCTGTAC-3′	5′-RACE of *lsrB*
P36	5′-TTGGCTTGTCCGTCGTCT-3′	5′-RACE of *lsrB*
P37	5′-AAGGTCGGCTCGGTTTCGAT-3′	5′-RACE of *trxB*
P38	5′-GGTTACGAGGTCGTTGAC-3′	5′-RACE of *trxB*
P39	5′-AAAGTCCGATTCCCCGGA-3′	5′-RACE of *lrp3*
P40	5′-TTGAGATTGGCGTCGGACTG-3′	5′-RACE of *lrp3*
P41	5′-CGATCTCGTCTGATGCTGC-3′	5′-RACE of *lpsC*
P42	5′-TACATGAAACGGATGGGCCA-3′	5′-RACE of *lpsC*
P43	5′-TGGGACAAACTGCGCATTT-3′	RT-PCR of *lsrB*
P44	5′-GCTTTCGAGCTTCATCAGCAC-3′	RT-PCR of *lsrB*
P45	5′-ACATCCGACATGGCAATCG-3′	RT-PCR of *trxB*
P46	5′-AGGTATTTCTCCGCTTCGAGC-3′	RT-PCR of *trxB*
P47	5′-ATCGTGCGCTTCTGAATGC-3′	RT-PCR of *lrp3*
P48	5′-TCCTGAAAGCTCGCAAGGTT-3′	RT-PCR of *lrp3*
P49	5′-GCCTATCTCGGCAAGTGCAT-3′	RT-PCR of *lpsC*
P50	5′-GATGTTGAAGCACCAGGGCT-3′	RT-PCR of *lpsC*
P51	5′-CATATTCACTTCGGCACGGAG-3′	RT-PCR of *lpsD*
P52	5′-GCAACTGCCAGGCCATTATG-3′	RT-PCR of *lpsD*
P53	5′-CAAGGTTTTTGTGCATGCAACTGC-3′	EMSA
P54	5′-GTTCCAAAAACACGTACGTTGACG-3′	EMSA

[a] Italics represent restriction endonuclease sites.

step in which the temperature was elevated on a gradient from 65°C to 95°C to dissociate the double-stranded DNA products. The primers P19 to P32 were used for amplification of *lsrB*, *lpsB*, *lpsC*, *lpsE*, *lrp3*, *trxB*, and *rpsF* fragments (listed in Table 2). The transcripts of *trxB*, *lsrB*, *lrp3*, *lpsC*, and *lpsD* were amplified with the primers P43 to P52 (Table 2).

Construction of promoter-*GUS* fusion and GUS activity assay. DNA fragments of approximately 300 bp, containing the *lsrB*, *trxB*, *lrp3*, and *lpsC* promoter regions, were amplified with the P7-P8, P9-P10, P11-P12, and P13-P14 primer pairs, respectively (Table 2). The amplicons were digested with BamHI, SmaI, or PstI and cloned into pRG960 (24), yielding the recombinant plasmids pLMG3, pLMG4, pLMG5, and pLMG6, respectively (Table 1). To assay β-glucuronidase activity, a rhizobial strain carrying an empty or a recombinant plasmid was grown in LB/MC medium to an OD_{600} of ~0.8, and cells were collected by centrifugation at 6,000 × *g* for 2 min (4°C). The cells were crushed with a pestle fitted to an electric drill (J1Z-GL-10; 220 V, 50 Hz, 400 W; Modong Company, Shanghai, China) for 30 s three times on ice. The cell lysate was retained on ice to analyze β-glucuronidase activity with the substrate of *p*-nitrophenyl-β-D-glucuronide (Sigma, USA) as described by Jefferson et al. (28).

Expression, purification of LsrB protein, preparation of anti-LsrB antibodies, and immunoblotting. The full-length *lsrB* ORF was amplified with the P17 and P18 primers (Table 2), purified, and ligated into pMD-18T (TaKaRa, Dalian, China). The foreign DNA was sequenced (Invitrogen, Shanghai, China). The correct plasmid was digested by SalI and NotI, and the released 900-bp DNA fragment was recycled and cloned into pET28b (+) (Novagen, Darmstadt, Germany) to construct a plasmid, pLMG8 (Table 1), which was used to transform competent cells of *E. coli* BL21(DE3)pLysS. This strain was cultured in 5 ml LB broth with kanamycin at 37°C overnight and then subcultured in 100 ml LB-kanamycin broth with 4 mg/ml isopropyl-β-D-thiogalactopyranoside (IPTG) at 28°C for 12 h. The bacterial cells were collected by centrifugation at 6,000 × *g* for 10 min (4°C) and washed once with 50 ml phosphate-buffered saline (PBS). The bacterial cells then were frozen with liquid nitrogen, ground to a powder, and resuspended in 10 ml PBS. The suspension was sonicated with a TY92-II sonifier (Scientz, Hangzhou, China) at a power of 400 W for 30 min in 10-s pulses. The supernatant was collected by centrifugation at 10,000 × *g* for 10 min and then loaded on a nickel-nitrilotriacetic acid (Ni-NTA) column at 4°C according to the Ni-NTA purification system procedure (Invitrogen, Shanghai, China). The eluted protein was analyzed using SDS-PAGE. The purified LsrB protein was diluted with 20% glycerol and stored at −80°C. The protein then was used to prepare anti-LsrB rabbit antibodies (Willget, Shanghai, China). Western blotting was carried out as previously described (29). The total cellular proteins were separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Pharmacia, Stockholm, Sweden). LsrB protein was detected with the prepared anti-LsrB specific polyclonal primary antibodies and the secondary antibody, goat anti-rabbit IgG-HRP (horseradish peroxidase), from Abmart (Shanghai, China). The blots were stained with a 3,3′,5,5′-tetramethylbenzidine (TMB)-stabilized substrate for HRP (Promega, Madison, WI).

ChIP. Chromatin immunoprecipitation (ChIP) was performed as described by Grainger et al. (30). In brief, 150 μl of formaldehyde was added to 5-ml volumes of fresh cultures (OD_{600}, ~0.8) of *S. meliloti* 1021 and the *lsrB1*$^{-2}$ mutant. Cells were shaken for 10 min at 28°C. Cells were pelleted from 1.5 ml of culture, washed twice with 1.5 ml of PBS (pH 7.4), frozen in liquid nitrogen, and then crushed with a pestle attached to the electric drill for 30 s, three times, on ice. The cell lysate was suspended in 5 ml of IP buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and then sonicated with a TY92-II sonifier at a power of 300 W for 20 min in 5-s pulses. After centrifugation at 4°C for 10 min, 100 μl of supernatant was stored at −20°C as a positive control for PCR. A 1-μl volume of anti-LsrB antibody was added to 4.9 ml of sonicated supernatant and the mixture shaken gently at room temperature for 1 h, and then 25 μl of IgA-agarose beads (Abmart, Shanghai, China) was added. Samples were shaken gently at room temperature for 1 h. Beads were collected by centrifugation at 13,000 × *g* for 1 min and washed five times with IP buffer and then twice with TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). Agarose beads were suspended in 95 μl of TE buffer and 5 μl of 2% SDS and then incubated at 65°C overnight for reverse cross-linking. The sample was used as a PCR template to amplify the *S. meliloti lrp3* promoter and *rpsF* (encoding a ribosome protein as a negative control) DNA fragments, using the primers P11 and P12 as well as P31 and P32 (Table 2), respectively.

Electrophoretic motility shift assay (EMSA). The oligonucleotides containing TN11A boxes (P53 and P54) (Table 2) of *lrp3* were synthesized by Invitrogen (Shanghai, China) and 3′ labeled using a DIG (digoxigenin) gel shift kit (Roche, Rotkreuz, Switzerland). The gel shift test of the LsrB-TN11A box was performed according to the manufacturer's protocol for the kit as well.

DNA sequence analysis. The deduced protein sequence of LsrB was downloaded from the *S. meliloti* genome site (http://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi). Homologs of LsrB were aligned and downloaded from the NCBI homepage using the BLAST microbial genome program (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi).

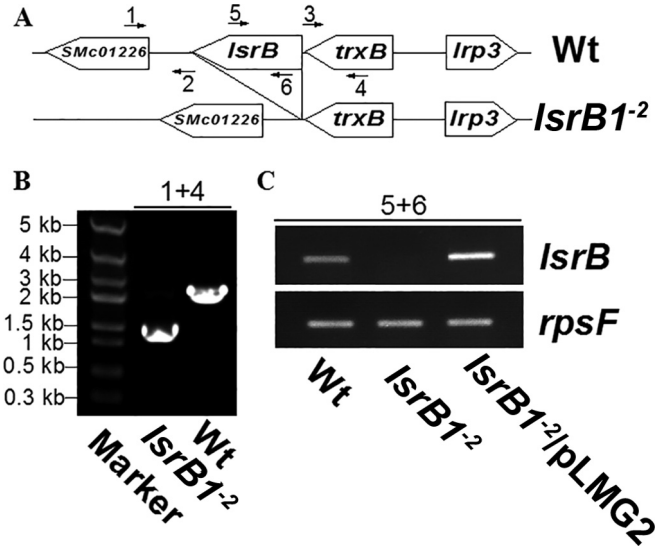

FIG 1 Verification of the constructed *S. meliloti lsrB* deletion mutant (*lsrB1*$^{-2}$ mutant). (A) Genomic organization of the wild type (Wt; *S. meliloti* 1021) and the *lsrB1*$^{-2}$ mutant. (B and C) The *lsrB1*$^{-2}$ mutant was confirmed using PCR (B) and RT-PCR (C). The numbers 1 to 6 indicate primers used for PCR.

The putative promoter DNA sequences were downloaded from the *S. meliloti* genome server, and potential promoters were predicted using the BDGP program (http://www.fruitfly.org/seq_tools/promoter.html). The BLAST nucleotide program from NCBI was used to analyze the DNA sequences.

RESULTS

Construction of an *S. meliloti lsrB* deletion mutant. In our previous work, a plasmid insertion mutant of the *S. meliloti lsrB* (*lsrB1*) strain showed apparent symbiotic deficiency (19). To exclude a polar effect of *lsrB1*, an in-frame deletion mutant was constructed and named the *lsrB1*$^{-2}$ mutant (Fig. 1A). *Sinorhizobium* genomic DNA was extracted, and PCR showed that a small DNA fragment (1.5 kb) was specifically amplified using the *lsrB1*$^{-2}$ mutant genomic DNA as a template, but a large DNA fragment (2.4 kb) was detected using genomic DNA from *S. meliloti* 1021, the parent strain (Fig. 1B). Sequencing both DNA fragments showed the absence of the *lsrB* open reading frame in the PCR fragment obtained from the *lsrB1*$^{-2}$ mutant. This result was confirmed by RT-PCR experiments (Fig. 1C). Primers P1 and P4 allowed the amplification of an internal fragment of the *lsrB* ORF in both the wild-type and the *lsrB1*$^{-2}$ complemented strain but not in the *lsrB1*$^{-2}$ mutant strain.

The *S. meliloti lsrB1*$^{-2}$ mutant was sensitive to acidic pH and SDS. The growth of the *lsrB1*$^{-2}$ mutant was analyzed under different conditions. First, this mutant grew more slowly than the wild-type strain in a complex medium (LB/MC, pH 7.0) (Fig. 2A). Second, LB/MC medium at different pHs was used to assay sensitivity to acidic pH. The growth curve showed that the cell density of the *lsrB1*$^{-2}$ mutant was clearly decreased in the medium at pH 6.0 and pH 6.5 compared to the density at pH 7.0 (Fig. 2A), indicating that this mutant was more sensitive to acidic pH. Third, when 0.1% SDS was added to the medium, the growth of *lsrB2* was inhibited (Fig. 2B), suggesting that the mutant was sensitive to detergents. The expression of the *lsrB* gene from the complementation plasmid, pLMG2, rescued the growth deficiency of the mu-

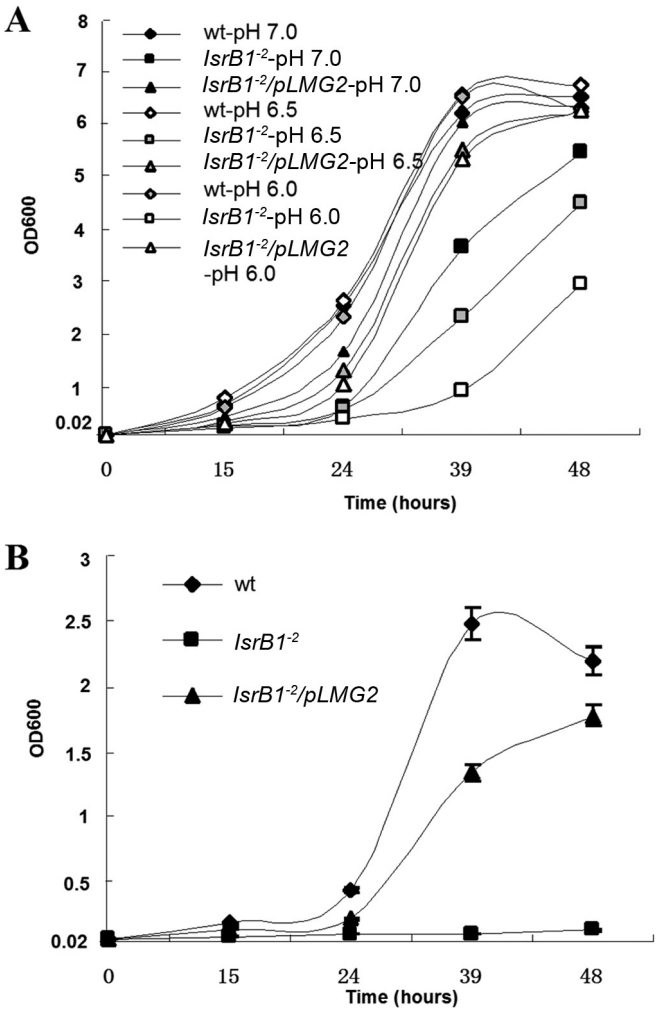

FIG 2 Growth of *S. meliloti lsrB1*$^{-2}$ mutant under different stress conditions. (A) Sensitivity of the *lsrB1*$^{-2}$ mutant to low pH in LB/MC broth; (B) sensitivity of the *lsrB1*$^{-2}$ mutant to 0.1% SDS in LB/MC broth. All data were derived from three independent experiments and are expressed as means ± standard deviations (SD).

tant under different conditions (Fig. 2A and B). These results suggested that the envelope of the *lsrB1*$^{-2}$ mutant was defective.

The *S. meliloti lsrB1*$^{-2}$ mutant produced slightly less LPS. Crude LPS from the *S. meliloti lsrB1*$^{-2}$ mutant, the complemented mutant, and the parent strain was extracted with both the hot phenol and boiling methods. Both LPS extraction methods gave similar results. Total sugar in the LPS extracts was quantified using the sulfate-anthrone method. The results showed that the level of LPS from the *lsrB1*$^{-2}$ mutant had a decreasing trend compared to the wild-type strain and the complemented mutant, although the difference was not very significant according to a Student *t* test. When DOC-PAGE was used to further analyze the LPS constituents, the *lsrB1*$^{-2}$ mutant showed the same types of smooth and rough LPSs, but decreased levels were observed compared to those of the other two strains (Fig. 3). Taken together, these results indicated that LPS production was slightly reduced in the *lsrB1*$^{-2}$ mutant and suggested that the *S. meliloti* LsrB protein was a positive regulator of LPS biosynthesis.

Identification of *lrp3-lpsCDE* and *trxB-lsrB* operons. Since a

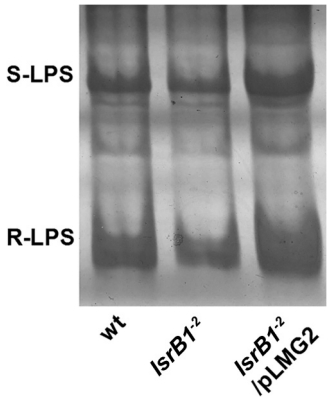

FIG 3 LPS produced by the *S. meliloti lsrB1*$^{-2}$ mutant. LPS was assayed by DOC-PAGE. LPS was extracted from different *S. meliloti* strains that were grown in LB/MC broth (OD_{600}, ~0.8) with the boiled method. S-LPS and R-LPS represent smooth-LPS and rough-LPS, respectively.

typical *lysR* gene often is located divergent from its target genes (16), the location and organization of *S. meliloti lsrB* and its adjacent genes were analyzed. In the genome of *S. meliloti* 1021, *lsrB* is adjacent to *trxB*, which is located divergent from *lrp3* and *lpsCDE*. To test the possibility that *lrp3-lpsC* and *trxB-lsrB* genes consist of two operons, the transcriptional start sites of *lsrB*, *trxB*, *lrp3*, *lpsC*, and *lpsD* were mapped using 5′-RACE. Each of these genes (except *lpsD*) is transcribed from its own transcriptional start site (Fig. 4A), indicating that each gene has its own promoter. To determine whether they are cotranscribed from one operon, their transcripts were analyzed by RT-PCR. The results showed that transcripts containing both *lrp3* and *lpsCD* were detected, just as *trxB* and *lsrB* were (Fig. 4B). These results suggest that *lrp3* and *lpsCDE* constitute one operon but *trxB* and *lsrB* constitute another.

The expression of *lpsCDE* was positively regulated by *S. meliloti* LsrB. Transcription of *trxB*, *lrp3*, *lsrB*, and the LPS core biosynthesis genes (*lpsB*, *lpsC*, and *lpsE*) was evaluated by qRT-PCR. The results showed that the transcript levels for *lrp3*, *lpsC*, and *lpsE* were significantly decreased in the *lsrB1*$^{-2}$ mutant (only 1/53, 1/13, and 1/12 of the level for the wild type), whereas those of *trxB* and *lpsB* remained unchanged from those of the wild-type strain. A ribosome gene, *rpsF*, was used as an internal standard (Fig. 5A). These results suggested that LsrB regulates the expression of *lrp3*, *lpsC*, and *lpsE*. To confirm this, four promoter DNA fragments were predicted by the BDGP program together with the deter-

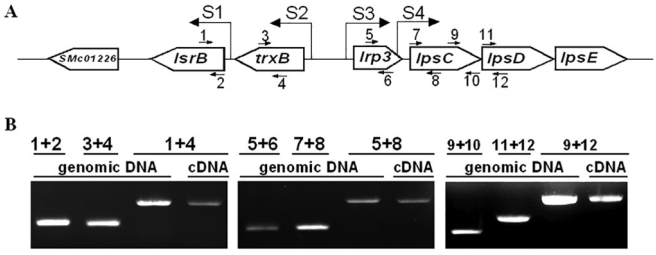

FIG 4 Transcripts and transcriptional start sites of *lsrB*, *trxB*, *lrp3*, *lpsC*, and *lpsD*. (A) Transcriptional start sites (S1 to S4) of *lsrB*, *trxB*, *lrp3*, and *lpsC* mapped by 5′-RACE are located at the nucleotide positions of 1700152, 1701200, 1701425, and 1701947 on the *S. meliloti* genome. TSSs of *lsrB* and *trxB* are almost consistent with published data (35). (B) The transcripts of the genes were determined by RT-PCR using the primers 1 to 12 and the template of genomic DNA and cDNA.

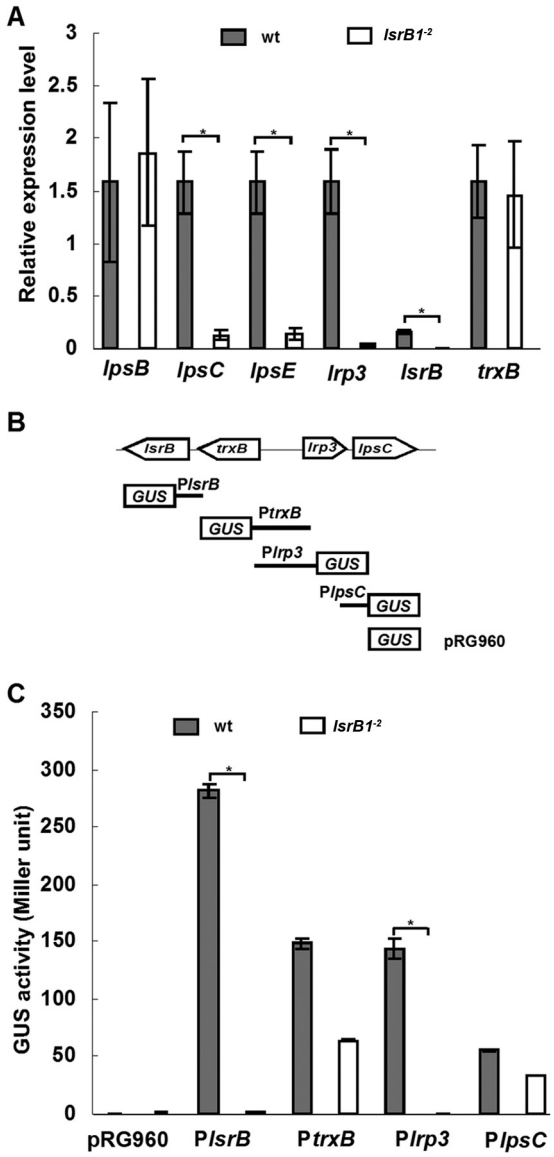

FIG 5 Expression of *lpsC*, *lpr3*, *trxB*, and *lsrB* genes in *S. meliloti* under free-living conditions. (A) Expression of LPS core biosynthesis genes (*lpsB*, *lpsC*, and *lpsE*) detected by qRT-PCR, with the internal standard of *rpsF*; (B) construction of promoter-*GUS* fusions; (C) β-glucuronidase activity of promoter-*GUS* fusions under free-living conditions. All data were derived from three independent experiments and are expressed as means ± SD. The *S. meliloti* strains were grown in LB/MC broth (OD_{600}, ~0.8).

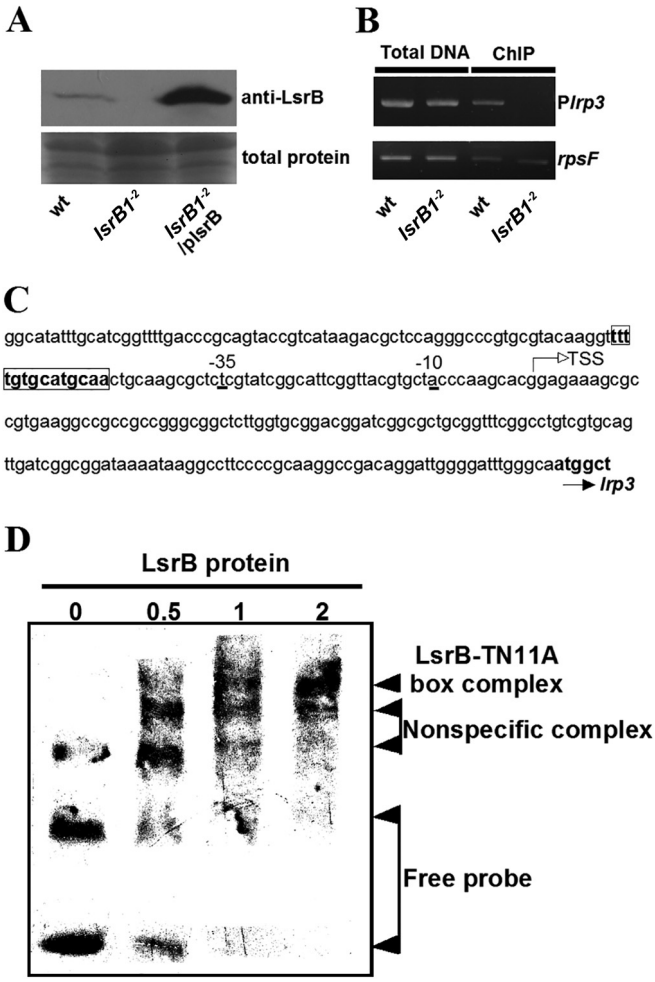

FIG 6 LsrB protein binds to the promoter of the *lrp3-lpsCDE* operon. (A) Immunoblot analysis of LsrB from the indicated *S. meliloti* strains. (B) ChIP assay of LsrB protein binding to the *lrp3* promoter *in vivo*. Total DNA from the sonicated samples; wt, *S. meliloti* 1021; P*lrp3*, *lrp3* promoter; *rpsF*, a ribosome protein gene used as a negative control. (C) The *lrp3* promoter sequence. TSS, transcriptional start sites mapped by 5′-RACE. Box, TN11A box. (D) LsrB protein binding to the TN11A box of the *lrp3* promoter *in vitro*. The amounts of loaded LsrB protein were 0, 0.5, 1, and 2 μg, respectively.

mined transcriptional start sites (Fig. 5A), fused to *GUS*, and introduced into *S. meliloti* strains (Fig. 5B). The GUS activities of these strains showed that the activities of the *lsrB* and *lrp3* promoters decreased 175- and 273-fold in the *lsrB1*$^{-2}$ mutant, but expression of the *trxB* and *lpsC* genes, in contrast to that determined by qRT-PCR, showed only 1.3- and 1.6-fold reductions (Fig. 5C). These results indicated that LsrB positively regulates the expression of *lrp3* (together with *lpsCDE*) and *lsrB* at the transcriptional level.

LsrB binds to the TN11A box on the *lrp3-lpsCDE* promoter. To determine whether LsrB regulates the transcription of the *lrp3-lpsCDE* operon by binding to the promoter DNA, we expressed the recombinant His-tagged LsrB protein in *E. coli*, and the purified protein was used to raise antibodies in two rabbits. The polyclonal antibodies then were applied to analyze the LsrB protein derived from *S. meliloti* strains. Immunoblotting confirmed the loss of LsrB protein in the *lsrB1*$^{-2}$ mutant, whereas one specific band (at about 35 kDa) was detected in the lysates of the parent strain and the complemented mutant (Fig. 6A). Therefore, this anti-LsrB antibody was used in a ChIP assay to determine whether LsrB binds to specific genomic DNA *in vivo*. The ChIP data indicated that the DNA fragment derived from the *lrp3* promoter was specifically enriched by the anti-LsrB antibodies *in vivo* (Fig. 6B). Hence, LsrB positively regulates the expression of the *lrp3* and *lpsCDE* genes by binding to the promoter DNA of the operon.

The target promoters of LysR family transcriptional factors usually contain at least one palindromic TN11A motif (17, 31). The putative overlapping TN11A boxes (TtttgtgcatgcA and TttgtgcatgcaA [uppercase letters represent the conserved nucleotides in a TN11A motif]) were found at position −47 from the transcriptional start site (Fig. 6C). Twenty-four-base-pair oligonucleotides

containing the TN11A boxes were synthesized and used in an EMSA. The data showed that the recombinant LsrB protein bound directly to the TN11A boxes of the *lrp3* promoter *in vitro* (Fig. 6D). These results indicate that LsrB regulates the expression of the *lpsCDE* genes by specifically binding to the TN11A box on the *lrp3* promoter.

DISCUSSION

To our knowledge, there are a few regulators involved in LPS biosynthesis in rhizobia, although some *S. meliloti* gene mutants produce defective LPS (11). SyrA in *S. meliloti* and TtsI in *S. fredii* NGR234 contribute to the regulation of LPS O-antigen synthesis (15, 16). It also has been reported that the transcription elongation factor GreA participates in LPS core biosynthesis in *S. meliloti* 1021 and *S. fredii* HH103 (7). However, regulation of the genes for the LPS core is not well understood. In the present study, we found that LsrB, the *S. meliloti* transcriptional factor from the LysR family, positively regulated transcription of the LPS core biosynthetic genes, *lpsCDE*, by binding to the TN11A box on the *lrp3-lpsC* operon promoter. Therefore, LsrB is a new LysR family regulator that is associated with LPS production. Moreover, LsrB appears to modify only the amount of LPS, not its constituents, as is the case with SyrA and TtsI (15, 16).

The defective growth of the *S. meliloti lsrB* mutant could result from modification of the cell envelope or the redox status. At first, we observed that the *lsrB1*$^{-2}$ mutant grew slowly on LB/MC or M9 agar plates. This result was consistent with the growth curve of the mutant in LB/MC broth (Fig. 2A). Importantly, this defective growth became more severe under stressful conditions, such as low pH and in the presence of a detergent (Fig. 2B), suggesting that the cell envelope of the mutant could be defective. This possibility was supported by LPS assays of *S. meliloti* cultures (Fig. 3). Additionally, the defective growth of the *lsrB* mutant could be associated with the cellular redox status. In our previously published data, the oxidative burst (reactive oxygen species accumulation) appeared in the *lsrB*-deleted cells because the catalase KatA was significantly increased and glutathione was somewhat decreased (32). Therefore, the growth defect of the *lsrB* mutant probably was caused by several factors.

The LPS decrease did not completely fit the expression of *lpsCDE*. We noticed from DOC-PAGE analysis that LPS production in the *lsrB1*$^{-2}$ mutant was slightly reduced (Fig. 3), and this was consistent with downregulation of the *lrp3-lpsCDE* operon (Fig. 5). The quantification of total sugar revealed a trend of decreasing LPS in the *lsrB1*$^{-2}$ mutant, but it was not very significant in a statistical analysis. It may be due to the upregulation of other polysaccharides (such as exopolysaccharide and cyclic-β-glucan) in the mutant. Importantly, the trend of decreasing LPS in the mutant was verified by another method, DOC-PAGE (Fig. 3). However, the extent of the LPS decrease in the *lsrB* mutant did not completely correspond with the amount of *lpsCDE* mRNA (Fig. 3 and 5). One possibility is that only a few LpsCDE proteins are required for LPS synthesis and that the expression of LpsCDE is regulated at the posttranscriptional level. The other is that other proteins play roles similar to those of LpsCDE in LPS core biosynthesis. These possibilities should be further verified.

Regulation of *lpsCDE* expression was complex under different conditions. The *lsrB*, *trxB*, *lrp3*, and *lpsCDE* genes can be organized into two different operons, but these genes are controlled by both their own promoters and the operon promoter (Fig. 4).

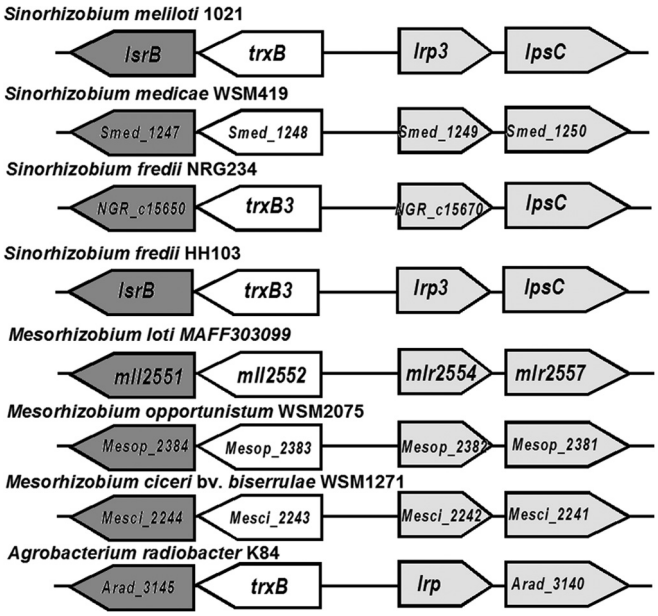

FIG 7 Organization of homologous genes to *lsrB* in various rhizobial genomes. Black boxes, *lsrB* homologs; gray boxes, the *lrp-lpsC* operon.

However, the expression data suggested that only two promoters (those for *lsrB* and *lrp3*) could be regulated by LsrB (Fig. 5). The expression of *lpsCDE* also is controlled by two promoters (P*lrp3* and P*lpsC*). Unlike P*lrp3*, P*lpsC* was not regulated by LsrB under free-living conditions, so the activity of the promoter-*GUS* fusion was not significantly altered between the wild type and the *lsrB* deletion mutant (Fig. 5C). This was supported by the EMSA data, as the P*lpsC* DNA was not able to bind to LsrB *in vitro* (data not shown), in contrast to P*lrp3* (Fig. 6). We also detected the expression of promoter-*GUS* fusions in bacteroids from alfalfa nodules (31). Compared to their activity under free-living conditions, the activity of all four promoters was decreased in alfalfa nodules induced by the *lsrB1*$^{-2}$ mutant (33), suggesting that the promoter activity for *lpsC* and *trxB* is regulated differently under free-living and symbiotic conditions.

The LsrB binding sites could be atypical. Two LsrB binding sites on the *lrp3* promoter were predicted from the presence of TN11A motifs typical of LysR family regulators, but a palindromic sequence has not been found in these motifs (Fig. 6C). Some LysR family regulators, such as OxyR, do not have the binding sites containing a palindromic sequence (34). We also analyzed the LsrB binding sites on the *lsrB* promoter, and a putative TN11A motif was found. However, EMSAs showed that LsrB weakly bound to the DNA fragment containing this motif (data not shown). We did not exclude that other LsrB binding sites could exist on the promoter region. In time, it is likely that the conserved binding sites of LsrB on the *S. meliloti* genome will be identified using ChIP sequencing.

Interestingly, regulation of LPS biosynthesis mediated by LsrB could be conserved in some *Rhizobium* species, because the organization of *lsrB*, *trxB*, *lrp3*, and *lpsC* is conserved in *S. meliloti* 1021, *S. medicae* WSM419, *S. fredii* NGR234/HH103, *Agrobacterium radiobacter* K84, and most *Mesorhizobium* species, as revealed by genomic DNA analysis (Fig. 7).

LPS production regulated by LsrB could be associated with

alfalfa nodule development. In our previous work, the *lsrB1*$^{-2}$ and *lsrB1* (plasmid insertion) mutants induced heterogeneous, ineffective nodules on alfalfa (19, 33). Premature senescence appeared in these nodules, and abnormal bacteroid differentiation was found (33). Correspondingly, oxidative bursts were observed in most defective nodules (33). Therefore, it is possible that LsrB is involved in suppression of the host defense response. It has been reported that purified *S. meliloti* LPS can suppress the oxidative burst of *Medicago* cell cultures induced by invertase (8), and the null mutant of *S. meliloti* 1021 LPS core biosynthesis genes (*lpsB* and *lpsCDE*) induces deficient nodules with premature senescence on some ecotypes of alfalfa (4, 11). These data, together with our results, supported the possibility that LsrB can positively regulate LPS biosynthesis to suppress host defense responses that contribute to nodule premature senescence.

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