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Involvement of *exo5* in Production of Surface Polysaccharides in *Rhizobium leguminosarum* and Its Role in Nodulation of *Vicia sativa* subsp. *nigra*

Marc C. Laus,[1]* Trudy J. Logman,[1] Anton A. N. van Brussel,[1] Russell W. Carlson,[2] Parastoo Azadi,[2] Mu-Yun Gao,[2] and Jan W. Kijne[1]

Institute of Biology Leiden, Leiden University, Leiden, The Netherlands,[1] and Complex Carbohydrate Research Center, The University of Georgia, Athens, Georgia[2]

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Analysis of two exopolysaccharide-deficient mutants of *Rhizobium leguminosarum*, RBL5808 and RBL5812, revealed independent Tn*5* transposon integrations in a single gene, designated *exo5*. As judged from structural and functional homology, this gene encodes a UDP-glucose dehydrogenase responsible for the oxidation of UDP-glucose to UDP-glucuronic acid. A mutation in *exo5* affects all glucuronic acid-containing polysaccharides and, consequently, all galacturonic acid-containing polysaccharides. Exo5-deficient rhizobia do not produce extracellular polysaccharide (EPS) or capsular polysaccharide (CPS), both of which contain glucuronic acid. Carbohydrate composition analysis and nuclear magnetic resonance studies demonstrated that EPS and CPS from the parent strain have very similar structures. Lipopolysaccharide (LPS) molecules produced by the mutant strains are deficient in galacturonic acid, which is normally present in the core and lipid A portions of the LPS. The sensitivity of *exo5* mutant rhizobia to hydrophobic compounds shows the involvement of the galacturonic acid residues in the outer membrane structure. Nodulation studies with *Vicia sativa* subsp. *nigra* showed that *exo5* mutant rhizobia are impaired in successful infection thread colonization. This is caused by strong agglutination of EPS-deficient bacteria in the root hair curl. Root infection could be restored by simultaneous inoculation with a Nod factor-defective strain which retained the ability to produce EPS and CPS. However, in this case colonization of the nodule tissue was impaired.

Bacterial surface polysaccharides are essential for the establishment of successful interactions between many pathogenic and symbiotic bacteria and their corresponding host plants. Polysaccharide-deficient bacteria often show reduced virulence and reduced attachment to host tissue and can induce a rapid defense reaction in the host, in contrast to wild-type bacteria (21, 49). However, the exact function of many of the different surface polysaccharides in these interactions remains unclear. In the case of the symbiotic interaction between the soil bacterium *Rhizobium leguminosarum* and the roots of the host plant *Vicia sativa* subsp. *nigra*, production of bacterial polysaccharides is crucial. During this symbiosis the bacteria induce, under nitrogen-limited conditions, expression of nodulation-related plant genes, resulting in the formation of nodules on the plant root. Bacteria attach to root hair tips, induce root hair curling, and ultimately invade the nodule cells via infection threads, which are tubular structures formed by invagination of root cell membranes (29). After being released from an infection thread into a nodule cell by endocytosis, the bacteria continue to divide and fill the cytoplasm of the nodule cell. Next, the bacteria develop pleomorphic forms called bacteroids which are capable of fixing atmospheric nitrogen into ammonia for the benefit of the plant (48). Inside nodule cells, the bacteria are individually surrounded by a membrane of plant origin. Bacterial growth in and release from the infection threads, as well as maintenance inside the nodule cells, require the production of bacterial surface polysaccharides.

The polysaccharide capsule of *R. leguminosarum* is composed of a number of different polysaccharides, such as the O chain of lipopolysaccharides (LPS), capsular polysaccharides (CPS), and neutral β-1,2-glucans. LPS are complex molecules containing a lipid A domain, which is embedded in the outer membrane layer of the bacterium, and a core, which links a polysaccharide O chain to the lipid part of the molecule. Negatively charged domains within the LPS molecule have been suggested to confer stability to the bacterial membrane, probably by cross-linking with divalent cations (30). The structure of *R. leguminosarum* LPS was elucidated recently, and the core and lipid A domains were shown to be identical to those of *Rhizobium etli* (18, 27). LPS molecules are strong inducers of the immune response in vertebrates and can induce defense responses in some plants as well (25, 31). Some rhizobia whose LPS are affected show sensitivity to antimicrobial compounds and altered nodulation phenotypes on the corresponding host plants (20). CPS are molecules that form a polysaccharide matrix surrounding the bacteria. In late-stationary-phase cultures, CPS are replaced by a polysaccharide with strong gel-forming properties having an unknown function (74). Neutral β-1,2-glucans are present primarily in the periplasm but are also found in the capsule, as well as in the culture medium (13). Another polysaccharide that is also secreted into the culture medium is exopolysaccharide (EPS). This acidic polysaccharide consists of octameric repeat units, and the size of the molecule is determined by the number of subunit repetitions (60). Production of EPS is essential for successful nodulation

* Corresponding author. Mailing address: Institute of Biology Leiden, Leiden University, Wassenaarseweg 64, 2333AL Leiden, The Netherlands. Phone: 31715275082. Fax: 31715274999. E-mail: laus@rulbim.leidenuniv.nl.

TABLE 1. Bacterial strains and plasmids used in this study

Strains or plasmid	Relevant characteristics	Reference(s)
Strains		
RBL5515	*R. leguminosarum* bv. trifolii RCR5 cured of Sym plasmid, *str rif*	51
RBL5523	*R. leguminosarum* RBL5515(pRL1JI) *Spc3*::Tn*1831*, *str rif*	70
LPR5045	*R. leguminosarum* bv. trifolii RCR5 cured of Sym plasmid, *rif*	35
RBL5808	*R. leguminosarum* RBL5523 *exo5*::Tn*5* (EXO5), *str rif*	This study
RBL5812	*R. leguminosarum* RBL5523 *exo5*::Tn*5* (EXO54), *str rif*	This study
RBL5833	*R. leguminosarum* RBL5523 *pssD111*::Tn*5*, *str rif*	68, 70
RBL5811	*R. leguminosarum* RBL5523 *exoB52*::Tn*5*, *str rif*	16
RBL5975	*R. leguminosarum* RBL5811 *pssD-E*Δ*gm*-GFP, *str rif*	This study
DH5α	*E. coli* DH5α *endA1 gyrSA96 hrd17*(r_K^- m_K^-) *supE44 recA1*	10
Plasmids		
pRK2013	Rep ColE1, Km	22
pLAFR1	IncP, Δ*cos* Mob^+ Tra^- cosmid vector, Tc	28
pBBR1-MCS5	*gm* mob^+ *lacZa*	39
pMP4692	pBBR1-MCS5, containing *exo5*, amplified from RBL5523	This study
pMP4694	pBBR1-MCS5, containing *rkpK*, amplified from pAT330	This study
pMP4695	pMP3 *pssD-E*Δ*gm*-GFP, Tc	This study
pMP3034	*pssD-E* operon from *R. leguminosarum*, Tc	68
pAT330	pLAFR1, containing the rkp-2 region of *S. meliloti* Rm41	37
pXLGD4	*hemA-lacZ*, Tc	43
12c2	pLAFR1 containing a genomic fragment of RBL5523	This study
8c2	pLAFR1 containing a genomic fragment of RBL5523	This study

of host plants and formation of indeterminate nodules (40, 70). Some authors have reported that addition of micromolar quantities of purified low-molecular-weight EPS restores infection thread formation in an EPS-deficient strain, which suggests that EPS has a signaling function rather than a structural function in the formation of an infection thread (4, 23, 63).

Mutants affected in polysaccharide production are useful tools for studying the role of these different polysaccharides in nodulation. The present study shows that transposon mutagenesis of *R. leguminosarum* resulted in identification of a gene involved in EPS and CPS production, as well as in LPS modification. This gene, designated *exo5*, is a homologue of the *rkpK* gene of *Sinorhizobium meliloti*. In *S. meliloti, rkpK* encodes a UDP-glucose (UDP-Glc) dehydrogenase, which is responsible for the oxidation of UDP-Glc to UDP-glucuronic acid (UDP-GlcA) (37). GlcA is primarily present in the backbone of the EPS molecule of *R. leguminosarum* (60). Moreover, UDP-GlcA is the precursor for the formation of UDP-galacturonic acid (UDP-GalA), which is present as GalA in the core of the *R. leguminosarum* LPS molecule. In this study the effect of Exo5 deficiency on the production of the different polysaccharides and on outer membrane permeability was examined. In addition, the nodulation of *V. sativa* subsp. *nigra* by *exo5* mutant bacteria was compared to that of wild-type *R. leguminosarum* bacteria.

MATERIALS AND METHODS

R. leguminosarum and *E. coli* strains and plasmids are shown in Table 1. *R. leguminosarum* was grown in YMB (34) and TY (5) culture media, and *E. coli* was grown in LC medium (44) containing the appropriate antibiotics. MICs were determined by an agar dilution method. Dilutions of sodium dodecyl sulfate (SDS) in YMB agar plates were prepared, and a bacterial suspension containing approximately 400 CFU was applied with a Drigalsky spatula. The plates were incubated for 6 days at 28°C.

Mutagenesis. Random Tn*5* mutagenesis of *R. leguminosarum* RBL5523 yielded colonies affected in the production of EPS, which were identified by reduced fluorescence in the presence of calcofluor, as described by Leigh et al. (42). Construction of a genomic library of RBL5515 has been described previously by Roest et al. (61). Transfer of plasmids was carried out by triparental conjugal mating as described previously (22); pRK2013 was used as a helper plasmid. Cosmids which restored EPS production were subjected to in vitro Tn*7* transposon mutagenesis by using the GPS-1 genome priming system (New England Biolabs, Beverly, Mass.). Sequencing from Tn*7* priming sites present in cosmids which had lost the ability to restore EPS production allowed construction of primers for amplification and cloning of the *exo5* gene (GenBank accession no. AY312509).

Plasmid pMP4695 contains a green fluorescent protein (GFP)-gentamicin resistance cassette flanked by 431 bp of the *pssD* open reading frame and 451 bp of the *pssE* open reading frame. EPS deficiency in an *exoB* mutant background was obtained upon introduction of plasmid pMP4695 by triparental conjugal mating as described above. *R. leguminosarum* is unable to replicate pMP4695, which resulted in integration of the GFP-gentamicin cassette into the genome based on homologous recombination, resulting in strain RBL5975. EPS-deficient, gentamicin-resistant colonies were selected from solid agar plates and screened for the presence of GFP fluorescence and tetracycline sensitivity. Introduction of pMP3034 (68) containing the *pssD-pssE* operon restored EPS production in this strain.

Isolation of polysaccharides. EPS present in the supernatant of a stationary culture grown in B^- medium (65) was precipitated by addition of 3 volumes of ice-cold ethanol, lyophilized, and analyzed on a DEAE-Sephadex A-25 column for the presence of acidic polysaccharides (24). The hexose contents of the different fractions were determined by the orcinol-sulfuric acid method (47). Supernatants of cultures of EPS-deficient strains were concentrated 10-fold, dialyzed extensively against water by using dialysis tubing with a 12- to 14-kDa cutoff (Medicell Int., London, United Kingdom), lyophilized, subjected to DEAE-Sephadex A-25 chromatography, and analyzed for the presence of hexoses as described above.

CPS were isolated from B^- medium-grown bacteria by the method of Breedveld et al. (13) and were analyzed by ion-exchange chromatography as described above for EPS.

LPS were isolated by the TEA-EDTA method, as described by Valverde et al. (64), dialyzed, lyophilized, and purified by affinity chromatography (Detoxigel; Pierce). Purified LPS were analyzed on 18% deoxycholic acid–polyacrylamide gel electrophoresis (PAGE) gels (57) and were visualized by silver staining (Bio-Rad).

Analytical procedures. Glycosyl composition analysis was performed by combined gas chromatography-mass spectrometry (GC-MS) of per-*O*-trimethylsilyl derivatives of the monosaccharide methyl glycosides produced from a sample by acidic methanolysis. A *myo*-inositol internal standard (20 μg) was added to each dried sample. Methyl glycoside derivatives were prepared from 0.10-mg portions

of dry samples by methanolysis with 1 M HCl in methanol at 80°C (18 to 22 h), followed by N acetylation with pyridine and acetic anhydride in methanol (for detection of amino sugars). The samples were then per-*O*-trimethylsilylated by treatment with Tri-Sil (Pierce) at 80°C (0.5 h). These procedures were carried out as previously described (46, 73). GC-MS analysis of the per-*O*-trimethylsilyl methyl glycosides was performed with an HP 5890 GC interfaced with a 5970 MSD by using an Alltech AT-1 fused silica capillary column. Methyl glycoside derivatives of various monosaccharide standards were also prepared and analyzed by GC-MS for comparison with the sample peaks.

Glycosyl linkages were determined by methylation analysis. The samples were methylated with methyl iodide in dimethyl sulfoxide containing dimethyl sulfoxide anion by using the Hakomori procedure, as previously described by York et al. (73). The methylated samples were then converted to partially methylated alditol acetates as previously described (73) and were analyzed by GC-MS.

Proton nuclear magnetic resonance (NMR) spectroscopy was performed by using a Varian 300-MHz instrument. The samples were prepared for NMR analysis by repeated (two times) lyophilization from D_2O and were dissolved in D_2O for analysis.

Plant assays. Seeds of *V. sativa* subsp. *nigra* were sterilized and allowed to germinate as previously described by van Brussel et al. (66). Germinated seeds were placed on top of a perforated cap on a 30-ml dark glass vial containing Jensen medium (71), from which the precipitate of insoluble salts was removed. To inhibit the formation of ethylene, the medium was supplemented with aminoethoxyvinylglycine at a final concentration of 0.1 mg/liter. The growth and nodulation conditions were identical to those described by van Brussel et al. (66). Reisolation of rhizobia from nodule tissue was carried out as described by van Workum et al. (69).

To enhance visualization of rhizobia on the roots, the bacteria carried a fusion of the *E. coli lacZ* reporter gene to the promoter of the *S. meliloti hemA* gene (43). Staining was carried out as described by Boivin and colleagues (8) with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as a substrate. Nodules stained for LacZ were embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) used according to the manufacturer's recommendations. Transverse serial 6-μm sections were stained with a 1% solution of toluidine blue and investigated by light microscopy. Images were recorded with a DKC-5000 digital photo camera (Sony, Tokyo, Japan), converted to grayscale if appropriate, and corrected for brightness and contrast with Adobe Photoshop software (Adobe, San Jose, Calif.).

RESULTS

Identification of the *exo5* gene. By using Tn*5* transposon mutagenesis, carried out in a *R. leguminosarum* RBL5523 background, several EPS-deficient mutants have been identified, some of which have been described previously (16, 68). This screening procedure also resulted in isolation of the nonmucoid mutant strains exo5 and exo54. Cosmids able to restore mucoidy were isolated, in all cases in both exo5 and exo54. This suggested that the two mutant strains contain a Tn*5* insertion in either the same gene or in two closely linked genes involved in EPS production. DNA from two of these cosmids, 12c2 and 8c2, was subjected to in vitro Tn*7* transposon mutagenesis and introduced into *E. coli* DH5α. Chloramphenicol-resistant clones were transferred to exo5 and exo54 by triparental mating and screened for the inability to restore EPS production. Three independently obtained cosmids, 12c2-31, 8c2-34, and 8c2-45, each of which lacked the ability to rescue the mutant phenotype in exo5 and exo54, were identified. Sequencing directly from priming sites in the boarders of the Tn*7* transposon resulted in overlapping sequences from which a single, full-length open reading frame (ORF) could be identified. This ORF is 1,326 bp long and has a deduced level of amino acid identity of 80.4% with the *S. meliloti rkpK* gene (37). Original Tn*5* insertions were identified by PCR by using Tn*5* and gene-specific primers, followed by sequencing. Insertion sites were located in a single ORF at 1,266 and 352 bp downstream of the start codon for exo5 and exo54, respectively. Introduction of the full-length ORF, including a 97-bp putative promoter region from *R. leguminosarum* RBL5523 on plasmid pMP4692, resulted in restoration of EPS production in exo5 and exo54. Both pAT330 and pMP4694, containing the *S. meliloti rkpK* gene, restored EPS production in the two mutant strains. In this paper, the gene identified is designated *exo5*, the apparent *rkpK* homologue in *R. leguminosarum*.

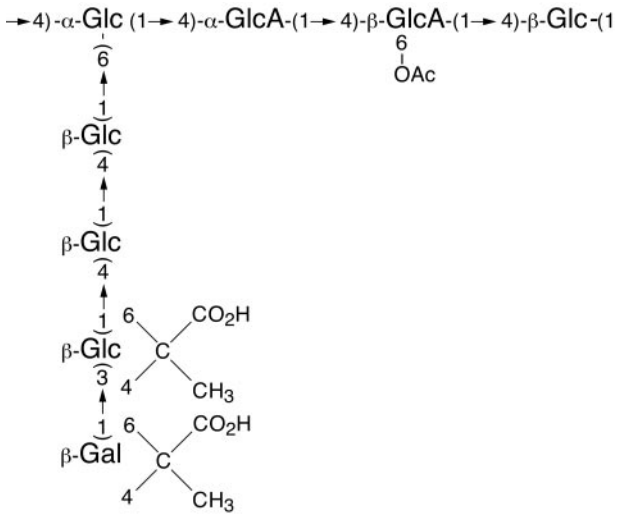

FIG. 1. Structure of EPS of *R. leguminosarum*. For details see references 50 and 60. Gal, galactose; OAc, *O*-acetyl.

Exo5 is involved in surface polysaccharide production. Mutations in *exo5* have a severe effect on the amount of EPS secreted. No acidic EPS could be isolated from the supernatant of an exo5 culture in B^- minimal medium, as judged by DEAE-Sephadex A-25 ion-exchange chromatography. Production of acidic EPS was completely restored after introduction of the *exo5* gene or *rkpK*. The *S. meliloti* RkpK protein has UDP-Glc dehydrogenase activity, which is responsible for the oxidation of UDP-Glc to UDP-GlcA (37). GlcA is specifically present in the backbone of the *R. leguminosarum* EPS repeat unit (Fig. 1), which explains the EPS deficiency of the *exo5* mutant.

Interestingly, when exo5 was grown on YMB medium plates supplemented with SDS, a decreased MIC of SDS (0.12 mg/ml) was observed. For wild-type bacteria and an exclusively EPS-deficient strain with a mutation in the *pssD* gene the MIC of SDS was 0.20 mg/ml. Introduction of *exo5* or *rkpK* into strain exo5 restored the MICs to those of wild-type bacteria. This suggested that there was an additional defect in the *exo5* mutant. Sensitivity to hydrophobic compounds, such as SDS, has been described previously for some rhizobia affected in the production of LPS (20). In the same genetic background as *exo5* and *pssD* mutants, an O-chain-defective LPS mutant has been identified previously (16). This *exoB* mutant is unable to convert UDP-Glc to UDP-galactose, which is necessary for addition of an O chain to the core of the LPS molecule (Fig. 2). The absence of an O chain in the *exoB* mutant did not seem to affect the sensitivity to SDS since the MICs were identical to those determined for wild-type bacteria. However, production of residual amounts of galactose-deficient EPS by the *exoB* mutant may have conferred some degree of SDS resistance. To

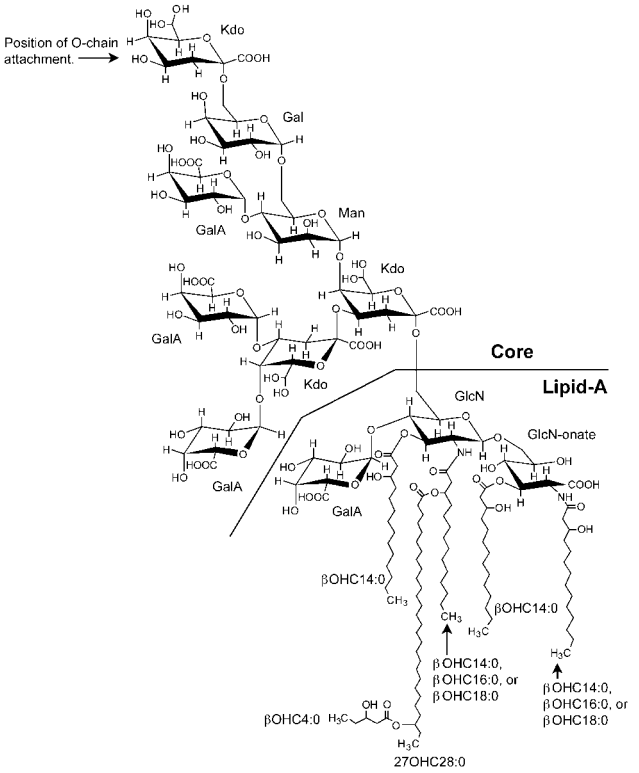

FIG. 2. Structure of the lipid A-core region of LPS from *R. leguminosarum*. Strains of *R. leguminosarum* and *R. etli* have been shown to produce this lipid A-core structure (27, 36). The lipid A portion is heterogeneous in its fatty acyl components; the N-fatty acyl substituents are β-hydroxymyristic acid, β-hydroxypalmitic acid, and β-hydroxystearic acid, and the predominant fatty acid is β-hydroxymyristic acid (6, 7). In addition, the fatty acyl residue at position 3 of the 2-aminogluconic acid residue is absent in some of the molecules (52). Also, the 2-aminogluconic acid residue is sometimes present as a glucosamine residue in some of the molecules (52–54). Kdo, 3-deoxy-D-*manno*-2-octulosonic acid; Man, mannose; Gal, galactose; GlcN, glucosamine; GlcN-onate, 2-aminogluconic acid; βOHC14:0, β-hydroxymyristic acid; βOHC16:0, β-hydroxypalmitic acid; βOHC18:0, β-hydroxystearic acid; 27OHC28:0, 27-hydroxyoctacosanoic acid; βOHC4:0, β-hydroxybutyric acid.

test for SDS resistance by production of EPS, an EPS-deficient strain was constructed by insertion of a GFP-gentamicin resistance cassette into the *pssD-pssE* operon in an *exoB* mutant background. This resulted in a lack of EPS production, together with an inability to produce the polysaccharide O chain of the LPS molecule. The *pssDE-exoB* double mutant was not affected in SDS sensitivity, demonstrating that neither EPS nor the O chain is involved in resistance to SDS.

LPS was isolated from exo5 and from the wild-type strain *R. leguminosarum* RBL5523. After PAGE of the LPS samples, the intensity of the O-chain-deficient LPS II band was increased compared to that of RBL5523 (Fig. 3). The intensity of this LPS II band could be returned to wild-type levels by introduction of plasmid pMP4692 into strain exo5. To identify differences in the exo5 LPS molecule compared to a wild-type LPS fraction, the purified LPS samples were subjected to a carbohydrate composition analysis. The results of this analysis are shown in Table 2. Most notably, the exo5 LPS sample appeared to lack GalA, whereas the wild-type LPS sample

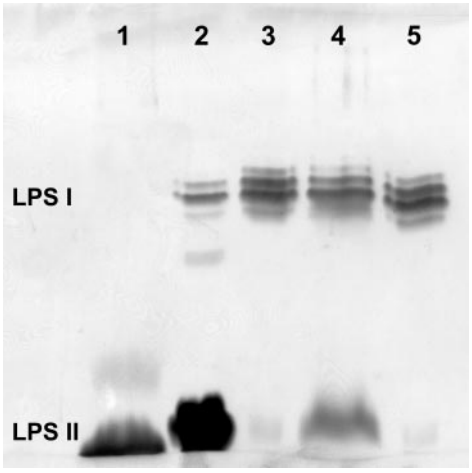

FIG. 3. PAGE profiles of affinity-purified LPS from various *R. leguminosarum* strains. Lane 1, *exoB* strain; lane 2, strain exo5; lane 3, strain exo5 restored by pMP4692; lane 4, *pssD* strain; lane 5, wild-type strain RBL5523.

contained a substantial amount of GalA. Studies of LPS from various *R. leguminosarum* strains revealed a core and lipid A structure similar to that identified for *R. etli* CE3 (36). We were able to attribute most of the carbohydrates identified, such as mannose, galactose, GalA, and 3-deoxy-D-*manno*-2-octulosonic acid, to the core of the *R. leguminosarum* RBL5523 LPS molecule (Fig. 2). Substantial amounts of Glc and *N*-acetylquinovosamine (QuiNAc) found in both samples could not be attributed to either the core or lipid A and therefore likely represent the O-chain portion of the LPS molecule. Small differences in the relative percentages of LPS carbohydrates between the wild type and exo5 may have been caused by differences in culture age. SDS sensitivity is likely to be caused by the absence of GalA moieties in the core and lipid A portions of the LPS molecule. Apparently, like the LPS of enteric bacteria, the negatively charged core of *Rhizobium* LPS is associated with resistance to hydrophobic compounds and outer membrane stability (26).

CPS production is affected in EPS-deficient strains. Production of other bacterial surface polysaccharides, such as neutral glucan and soluble and insoluble CPS, was also examined. Neutral glucan was present in the culture medium of the *exo5*

TABLE 2. Relative percentages of carbohydrates detected in hydrolyzed LPS samples of *R. leguminosarum* RBL5523 and exo5

Carbohydrate	% in[a]:	
	RBL5523	exo5
Xylose	1.8	0.8
Rhamnose	ND	1.1
3-Deoxy-D-*manno*-2-octulosonic acid	11.1	16.7
Galacturonic acid	12.6	ND
Mannose	7.0	6.8
Galactose	6.3	3.9
Glucose	29.2	34.0
Quinovosamine	38.0	34.9
N-Acetylglucosamine	Tr	1.8

[a] ND, not detected; Tr, trace detected (error, ±5%).

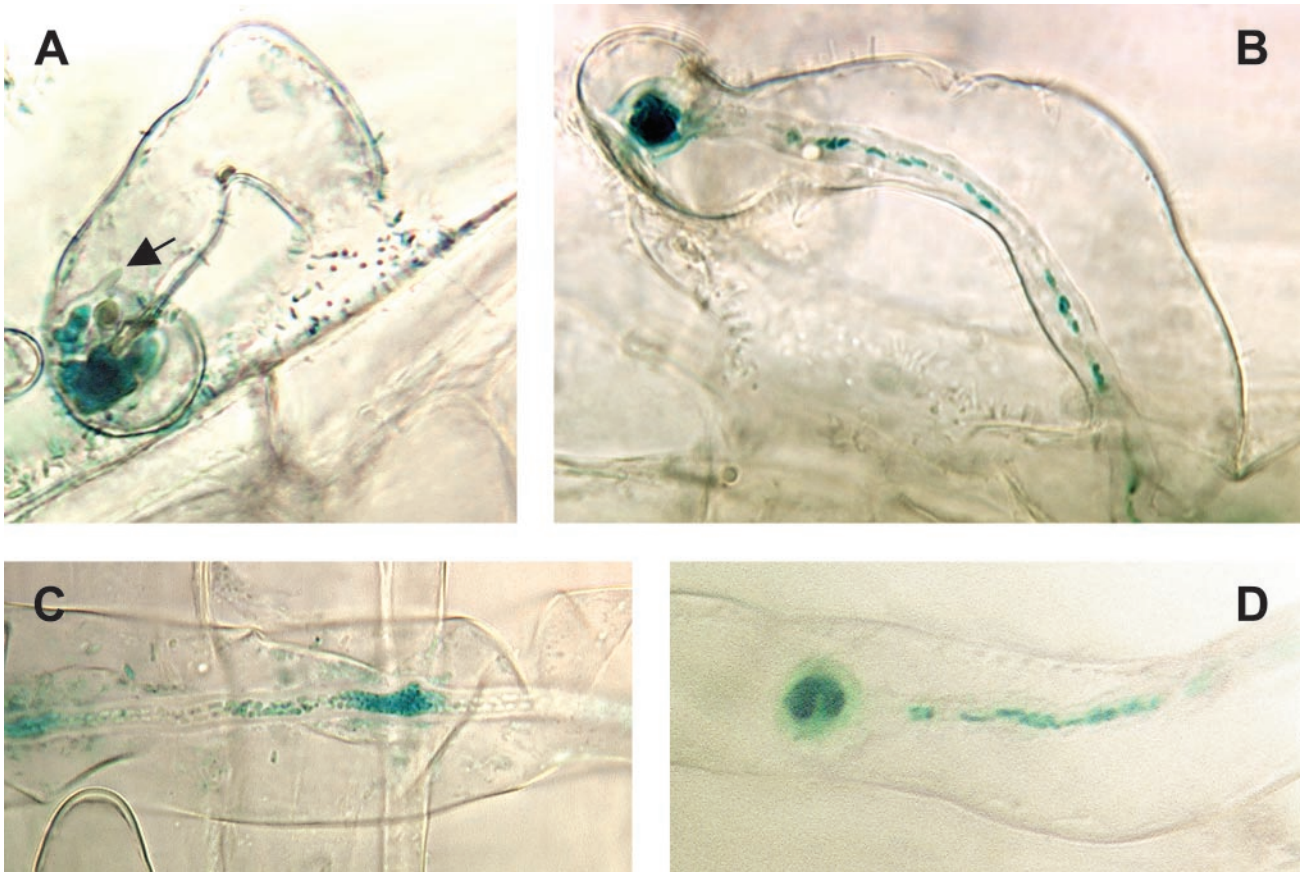

FIG. 4. Infection threads induced in *V. sativa* by LacZ-stained EPS-deficient *R. leguminosarum* strain exo5. (A) exo5 bacteria densely packed in a root hair curl. The infection thread (arrow) aborted shortly after initiation. Colonization of the infection thread was unsuccessful since part of the infection thread is devoid of bacteria. (B) Successful infection thread formation after coinoculation of *R. leguminosarum* exo5 (LacZ stained) and *R. leguminosarum* LPR5045 (unstained). The ratio of bacteria in this infection thread prevented agglutination of exo5 due to production of EPS by LPR5045 and allowed infection thread extension by the production of Nod factors by exo5. (C) Agglutination of exo5 bacteria without affecting growth of the infection thread. LPR5045 bacteria are single white spots in the infection thread. (D) Absence of LPR5045 bacteria resulted in agglutination of exo5 bacteria in the infection thread. As a result, infection thread formation was blocked. The average root hair diameter is 13 μm.

mutant strain, and production in this strain did not appear to be affected when it was compared to that in wild-type strain RBL5523. CPS fractions of the RBL5523, EXO5, and PSSD strains were isolated by the method of Breedveld et al. (13), and this was followed by DEAE-Sephadex A-25 ion-exchange chromatography. In the case of RBL5523, this procedure resulted in two fractions, a large neutral glucan fraction present in the void volume of the column and an acidic CPS fraction which could be eluted from the column with a sodium chloride gradient. In the case of the *exo5* and *pssD* mutants, only a neutral glucan fraction was found, and no acidic polysaccharides were eluted from the column with a sodium chloride gradient up to a concentration of 1 M. Both the CPS and glucan samples of RBL5523 were subjected to a carbohydrate composition analysis. The neutral glucan sample consisted of 100% Glc, whereas the acidic CPS fraction had a carbohydrate composition similar to that described previously for EPS of *R. leguminosarum* (data not shown) (Fig. 1) (50, 60). In order to obtain additional structural data, the acidic CPS sample was characterized by NMR and methylation analyses (data not shown). Both the NMR spectrum and the methylation analysis confirmed that acidic CPS has the same characteristics as those reported for EPS from *R. leguminosarum* (50, 60). This explains the absence of CPS in the *exo5* and *pssD* mutants. Insoluble CPS, such as those described by Zevenhuizen and van Neerven (74), could not be isolated from the bacteria until the culture reached the late stationary phase, and they were found in capsular fractions of both RBL5523 and exo5.

Infection studies. Inoculation of *V. sativa* subsp. *nigra* seedlings with wild-type *R. leguminosarum* RBL5523 resulted in root nodule formation within 7 days. Seedlings inoculated with the *exo5* mutant did not develop nodules within 21 days. After 21 days, the main root was covered with primordia and had severe swellings likely to be caused by Nod factor-induced production of ethylene (67). Introduction of plasmid pMP4692, containing *exo5*, into the mutant restored nodulation to wild-type levels. The stage at which exo5-induced nodulation of *V. sativa* roots was aborted was examined further by using *lacZ*-labeled bacteria. Staining of the roots for LacZ activity revealed that exo5 bacteria were densely packed and fixed inside the root hair curls. In some cases, an infection thread was initiated which appeared to be aborted shortly after initiation (Fig. 4A). EXO5 bacteria failed to successfully colonize the initiated infection thread, resulting in an area at the tip of

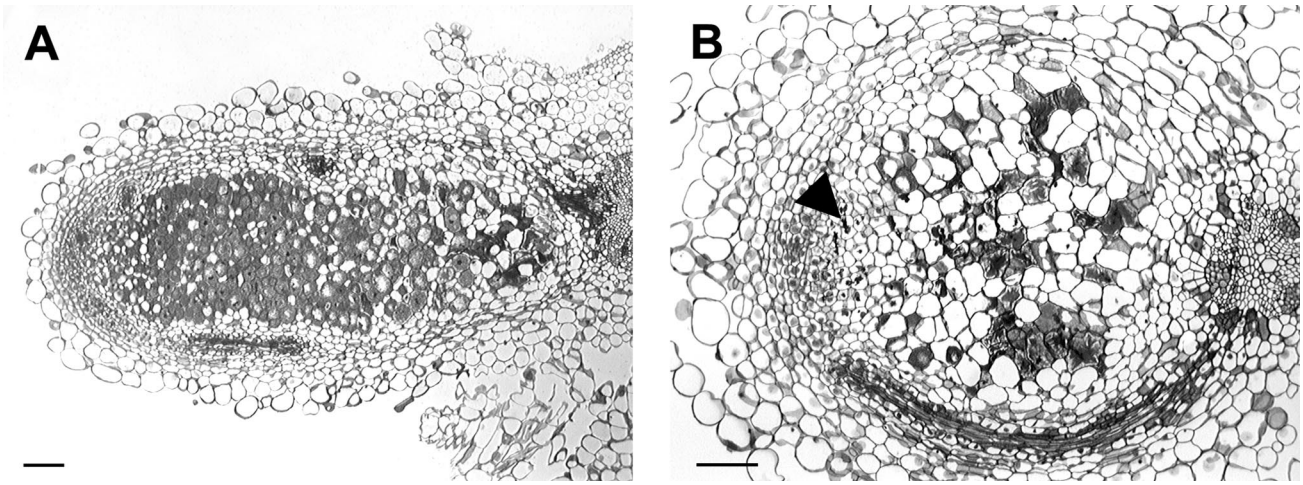

FIG. 5. Six-micrometer sections of nodules induced by RBL5523 (A) and exo5 (B). Infection threads present in the exo5-induced nodule are indicated by an arrowhead. Cells inside the exo5 nodule closely resemble senescent cells present at the base of RBL5523-induced nodules. Bars = 0.1 mm.

the infection thread devoid of bacteria (Fig. 4A). Attempts to restore infection thread formation by adding purified EPS from cultures of RBL5523 bacteria at concentrations ranging from 25 μg/ml to 2 mg/ml were unsuccessful.

Early infection thread abortion and lack of colonization of the infection thread could be prevented by coinoculation of exo5 with *R. leguminosarum* LPR5045, which normally produces EPS but lacks the pRL1JI Sym plasmid. Nodulation of coinoculated roots was studied by using *lacZ*-labeled exo5 bacteria and unlabeled LPR5045 bacteria. The ratio of EPS-producing bacteria to Nod factor-producing bacteria inside the infection thread appeared to be a critical factor for successful infection thread formation. Many of the induced infection threads aborted either in the root hair or in the first cortical cell layers. A majority of these aborted infection threads were ultimately populated by one of the two bacterial strains. If large numbers of exo5 cells were present in the infection thread, the bacteria were strongly agglutinated (Fig. 4C and D). In some cases, infection thread formation was successful (Fig. 4B), and small white nodules formed along the root. After 21 days, both small white nodules and elongated pink nodules were present on a coinoculated root. The pink color of some root nodules indicated the presence of leghemoglobin, which is necessary for nitrogen fixation in an oxygen-deprived environment (3). To test whether nitrogen-fixing nodules on coinoculated roots resulted from conjugation of the pRL1JI Sym plasmid to strain LPR5045, pink nodules were surface sterilized and crushed to release the bacteria. The bacteria were transferred to YMB medium plates containing spectinomycin, the resistance marker of the pRL1IJ Sym plasmid, and screened for the production of EPS. EPS-producing, spectinomycin-resistant bacteria could be isolated from pink nodules, indicating that the Sym plasmid was transferred from strain exo5 to strain LPR5045. Inoculation of *V. sativa* roots with these reisolated bacteria resulted in nodule formation within 7 days. Surface-sterilized small white nodules did not release EPS-producing, spectinomycin-resistant bacteria. The absence of leghemoglobin and the lack of an elongated nodule shape suggested that exo5 bacteria were not successfully released into the nodule cells. Impaired release of bacteria can be caused by the absence of uronic acid in the LPS molecule, as described previously for *S. meliloti* (14, 41), or by the absence of CPS and EPS (40). Coinoculation of LPR5045 with another EPS-deficient strain with intact LPS, such as the *pssD* mutant, gave results identical to those observed with exo5 and LPR5045. Both small white nodules and elongated pink nodules were present on the main roots, and the pink nodules were the result of transfer of the Sym plasmid.

Sections of 21-day-old, small, white nodules induced either by exo5 and LPR5045 or by the *pssD* mutant and LPR5045 showed that there were infection threads in the infection zones of the nodules. Figure 5 shows that in contrast to wild-type strain RBL5523-induced nodules, only a few cells inside a nodule were infected with bacteria. This indicates that there was inefficient endocytosis of EPS-deficient strains, such as the EXO5 and PSSD strains, resulting in reduced host cell invasion. Some cells had a central vacuole, and the cytoplasm was packed with bacteria. However, differentiation into bacteroids could not be detected. Lack of bacteroid differentiation coincided with senescence of the infected cells. Most of these cells closely resembled degenerate cells present in the zone of senescence in wild-type nodules (Fig. 5).

DISCUSSION

The EPS deficiency of *R. leguminosarum* strains exo5 and exo54 appeared to be caused by Tn*5* integration in a single gene, *exo5*, which has high structural and functional homology to the *S. meliloti rkpK* gene (37). The *rkpK* gene encodes a UDP-Glc dehydrogenase involved in the production of *E. coli* K-antigen-related CPS, which is present in some rhizobial species, such as *S. meliloti* and *Sinorhizobium fredii* (37, 58). However, until now K-antigen-like polysaccharides in *R. leguminosarum* have not been reported (38). The observed homology between *exo5* and *rkpK* suggests that *exo5* encodes a UDP-Glc dehydrogenase. A putative dehydrogenase function for *exo5* (as well as for *rkpK*) is supported by the presence of 20 of 22 strictly conserved amino acid residues identified in 47 different

dehydrogenases (15). Amino acid alignment data for these dehydrogenases suggested that an arginine around position 244 is a determinant of substrate specificity that is conserved in UDP-Glc and UDP–*N*-acetylmannosamine dehydrogenases (15). The proteins encoded by both *exo5* and *rkpK* contain this amino acid at positions 247 and 249, respectively. Since *N*-acetylmannosaminuronic acid has not been identified in the different polysaccharides of *R. leguminosarum*, *exo5* likely encodes a UDP-Glc dehydrogenase.

UDP-Glc dehydrogenase is responsible for the oxidation of UDP-Glc to UDP-GlcA. This oxidized saccharide can be converted into UDP-GalA, for example, by a gene product equivalent to that of the *S. meliloti lpsL* gene (37). As a result, an *exo5* mutant has a pleiotropic phenotype and is affected in GlcA- and GalA-containing polysaccharides, such as EPS, CPS, and LPS. In contrast to the *pss* and *psi* genes (9, 40, 45, 68), *exo5* is not directly involved in the production of EPS in *R. leguminosarum*. The absence of GlcA in exo5 bacteria, specifically in the backbone of the EPS molecule, blocks assembly of the EPS molecule, resulting in an EPS-deficient phenotype.

Polysaccharides isolated from washed *R. leguminosarum* cells by the method of Breedveld et al. (13) are often referred to as CPS. Our results show that depending on the age of the bacterial culture, CPS can consist of different polysaccharides. Until the early stationary phase of a culture of wild-type bacteria, a neutral glucan consisting of 100% glucose and an acidic polysaccharide whose carbohydrate composition closely resembles that of EPS are both present in the CPS fraction. The neutral glucan may be a cyclic β-1,2-glucan, as previously identified for different *Rhizobium* species (12), and its secretion has been reported to be enhanced in cultures of EPS-deficient rhizobia (11). As judged from the data obtained from NMR studies and methylation analysis of the acidic CPS, we concluded that CPS is similar if not identical to EPS (Fig. 1). In the early 1980s, anionic CPS and EPS were reported to be strikingly similar in terms of glycosyl composition and structure (1, 17). Differences in noncarbohydrate substitutions, such as *O*-acetyl, pyruvate, and 3-hydroxybutyrate, may distinguish anionic capsule-bound polysaccharides from secreted EPS (32). Since the sugar compositions of EPS and anionic CPS are similar, mutations that normally affect EPS production also affect the production of the anionic CPS. As a result, a CPS fraction from EXO5 or PSSD bacteria consists solely of neutral glucan. In late-stationary-phase cultures, the anionic CPS is replaced by capsule-bound, gel-forming polysaccharides, which we found in capsular fractions of both wild-type bacteria and EPS-deficient strains, such as the PSSD and EXO5 strains. This is consistent with the structure of the gel-forming polysaccharide molecule, which lacks both GlcA and GalA (74).

Carbohydrate composition analysis of exo5 LPS revealed that the molecule is devoid of GalA, which is normally present in the core and lipid A of *R. leguminosarum* and *R. etli* LPS molecules (18, 27, 33). Glc and QuiNAc identified in both wild-type and exo5 LPS samples likely originated from the O-chain part of the molecule. QuiNAc has been identified previously as a component of *R. leguminosarum* bv. *trifolii* LPS (19). From the carbohydrate composition analysis and the LPS PAGE profiles, we concluded that the exo5 strain is able to produce a smooth-type LPS I molecule similar to that of the wild-type bacterium RBL5523. An increased amount of O-chain-deficient LPS II, identified in LPS samples from exo5 bacteria, suggests that addition of an O chain to an LPS core, lacking GalA, is less efficient. A similar situation has been described for *R. etli* mutant CE358, which lacks a single GalA molecule in the LPS core and shows reduced O-chain addition (18, 59).

EXO5 bacteria showed increased sensitivity to SDS (MIC, 0.12 mg/ml). Wild-type MICs (0.20 mg of SDS per ml) were observed after introduction of either *exo5* or *rkpK* and with the EPS-deficient *pssD* mutant, whose LPS is not affected. Also, the O-chain-defective *exoB* mutant, whose acidic LPS core is largely intact, did not show increased sensitivity to SDS, indicating that the polysaccharide O chain is not involved in SDS resistance. The presence of GalA moieties in the core of *R. leguminosarum* LPS probably confers stability to the outer membrane of the bacterium. The presence of a negatively charged inner core in the LPS molecule is common among a wide range of bacterial species and is generally associated with resistance to antimicrobial compounds (26). Our data are strikingly similar to those obtained for some enteric bacteria, such as *E. coli* and *Salmonella*, which obtain a negatively charged LPS core by phosphorylation of inner core heptose residues. Bacteria deficient in core phosphorylation showed increased sensitivity to hydrophobic compounds and only a slightly decreased efficiency for O-chain addition (55, 72). Divalent cations, which have been suggested to be incorporated into the LPS layer (30), could be provided either by the environment or by a specific cation exporter, such as CpaA (2).

Strains of *R. leguminosarum* completely deficient in EPS production induce formation of a few or no aborted infection threads in their host plants. Mutant exo5 is not different in this respect. exo5 bacteria stay fixed inside a root hair curl and fail to colonize an infection thread, and there is limited growth of the infection thread as a result. Successful infection thread formation could be obtained only by coinoculation with a Sym plasmid-cured, EPS-producing strain. Infection thread formation via coinoculation was shown to be a delicate process. If one of the two strains takes over colonization of the infection thread, the infection process is aborted, with strong agglutination of the EPS-deficient strain as a result. Apparently, an EPS or CPS capsule surrounding the bacteria prevents agglutination of the bacteria and stimulates colonization of the infection thread. Secreted EPS produced inside the infection thread might prevent the bacteria from being cross-linked in the infection thread matrix by plant-derived molecules, such as matrix glycoproteins. These glycoproteins are thought to adhere to negatively charged residues, such as those present on the bacterial surface and also in EPS (56). EPS and CPS might also suppress host plant defense responses which could be elicited by components of the bacterial cell wall, such as LPS (25, 49). Attempts to restore infection thread formation by exogenously adding purified EPS were unsuccessful. Apparently, purified EPS did not reach entrapped EPS-deficient rhizobia to prevent agglutination.

V. sativa subsp. *nigra* roots coinoculated with both the EPS-deficient *pssD* mutant and LPR5045 showed the same nodulation phenotype as roots coinoculated with EXO5 and LPR5045. Sections of the nodules showed that there was severely reduced release of bacteria inside the nodule cells. The bacteria failed to differentiate into bacteroids. Since the nodule

cells closely resemble the cells found in the senescent zone of a wild-type nodule, the infected cells apparently lost their viability shortly after release of the bacteria. Possibly, EPS production or a CPS capsule is required during endocytosis and bacteroid formation. Reduced endocytosis has also been described for an EPS-deficient *pssD* mutant of *R. leguminosarum* bv. *trifolii* TA1 which induces formation of empty nodules on red clover (40). Due to the reduced release of EPS-deficient bacteria in nodules, we were unable to attribute a specific nodulation phenotype to the absence of GalA in the LPS core of exo5. An *lpsB* mutant of *S. meliloti*, in which the core of the LPS molecule lacks uronic acids, failed to colonize nodule cells. *lpsB* mutant bacteria were unable to survive under the conditions encountered in the nodule cells and were degraded (14, 41).

Our results suggest that EPS or CPS is necessary for successful infection thread colonization because it prevents autoagglutination of bacteria. Smit et al. (62) have shown that *R. leguminosarum* RBL5523 produces a large amount of cellulose fibrils on the bacterial surface, which can cause bacterial agglutination in liquid culture. Production of EPS in the infection thread by the coinoculated partner might prevent cellulose-mediated agglutination of the EPS-deficient bacteria. At the point of release inside the nodule cells, each bacterium is separately enclosed by a plant membrane, making EPS complementation impossible and resulting in subsequent degradation of the bacteria. The presence of EPS or a CPS capsule surrounding the released bacteria may prevent direct contact between the rhizobia and the plant membrane, thereby preventing a possible plant defense response. The data presented here indicate that EPS and CPS production is essential for infection thread colonization, as well as survival inside the nodule cells.

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REFERENCES

1. **Abe, M., J. E. Sherwood, R. I. Hollingsworth, and F. B. Dazzo.** 1984. Stimulation of clover root hair infection by lectin-binding oligosaccharides from the capsular and extracellular polysaccharides of *Rhizobium trifolii*. J. Bacteriol. **160:**517–520.
2. **Allaway, D., L. Calvaco, S. Saini, P. Hocking, E. M. Lodwig, M. E. Leonard, and P. S. Poole.** 2000. Identification of a putative LPS-associated cation exporter from *Rhizobium leguminosarum* bv. *viciae*. FEMS Microbiol. Lett. **186:**47–53.
3. **Appleby, C. A.** 1984. Leghemoglobin and *Rhizobium* respiration. Annu. Rev. Plant Physiol. **35:**443–478.
4. **Battisti, L., J. C. Lara, and J. A. Leigh.** 1992. Specific oligosaccharide form of the *Rhizobium meliloti* exopolysaccharide promotes nodule invasion in alfalfa. Proc. Natl. Acad. Sci. USA **89:**5625–5629.
5. **Beringer, J. E.** 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84:**188–198.
6. **Bhat, U. R., and R. W. Carlson.** 1992. A new method for the analysis of amide-linked hydroxy fatty acids in lipid-As from gram-negative bacteria. Glycobiology **2:**535–539.
7. **Bhat, U. R., L. S. Forsberg, and R. W. Carlson.** 1994. Structure of lipid A component of *Rhizobium leguminosarum* bv. *phaseoli* lipopolysaccharide. Unique nonphosphorylated lipid A containing 2-amino-2-deoxygluconate, galacturonate, and glucosamine. J. Biol. Chem. **269:**14402–14410.
8. **Boivin, C., S. Camut, C. A. Malpica, G. Truchet, and C. Rosenberg.** 1990. *Rhizobium meliloti* genes encoding catabolism of trigonelline are induced under symbiotic conditions. Plant Cell **2:**1157–1170.
9. **Borthakur, D., R. F. Barker, J. W. Latchford, L. Rossen, and A. W. B. Johnston.** 1988. Analysis of *pss* genes in *Rhizobium leguminosarum* required for exopolysaccharide synthesis and nodulation of peas: their primary structure and their interaction with *psi* and other nodulation genes. Mol. Gen. Genet. **213:**155–162.
10. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–472.
11. **Breedveld, M. W., H. C. J. Canter Cremers, M. Batley, M. A. Posthumus, L. P. T. M. Zevenhuizen, C. A. Wijffelman, and A. J. B. Zehnder.** 1993. Polysaccharide synthesis in relation to nodulation behavior of *Rhizobium leguminosarum*. J. Bacteriol. **175:**750–757.
12. **Breedveld, M. W., and K. J. Miller.** 1994. Cyclic beta-glucans of members of the family *Rhizobiaceae*. Microbiol. Rev. **58:**145–161.
13. **Breedveld, M. W., L. P. T. M. Zevenhuizen, and A. J. B. Zehnder.** 1990. Excessive excretion of cyclic beta-(1–2)-glucan by *Rhizobium trifolii* TA1. Appl. Environ. Microbiol. **56:**2080–2086.
14. **Campbell, G. R. O., B. L. Reuhs, and G. C. Walker.** 2002. Chronic intracellular infection of alfalfa nodules by *Sinorhizobium meliloti* requires correct lipopolysaccharide core. Proc. Natl. Acad. Sci. USA **99:**3938–3943.
15. **Campbell, R. E., S. C. Mosimann, I. Van de Rijn, M. E. Tanner, and N. C. Strynadka.** 2000. The first structure of UDP-glucose dehydrogenase reveals the catalytic residues necessary for the two-fold oxidation. Biochemistry **39:**7012–7023.
16. **Canter Cremers, H. C. J., M. Batley, J. W. Redmond, L. Eydems, M. W. Breedveld, L. P. T. M. Zevenhuizen, E. Pees, C. A. Wijffelman, and B. J. J. Lugtenberg.** 1990. *Rhizobium leguminosarum exoB* mutants are deficient in the synthesis of UDP-glucose 4′-epimerase. J. Biol. Chem. **265:**21122–21127.
17. **Carlson, R. W., and R. P. Lee.** 1983. A comparison of the surface polysaccharides from *Rhizobium leguminosarum* 128C53 Sm^r Rif^r with the surface polysaccharides from its exo^{-1} mutant. Plant Physiol. **71:**223–228.
18. **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.
19. **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, D. Gerhold, and G. Orgambide.** 1991. *Rhizobium* lipopolysaccharide modulates infection thread development in white clover root hairs. J. Bacteriol. **173:**5371–5384.
20. **de Maagd, R. A., A. S. Rao, I. H. M. Mulders, L. Goosen-de Roo, M. van Loosdrecht, C. A. Wijffelman, and B. J. J. Lugtenberg.** 1989. Isolation and characterization of mutants of *Rhizobium leguminosarum* biovar *viciae* strain 248 with altered lipopolysaccharides: possible role of surface charge or hydrophobicity in bacterial release from infection threads. J. Bacteriol. **171:**1143–1150.
21. **Denny, T. P.** 1995. Involvement of bacterial polysaccharides in plant pathogenesis. Annu. Rev. Phytopathol. **33:**173–197.
22. **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.
23. **Djordjevic, S. P., H. Chen, M. Batley, J. W. Redmond, and B. G. Rolfe.** 1987. Nitrogen fixation ability of exopolysaccharide synthesis mutants of *Rhizobium* sp. NGR234 and *Rhizobium trifolii* is restored by the addition of homologous exopolysaccharides. J. Bacteriol. **169:**53–60.
24. **Djordjevic, S. P., B. G. Rolfe, M. Batley, and J. R. Redmond.** 1986. The structure of the exopolysaccharide from *Rhizobium* sp. strain ANU280 (NGR234). Carbohydr. Res. **148:**87–99.
25. **Dow, M., M. A. Newman, and E. von Roepenack.** 2000. The induction and modulation of plant defense responses by bacterial lipopolysaccharides. Annu. Rev. Phytopathol. **38:**241–261.
26. **Epand, R. M., and H. J. Vogel.** 1999. Diversity of antimicrobial peptides and their mechanisms of action. Biochim. Biophys. Acta **1462:**11–28.
27. **Forsberg, L. S., and R. W. Carlson.** 1998. The structures of the lipopolysaccharides from *Rhizobium etli* strains CE358 and CE359. The complete structure of the core region of *R. etli* lipopolysaccharides. J. Biol. Chem. **273:**2747–2757.
28. **Friedman, A. M., S. R. Long, S. E. Brown, W. J. Buikema, and F. M. Ausubel.** 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene **18:**289–296.
29. **Gage, D. J., and W. Margolin.** 2000. Hanging by a thread: invasion of legume plants by rhizobia. Curr. Opin. Microbiol. **3:**613–617.
30. **Hancock, R. E. W.** 1984. Alterations in outer-membrane permeability. Annu. Rev. Microbiol. **38:**237–264.
31. **Heumann, D., and T. Roger.** 2002. Initial responses to endotoxins and Gram-negative bacteria. Clin. Chim. Acta **323:**59–72.
32. **Hollingsworth, R., M. Abe, J. E. Sherwood, and F. B. Dazzo.** 1984. Bacteriophage-induced acidic hetropolysaccharide lyases that convert the acidic heteropolysaccharides of *Rhizobium trifolii* into oligosaccharide units. J. Bacteriol. **160:**510–516.
33. **Hollingsworth, R. I., R. W. Carlson, F. Garcia, and D. A. Gage.** 1989. A new core tetrasaccharide component from the lipopolysaccharide of *Rhizobium trifolii* ANU 843. J. Biol. Chem. **264:**9294–9299.

34. **Hooykaas, P. J. J., P. M. Klapwijk, M. P. Nuti, R. A. Schilperoort, and A. Rörsch.** 1977. Transfer of the *Agrobacterium tumefaciens* TI plasmid to avirulent agrobacteria and to rhizobia *ex planta*. J. Gen. Microbiol. **98:**477–484.
35. **Hooykaas, P. J. J., F. G. M. Schnijdewindt, and R. A. Schilperoort.** 1982. Identification of the Sym plasmid of *Rhizobium leguminosarum* strain 1001 and its transfer to and expression in other rhizobia and *Agrobacterium tumefaciens*. Plasmid **8:**73–82.
36. **Kannenberg, E. L., B. L. Reuhs, L. S. Forsberg, and R. W. Carlson.** 1998. Lipopolysaccharides and K-antigens: their structures, biosynthesis and function, p. 119–154. *In* H. P. Spaink, A. Kondorosi, and P. J. J. Hooykaas (ed.), The *Rhizobiaceae*: molecular biology of model plant-associated bacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
37. **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.
38. **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.
39. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop, and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175–176.
40. **Król, J., J. Wielbo, A. Mazur, J. Kopcinska, B. Lotocka, W. Golinowski, and A. Skorupska.** 1998. Molecular characterization of *pssCDE* genes of *Rhizobium leguminosarum* bv. *trifolii* strain TA1: *pssD* mutant is affected in exopolysaccharide synthesis and endocytosis of bacteria. Mol. Plant-Microbe Interact. **11:**1142–1148.
41. **Lagares, A., D. F. Hozbor, K. Niehaus, A. J. L. Pich Otero, J. Lorenzen, W. Arnold, and A. Pühler.** 2001. Genetic characterization of a *Sinorhizobium meliloti* chromosomal region in lipopolysaccharide biosynthesis. J. Bacteriol. **183:**1248–1258.
42. **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.
43. **Leong, S. A., P. H. Williams, and G. S. Ditta.** 1985. Analysis of the 5′ regulatory region of the gene for δ-aminolevulinic acid synthase of *Rhizobium meliloti*. Nucleic Acids Res. **13:**5965–5976.
44. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
45. **Mazur, A., J. E. Król, and A. Skorupska.** 2001. Isolation and sequencing of *Rhizobium leguminosarum* bv. *trifolii PssN*, *PssO* and *PssP* genes encoding the proteins involved in polymerization and translocation of exopolysaccharide. DNA Seq. **12:**1–12.
46. **Merkle, R. K., and I. Poppe.** 1994. Carbohydrate composition analysis of glycoconjugates by gas-liquid chromatography/mass spectrometry. Methods Enzymol. **230:**1–15.
47. **Monsigny, M., C. Petit, and A. C. Roche.** 1988. Colorimetric determination of neutral sugars by a resorcinol sulfuric acid micromethod. Anal. Biochem. **175:**525–530.
48. **Mylona, P., K. Pawlowski, and T. Bisseling.** 1995. Symbiotic nitrogen fixation. Plant Cell **7:**869–885.
49. **Niehaus, K., D. Kapp, and A. Pühler.** 1993. Plant defence and delayed infection of alfalfa pseudonodules induced by an exopolysaccharide (EPSI)-deficient *Rhizobium meliloti* mutant. Planta **190:**415–425.
50. **O'Neill, M. A., A. G. Darvill, and P. Albersheim.** 1991. The degree of esterification and point of substitution by *O*-acetyl and *O*-(3-hydroxybutanoyl) groups in the acidic extracellular polysaccharides secreted by *Rhizobium leguminosarum* biovars *viciae*, trifolii, and *phaseoli* are not related to host-range. J. Biol. Chem. **266:**9549–9555.
51. **Priem, W. J. E., and C. A. Wijffelman.** 1984. Selection of strains cured of the *Rhizobium leguminosarum* Sym plasmid pRL1JI by using small bacteriocin. FEMS Microbiol. Lett. **25:**247–251.
52. **Que, N. L. S., S. H. Lin, R. J. Cotter, and C. R. H. Raetz.** 2000. Purification and mass spectrometry of six lipid A species from the bacterial endosymbiont *Rhizobium etli*. Demonstration of a conserved distal unit and a variable proximal portion. J. Biol. Chem. **275:**28006–28016.
53. **Que-Gewirth, N. L. S., M. J. Karbarz, S. R. Kalb, R. J. Cotter, and C. R. H. Raetz.** 2003. Origin of the 2-amino-2-deoxy-gluconate unit in *Rhizobium leguminosarum* lipid A. Expression cloning of the outer membrane oxidase LpxQ. J. Biol. Chem. **278:**12120–12129.
54. **Que-Gewirth, N. L. S., S. Lin, R. J. Cotter, and C. R. H. Raetz.** 2003. An outer membrane enzyme that generates the 2-amino-2-deoxy-gluconate moiety of *Rhizobium leguminosarum* lipid A. J. Biol. Chem. **278:**12109–12119.
55. **Raetz, C. R. H., and C. Whitfield.** 2002. Lipopolysaccharide endotoxins. Annu. Rev. Biochem. **71:**635–700.
56. **Rathbun, E. A., M. J. Naldrett, and N. J. Brewin.** 2002. Identification of a family of extensin-like glycoproteins in the lumen of *Rhizobium*-induced infection threads in pea root nodules. Mol. Plant-Microbe Interact. **15:**350–359.
57. **Reuhs, B. L., D. P. Geller, J. S. Kim, J. E. Fox, V. S. K. 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.
58. **Reuhs, B. R., 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.
59. **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.
60. **Robertsen, B. K., P. Åman, A. G. Darvill, M. McNeil, and P. Albersheim.** 1981. Host-symbiont interactions. V. The structure of acidic extracellular polysaccharides secreted by *Rhizobium leguminosarum* and *Rhizobium trifolii*. Plant Physiol. **67:**389–400.
61. **Roest, H. P., C.-J. P. Bloemendaal, C. A. Wijffelman, and B. J. J. Lugtenberg.** 1995. Isolation and characterization of *ropA* homologous genes from *Rhizobium leguminosarum* biovars *viciae* and *trifolii*. J. Bacteriol. **177:**4985–4991.
62. **Smit, G., J. W. Kijne, and B. J. J. Lugtenberg.** 1987. Involvement of both cellulose fibrils and a Ca^{2+}-dependent adhesin in the attachment of *Rhizobium leguminosarum* to pea root hair tips. J. Bacteriol. **169:**4294–4301.
63. **Urzainqui, A., and G. C. Walker.** 1992. Exogenous suppression of the symbiotic deficiencies of *Rhizobium meliloti exo* mutants. J. Bacteriol. **174:**3403–3406.
64. **Valverde, C., D. F. Hozbor, and A. Lagares.** 1997. Rapid preparation of affinity-purified lipopolysaccharide samples for electrophoretic analysis. BioTechniques **22:**230–236.
65. **van Brussel, A. A. N., K. Planqué, and A. Quispel.** 1977. The wall of *Rhizobium leguminosarum* in bacteroid and free-living forms. J. Gen. Microbiol. **101:**51–56.
66. **van Brussel, A. A. N., T. Tak, K. J. M. Boot, and J. W. Kijne.** 2002. Autoregulation of root nodule formation: signals of both symbiotic partners studied in a split-root system of *Vicia sativa* subsp. *nigra*. Mol. Plant-Microbe Interact. **15:**341–349.
67. **van Spronsen, P. C., A. A. N. van Brussel, and J. W. Kijne.** 1995. Nod factors produced by *Rhizobium leguminosarum* biovar *viciae* induce ethylene-related changes in root cortical cells of *Vicia sativa* ssp. *nigra*. Eur. J. Cell Biol. **68:**463–469.
68. **van Workum, W. A. T., H. C. J. Canter Cremers, A. H. M. Wijfjes, C. van der Kolk, C. A. Wijffelman, and J. W. Kijne.** 1997. Cloning and characterization of four genes of *Rhizobium leguminosarum* bv. *trifolii* involved in exopolysaccharide production and nodulation. Mol. Plant-Microbe Interact. **10:**290–301.
69. **van Workum, W. A. T., A. A. N. van Brussel, T. Tak, C. A. Wijffelman, and J. W. Kijne.** 1995. Ethylene prevents nodulation of *Vicia sativa* ssp. *nigra* by exopolysaccharide-deficient mutants of *Rhizobium leguminosarum* bv. *viciae*. Mol. Plant-Microbe Interact. **8:**278–285.
70. **van Workum, W. A. T., S. van Slageren, A. A. N. van Brussel, and J. W. Kijne.** 1998. Role of exopolysaccharides of *Rhizobium leguminosarum* bv. *viciae* as host plant-specific molecules required for infection thread formation during nodulation of *Vicia sativa*. Mol. Plant-Microbe Interact. **11:**1233–1241.
71. **Vincent, J. M.** 1970. A manual for the practical study of root-nodule bacteria. I.B.P. handbook no. 15. Blackwell Scientific Publications, Oxford, United Kingdom.
72. **Yethon, J. A., J. S. Gunn, R. K. Ernst, S. I. Miller, L. Laroche, D. Malo, and C. Whitfield.** 2000. *Salmonella enterica* serovar Typhimurium *waaP* mutants show increased susceptibility to polymyxin and loss of virulence in vivo. Infect. Immun. **68:**4485–4491.
73. **York, W. S., A. G. Darvill, M. McNeil, T. T. Stevenson, and P. Albersheim.** 1985. Isolation and characterization of plant cell walls and cell wall components. Methods Enzymol. **118:**3–40.
74. **Zevenhuizen, L. P. T. M., and A. R. W. van Neerven.** 1983. Surface carbohydrates of *Rhizobium*. IV. Gel-forming capsular polysaccharide of *Rhizobium leguminosarum* and *Rhizobium trifolii*. Carbohydr. Res. **124:**166–171.