Lipopolysaccharide O-Chain Core Region Required for Cellular Cohesion and Compaction of *In Vitro* and Root Biofilms Developed by *Rhizobium leguminosarum*

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The formation of biofilms is an important survival strategy allowing rhizobia to live on soil particles and plant roots. Within the microcolonies of the biofilm developed by *Rhizobium leguminosarum*, rhizobial cells interact tightly through lateral and polar connections, forming organized and compact cell aggregates. These microcolonies are embedded in a biofilm matrix, whose main component is the acidic exopolysaccharide (EPS). Our work shows that the O-chain core region of the *R. leguminosarum* lipopolysaccharide (LPS) (which stretches out of the cell surface) strongly influences bacterial adhesive properties and cell-cell cohesion. Mutants defective in the O chain or O-chain core moiety developed premature microcolonies in which lateral bacterial contacts were greatly reduced. Furthermore, cell-cell interactions within the microcolonies of the LPS mutants were mediated mostly through their poles, resulting in a biofilm with an altered three-dimensional structure and increased thickness. In addition, on the root epidermis and on root hairs, O-antigen core-defective strains showed altered biofilm patterns with the typical microcolony compaction impaired. Taken together, these results indicate that the surface-exposed moiety of the LPS is crucial for proper cell-to-cell interactions and for the formation of robust biofilms on different surfaces.

During legume-rhizobial interactions, bacteria invade the legume plant roots, leading to the formation of nodules in which atmospheric nitrogen is reduced to ammonia that is ultimately used by the host to grow in nitrogen-depleted soils. Only a fraction of soil rhizobia infect and colonize host plants (1, 2), suggesting that they must have alternative strategies, such as biofilm formation, to survive in different environments and under different conditions (3, 4).

Biofilms are structures in which microorganisms are encased in a matrix of polymeric substances and grow attached to biotic or abiotic surfaces. Biofilm formation requires initial attachment to a surface, microcolony formation, maturation, dispersion, and migration (5–7). Structured microbial communities attached to plant roots, and the surrounding soil particles can be viewed as biofilm communities (3, 8). Rhizobia and the closely related agrobacteria develop structured biofilms *in vitro* consisting of layers of bacteria in contact with each other and interlaced with water channels (9, 10). Within curled root hairs, *Sinorhizobium meliloti* cells form small biofilm-type aggregates that provide the inoculum for root invasion (1). For *S. meliloti* strain 1021, attachment to polystyrene and growth as a biofilm depend on the environmental conditions (11), and biotic and abiotic surface colonization is affected by succinoglycan production (12). Establishment of a three-dimensional biofilm structure and autoaggregation depend on the production of another exopolysaccharide (EPS), termed EPS II (13, 14), and also on core nodulation (Nod) factor (15).

Mutants of *Rhizobium leguminosarum* bv. viciae strain A34, defective in the production of the acidic EPS and the capsular polysaccharide (CPS), were unable to develop typical microcolonies and a structured biofilm *in vitro* (9). Two EPS- β -1,4 glycanases and several proteins from the Rap (*Rhizobium* adhering protein) family, secreted by the PrsDE system, were proposed to be

involved in the maturation of an organized biofilm structure (9, 16). One of the Rap proteins, RapA2, is a calcium-dependent lectin that specifically interacts with the EPS and CPS of *R. leguminosarum*, supporting a role for Raps in the development of the biofilm matrix (17). Recently, overexpression of several Raps was observed in a mutant defective in the transcriptional repressor PraR, resulting in enhanced root attachment and nodule competitiveness (18). The development of an *in vitro* biofilm by the sequenced strain 3841 requires EPS, but not cellulose, glucomannan, or gel-forming polysaccharide, whereas glucomannan and cellulose were required for biofilm formation on root hairs (19). In addition, calcium seems to play an important role in the adhesion of *R. leguminosarum* to hydrophilic abiotic surfaces by remodeling higher-order structures of polysaccharides. It was proposed that

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calcium influences surface roughness and the hydrophilic character, which ultimately affects cell adhesion properties (20).

The external leaflet of the outer membrane of Gram-negative bacteria is built of lipopolysaccharide (LPS), which is in rhizobia as in other bacteria a key determinant of the bacterial cell surface antigenicity. LPS is made up of the lipid A, which anchors the molecule to the external membrane, the connecting core oligosaccharide, and the distal O-chain polysaccharide. The LPSs from *R. leguminosarum* and *R. etli* share a lipid A-core structure and vary in their O-chain structures (21). The lipid A structure of rhizobial LPSs differs from those of enteric bacteria, in that it lacks phosphate groups and is acylated with hydroxylated fatty acids of variable lengths, one of which is an unusual very-long-chain fatty acid, 27-hydroxyoctacosanoic (21–23). The core oligosaccharide of *R. leguminosarum* species and *R. etli* consists of an octasaccharide of mannose (Man), galactose (Gal), galacturonic acid (GalA), and 3-deoxy-D-manno-2-octulosonic acid (Kdo) residues in a 1:1:3:3 molar ratio, arranged in the structure lipid A-(Kdo)2-Man-Gal-Kdo-O antigen with two GalA residues linked to an internal Kdo and another to the Man residue (21, 23, 24). In *Rhizobium* spp., neutral O-antigen polysaccharides which are relatively hydrophobic are favored; residues imparting net negative charge are absent or, when present, are blocked by esterification or neutralized with a positive substituent (21). The main glycosyl residues present are deoxyhexoses and methylated glycosides (25). The O antigen of *R. leguminosarum* strain 3841 is formed by a branched tetraheteroglycan consisting of three or four repeating units of 6-deoxy-3-O-methyltalose (3Me-6dTal), 2-acetamido-2-deoxy-L-quinovosamine (QuiNAc), 3-acetimidoylamino-3-deoxy-D-glucosyl-hexuronic acid (Glc3NAc; rhizoaminuronic acid), and fucose (Fuc) residues with endogenous O-methylation and O-acetylation (26).

An intact LPS plays an important role in infection of both determinate and indeterminate nodules in legumes (21, 27–29). Rhizobial LPS exhibits considerable heterogeneity in different plant microhabitats and soil environments (30), and several factors induce modifications in LPS structure (28, 31–35). This suggests that variation in the LPS may have a role in survival and adaptation to local microenvironments. A role for LPS in biofilm structures has been shown in several pathogenic or opportunistic pathogenic bacteria (36–39). In *R. leguminosarum*, the participation of the lipid A component of the LPS in desiccation tolerance, biofilm formation, and motility has been reported (34). In this work, we show that the O-antigen core region of LPS of *R. leguminosarum* is essential for the establishment of lateral and intimate cell-to-cell interactions and is required for the formation of a compact biofilm structure. In addition, the outermost part of the LPS influences adhesion properties on both abiotic and root surfaces.

MATERIALS AND METHODS

Microbiological techniques and phenotypic analysis. Bacterial strains and plasmids are described in Table 1. *R. leguminosarum* strains were grown at 28°C in tryptone-yeast extract (TY) medium (40) or Y-minimal medium (41) containing mannitol (0.2%, wt/vol) as a carbon source. *Escherichia coli* cultures were grown at 37°C in LB medium (42). Bacterial growth was monitored at 600 nm using an Amersham Pharmacia spectrophotometer. Plasmids were mobilized into *Rhizobium* by triparental mating using a helper strain of *E. coli*. Cellulose production was detected using Y-mannitol minimal medium agar plates containing 0.001% (wt/vol) Congo red. *R. leguminosarum* strains were inoculated using a toothpick and cultured for 3 days at 28°C. Red or pink colonies are indicative of

TABLE 1 Strains and plasmids used in this work

Strain or plasmid	Description	Reference or source
Strains		
3841	<i>R. leguminosarum</i> bv. viciae 3841 (Str ^r)	76
A34	<i>R. leguminosarum</i> bv. viciae 8401/pRL1JI (Str ^r)	77
A950	Mutant of 3841, <i>lpsD</i> ::Tn5	This work
A951	Mutant of 3841, <i>lpcB</i> ::Tn5	This work
B772	Mutant of A34, <i>lpcA</i> ::Tn5	This work
Plasmids		
pRU1319	Plasmid pOT1 carrying green fluorescent protein (GFPuv)	47
pJB4JI	pPH1JI derivative plasmid carrying Mu and Tn5	44
pGEM-T Easy	Cloning vector for PCR products	Promega
pLAFR3	Broad-host-range cosmid cloning vector	78
pFC222	pLAFR3 cosmid carrying the <i>lpcA</i> and <i>lpcB</i> genes and the upstream regulatory sequences from 3841	This work
pFC224	pLAFR3 cosmid carrying the <i>lpsD</i> gene and the upstream regulatory sequences from 3841	This work

cellulose production in *Rhizobium leguminosarum* (19, 43). Swimming motility was assayed (10) by inoculating bacteria from cultures (optical density at 600 nm [OD₆₀₀] adjusted to 1.0) on Y-mannitol minimal medium containing 0.3% agar and measuring the colony diameters after 4 days of growth. Statistical analysis was done using GraphPad Prism 5 software.

Tn5 mutagenesis and screening of the LPS mutants. A suicide plasmid, pJB4JI, containing Tn5 was conjugated from *E. coli* into *R. leguminosarum* bv. viciae A34 by filter mating as described previously (44). The *lpcA* mutant was identified by screening for colonies with a rough morphology on TY agar. To determine the Tn5 insertion site, an EcoRI fragment containing the transposon from genomic DNA of the mutant was cloned into pBluescript. A BamHI fragment was subcloned into pBluescript and PCR amplified using primers from the end of IS50 (TTCCGT TCAGGACGCTA) and the T7 (GTAATACGACTCACTATAGGCG) site from pBluescript. The PCR product was sequenced to identify the transposon insertion point. The *lpcB* and *lpsD* mutant derivatives of strain 3841 were isolated by gene-specific PCR amplification using pools of Tn5 mutants as previously described (19) and the insertion sites confirmed by DNA sequencing using products amplified by Tn5 and gene-specific primers.

Complementation of mutant strains. To clone the *lpcA-lpcB* and *lpsD* genes, the regions indicated in Fig. 1 were amplified by PCR using specific oligonucleotides listed below. Primers were designed using gene sequences of the *R. leguminosarum* 3841 strain (45). The *lpcA-lpcB* locus was amplified from 3841 DNA using sense and antisense primers containing a BamHI restriction site (italics): *lpcAB*for, CAGGATCCTCTAAGTTCAC GTTCCGATTC, and *lpcAB*rev, AGGGATCCGCCACGTAGCGTCAACT CAAAG. A PCR product of 2,074 bp, including the complete coding sequence of *lpcA* (RL3440) and *lpcB* (RL3439) and the upstream putative regulatory sequences, was cloned into BamHI-digested pLAFR3 to generate pFC222. The *lpsD* gene was PCR amplified from 3841 DNA using sense and antisense primers containing BglII restriction sites (italics): *lpsD*for, CAAGATCTGAAGGTTTCGACACGCCCATATTG, and *lpsD*rev, CAAGA TCTCGAGCCAATACGGCTACCTCAG. A PCR product of 1,600 bp, including the coding and regulatory sequences of *lpsD* (pRL90053), was cloned into pGEM-T Easy (pGEM-T Easy cloning kit; Promega) to generate pFC219. The 1,600-bp BglII fragment from pFC219 was subcloned into the BamHI site of the pLAFR3 cosmid to make pFC224.

FIG 1 (A) Diagram of the *R. leguminosarum* strain 3841 locus organization involved in LPS biosynthesis. The location of each gene on the 3841 chromosome (or pRL9 plasmid) is indicated by numbers relating to the genome sequence. The positions of the Tn5 insertions in mutants are shown by black (A950 and A951) or white (B772) arrows. The lower bold lines indicate the amplified products used to generate the complementation plasmids. In the δ -*lps* locus, *cpaA* encodes an LPS-associated cation exporter, *lpcB* encodes a CMP-Kdo transferase, and *lpcA* encodes a galactosyl transferase. In the pRL9-borne β locus, *lpsB2* encodes a hypothetical O-antigen biosynthesis-related protein, *lpsB1* encodes a putative galactosyl transferase protein, and *lpsD* encodes a putative O-antigen ligase. (B) SDS-12% PAGE-silver periodate oxidation (left) or immunoblot (right) analysis of the LPS extracted from A34; 3841; *lpcA*, *lpcB*, and *lpsD* mutants; and complemented strains. O chains were detected by immunoblotting using the specific monoclonal antibody MAC 114 or MAC57, which recognizes the O-chain LPS from A34 or 3841, respectively. LPS I and LPS II components of the LPS are indicated.

Analysis of biofilms *in vitro*. To analyze biofilms, bacteria grown in TY medium containing appropriate antibiotics (OD_{600} of about 1.5) were inoculated at a 1:1,000 dilution into 100 ml of Y-mannitol medium in a 300-ml conical glass flask with shaking at 250 rpm in an orbital shaker (9). Rings of biofilms at the air-liquid interface were qualitatively scored after 5 days of growth. For quantification of biofilms in microtiter plates, rhizobia were inoculated as described above and cultured in 96-well flat-bottom polystyrene (PE) culture plates (Greiner; CellStar number 655180) for 3 days without shaking. Unbound bacteria were removed by gentle washing with 0.9% NaCl, and attachment was quantified by staining with 0.1% crystal violet (46). To analyze the biofilm structures, bacteria carrying plasmid pRU1319, which expresses the green fluorescent protein (GFPuv) (47), were cultured for 3 days at 28°C in 5 ml of TY

medium. After centrifugation, bacteria were washed and suspended in Y-mannitol medium at a 1:1,000 dilution; 0.5 ml of this bacterial suspension was cultured statically in chambered glass cover slides (Nunc; Lab Tek number 155411) at 28°C (9). Observation of biofilm formation in a 4-day time course experiment was done as previously described using Plan-Apochromat 100 $\times$ /1.40 oil or C-Apochromat 40 $\times$ /1.2 W objectives from a Carl Zeiss Pascal LSM 5-Axioplan 2 laser scanning confocal microscope (488-nm argon laser excitation and 500-nm long-pass emission filter) (9). Representative horizontal projections of confocal laser scanning microscopy (CLSM) image stacks taken from five independent experiments are shown. To quantify the biofilm structures developed in the chambers, at least five 40 $\times$ image stacks taken from three independent experiments were analyzed by COMSTAT software (48). Videos in the

supplemental material are representative image stacks of biofilms developed after 4 days in chambered cover slides from A34 and *lpcA* strains observed from the base to the top using a Plan-Apochromat 100×/1.40 oil objective.

Preparation and analysis of LPS. LPS was extracted by the hot-phenol method (49), modified for rhizobia (28). Briefly, *R. leguminosarum* strains were cultured for 72 h in TY medium, harvested, and washed with 0.9% NaCl. The pellet (1 g of wet cells) was suspended in sterile Milli-Q water and phenol (1:1) at 70°C as described previously (28). After mixing with Laemmli's solubilization buffer, the LPS suspension was analyzed by SDS-PAGE (12%) in Tris-glycine running buffer and visualized by carbohydrate-specific periodate oxidation and silver staining as described previously (50). Immunochemical analysis of the LPS was performed by immunoblotting on nitrocellulose membranes using *R. leguminosarum* O-antigen monoclonal antibodies MAC 57 and MAC 114 as previously reported (51, 52) and anti-rat horseradish peroxidase-conjugated secondary antibody (Sigma). The ECL Plus Western blotting detection reagents (GE Healthcare, United Kingdom) were used to detect the signals using a Storm 840 imager (Amersham Pharmacia Biotech) by following the manufacturer's instructions.

Quantification of EPS and CPS production. To obtain the EPS and CPS, rhizobia were cultured for 5 days at 28°C in 100 ml of Y-mannitol minimum medium and centrifuged at $8,000 \times g$ for 1 h at 4°C (53). The supernatants were recentrifuged to remove remaining cells and then the EPS was precipitated with 2 volumes of cold ethanol, dissolved in water, and quantified by the *meta*-hydroxy-diphenyl-sulfuric acid method (54). The bacterial pellets were washed twice with 10 mM phosphate-buffered saline (PBS; pH 7.4) containing 1 mM $MgSO_4$ and centrifuged at $10,000 \times g$ for 15 min at 4°C. The cells were suspended in PBS containing 1 mM $MgSO_4$ and 0.5 M NaCl and stirred vigorously for 1 h at room temperature. After centrifugation, the CPS was precipitated with 3 volumes of cold ethanol, dissolved in water, and quantified by the *meta*-hydroxy-diphenyl-sulfuric acid method (54). Means and standard errors of replicated samples of EPSs and CPSs from two independent experiments are shown.

Autoaggregation assay. To monitor differences in autoaggregation, each rhizobial strain from a TY medium starter culture of 4 days was diluted to 1:100 in 5 ml of Y-mannitol or TY medium (inoculum $OD_{600} = 0.01$) and shaken (200 rpm) at 28°C. After 5 days, the cultures were mixed vigorously for 15 s and the suspensions were left standing to start the assay. At regular time intervals, a 150- μ l sample was taken at 0.5 cm from the liquid surface and the OD_{600} quantified in a microtiter plate in a Beckman Coulter DTX880 multimode detector as previously described (55). The results of two independent experiments using replicated cultures of each strain are shown.

Initial attachment, biofilm formation, and nodulation tests on pea roots. To evaluate initial attachment to root surfaces, 10-day-postgermination *Pisum sativum* variety Frisson (pea) plantlets were dissected and roots sectioned in 1-cm segments. Root sections were placed on a Fahræus chamber containing 0.5 ml of 0.3% Fahræus plant medium (FP) agar and incubated for 45 min in 20 ml of GFP-tagged bacterial suspension ($OD_{600} = 0.06$) in darkness at room temperature (56). The pea root sections were observed by scanning different focal planes of the root surface using a C-Apochromat 40×/1.2 W objective from a Carl Zeiss Pascal LSM 5-Axioplan 2 microscope (see above). The estimation of the total number of bacteria associated with the root section per square centimeter was calculated using Carl Zeiss Browser software by counting total bacteria in each layer of at least six z-stack images obtained from two independent experiments. The proportion of the number of bacteria that are in direct contact with the epidermis in relation to the total number of bacteria associated with the epidermis in the same image was calculated as the root attachment index (AI).

To analyze biofilm development on root surfaces, pea plantlets were inoculated with a suspension of GFP-labeled bacteria. *Rhizobium* strains cultured in TY medium were centrifuged, and pelleted bacteria were washed and suspended in FP. Ten milliliters of the bacterial suspension

($OD_{600} = 0.06$) was used to inoculate each plantlet grown in FP and incubated at 22°C in a plant growth chamber (16 h of light/8 h of darkness). After 5 days, the entire plant was removed and the roots were washed twice in FP liquid medium with shaking to remove loosely associated cells. Then, roots were weighed and crushed to estimate root-associated bacteria by plating serial dilutions of smashed roots on TY agar containing streptomycin and counting the CFU per gram of root tissue. At least four whole-pea roots per strain from two independent experiments were analyzed. To visualize the biofilms, roots were washed and dissected in 1-cm sections and placed on a slide containing 0.5 ml of 0.5% FP agar. CLSM stack images were obtained by scanning different focal planes of the root surface. At least six whole-pea roots per strain from five independent experiments were analyzed. Images were projected and processed using Carl Zeiss confocal image browser software and Adobe Photoshop CS 8.01.

Nodulation tests were done using pea plants (*Pisum sativum* variety Frisson) in at least two independent experiments as previously described (57).

RESULTS

Genetic and phenotypic characterization of LPS mutants. To analyze the contribution of the LPS in the formation of an organized biofilm, we isolated mutants impaired in LPS biosynthesis in two different *R. leguminosarum* bv. viciae genetic backgrounds: one mutant (B772) is a derivative of strain A34, which has been used for related studies in our laboratory, and two mutants (A950 and A951) are derivatives of strain 3841, the genome of which has been sequenced. The gene mutated in B772 is 99% similar to *lpcA* from *Rhizobium leguminosarum* bv. phaseoli 8002 (GenBank accession number X94963.1) and is 90% similar to RL3440 from *R. leguminosarum* bv. viciae 3841. The *lpcA* gene encodes a galactosyl transferase that adds a galactose residue to the mannose residue of the core oligosaccharide (58, 59). In 3841, *lpcA* (RL3440) is upstream of and probably cotranscribed with *lpcB* (Fig. 1A), which encodes a putative CMP Kdo transferase that adds the most external Kdo residue of the core region to the galactose residue. A951 carries Tn5 in *lpcB* (RL3439) (Fig. 1A). The *lpcA* and *lpcB* genes were previously described as locus δ , involved in the biosynthesis of the core region of the LPS in *R. leguminosarum* (58–60).

A950 carries Tn5 in pRL90053, a gene encoding a putative O-antigen ligase that shares 81% identity with the gene of a putative O-antigen polymerase from *R. etli* CFN42 (RHE_PB00003). The pRL90053 gene (*lpsD* in the new annotation [http://bacteria.ensembl.org/rhizobium_leguminosarum_bv_viciae_3841]) is on plasmid pRL9 and adjacent to and transcribed divergently from *lpsB1* (pRL90051) and *lpsB2* (pRL90052) (Fig. 1A). *LpsB1* (RHE_PB00001) and *LpsB2* (RHE_PB00002) from *R. etli* CFN42 are implicated in O-chain synthesis and are localized in locus β from the p42b symbiotic plasmid (61, 62).

Thus, the LPS mutants we used have mutations in two different regions associated with LPS biosynthesis; one is on the chromosome and the other on a plasmid. The *lpcA* and *lpcB* mutants would be expected to produce LPS lacking the O chain and with an incomplete core oligosaccharide. On the other hand, the *lpsD* mutant would be predicted to have a complete core oligosaccharide that should lack the O-antigen repeat units.

The LPS obtained by hot phenol-water extraction from cultured *lpcA*, *lpcB*, and *lpsD* mutants lacked LPS I, but a band of higher mobility corresponding to LPS II was observed (Fig. 1B). By immunoblotting using MAC 114 or MAC 57 antibodies, we confirmed that the O antigen is absent in the LPS fraction of the

TABLE 2 EPS and CPS production^a

Strain	EPS (mg of GlcA equivalents/100 ml)	CPS (μ g of GlcA equivalents/100 ml)
A34	17 $\pm$ 2	127 $\pm$ 4
<i>lpcA</i> mutant	17 $\pm$ 7	73 $\pm$ 23 (–42%)
3841	27 $\pm$ 6	113 $\pm$ 6
<i>lpcB</i> mutant	22 $\pm$ 8	69 $\pm$ 5 (–39%)
<i>lpsD</i> mutant	23 $\pm$ 3	75 $\pm$ 5 (–34%)

^a EPS and CPS produced by *Rhizobium* strains were estimated as glucuronic acid equivalents quantified by the meta-hydroxybiphenyl method (54).

lpcA, *lpcB*, and *lpsD* mutants (Fig. 1B). Silver-periodate staining and immunoblot analysis showed that *lpcA* and *lpcB* cloned into pFC222 complemented the LPS pattern of both the *lpcA* and *lpcB* mutants (Fig. 1B) and that *lpsD* cloned into pFC224 restored LPS I in the *lpsD* mutant (Fig. 1B).

Since LPS mutations may affect the production or stability of other surface polysaccharides, the EPS, CPS, and cellulose contents of the mutants were assayed. Similar amounts of EPS, referred as glucuronic acid equivalents, were obtained from the supernatant of the *lpcA*, *lpcB*, and *lpsD* mutants compared with isogenic wild-type (WT) strains grown in Y-mannitol minimal medium (Table 2). In Y-mannitol semisolid medium in the presence of Congo red (43), the colony phenotype observed was also indistinguishable between the mutants and the isogenic WT strains (see Fig. S1 in the supplemental material). These observations suggest that neither the production of EPS nor that of cellulose was greatly altered in the LPS mutants.

R. leguminosarum strains are surrounded by the acidic CPS, whose structure and genetic determinants are shared with the EPS and differ only in their degree of noncarbohydrate substitutions (53, 63). A defective LPS could affect the interaction of CPS with the cell surface. We observed a reduction of 30 to 40% in the amount of glucuronic acid equivalents extracted from the cell surface of the LPS mutants compared with the isogenic WT strains (Table 2). These observations suggest that absence of the outermost region of the LPS decreases the amount of CPS associated with the rhizobial surface.

Alterations in flagellar motility have been observed with some rhizobial LPS mutants (64, 65), but the swimming halo diameters of the *lpcA*, *lpcB*, and *lpsD* mutants were similar to those of the isogenic WT strains (see Fig. S2 in the supplemental material), suggesting that flagellum integrity and functionality were unaffected.

Role of the LPS O-chain core region in surface attachment and biofilm development in *R. leguminosarum*. In liquid TY cultures, the *lpcA*, *lpcB*, and *lpsD* mutants showed an increased sedimentation rate, suggesting that the absence of the surface-exposed moiety of the LPS enhances autoaggregation (Fig. 2). In Y-mannitol minimal medium, no significant differences in sedimentation kinetics were observed between the mutants and the isogenic WT strains (see Fig. S3 in the supplemental material). The high carbon/nitrogen ratio of the Y-mannitol minimal medium stimulates CPS and EPS synthesis (66), which increases the viscosity of bacterial cultures. This effect may prevent differential sedimentation phenotypes in the LPS mutants and wild-type strains.

The absence of the hydrophobic O chain in rhizobial LPS may

result in a reduction in cell surface hydrophobicity (26), causing a decrease in initial attachment to hydrophobic surfaces. After 3 days, the *lpcA*, *lpsD*, and *lpcB* mutants showed 63%, 62%, and 52% reductions, respectively, in the biofilms attached to polystyrene (PE) compared with the isogenic WT strains (Fig. 3); the biofilms were restored to normal by complementation with pFC222 (*lpcA* and *lpcB*) or pFC224 (*lpsD*) (Fig. 3). The influence of the O-chain core region in the attachment to glass (a hydrophilic surface) was analyzed using shaking-flask cultures in Y-mannitol medium (9). Under these conditions, the *lpcA*, *lpcB*, and *lpsD* strains showed thicker rings of biofilms (see Fig. S4 in the supplemental material) than WT strains, while pFC222 (*lpcA* and *lpcB*) or pFC224 (*lpsD*) complemented the mutants to normal (data not shown).

A possible interpretation of these results is that absence of the outermost part of the LPS makes rhizobia more proficient in attaching to hydrophilic surfaces but less capable of attaching to hydrophobic surfaces. Another possibility is that cell-cell interactions in biofilms grown with aeration could be particularly favored in the mutants compared with the wild type. Alternatively, the attachment phenotypes could be explained by a combination of several effects.

Role of LPS in cell-to-cell interactions during biofilm formation. Strains A34 and 3841 develop organized and compact microcolonies with most bacteria attached to each other side by side in static cultures in Y medium (9, 19). CLSM of the GFP-labeled *lpcA* mutant grown for 1 day in chambered glass slides revealed premature formation of microcolonies, in which abnormal interactions between bacteria occurred, with abundant chains of cells interacting through their poles (Fig. 4). After 2 or 3 days, the *lpcA* mutant formed unusual nets of bacteria connected mostly

FIG 2 Autoaggregation assays. The sedimentation profiles of liquid suspensions of *R. leguminosarum* strain A34 (A) or 3841 (B) derivative strains in TY medium are shown. Each point corresponds to average of replicated samples from two independent experiments.

FIG 3 Rhizobial adhesion to a hydrophobic abiotic surface. *R. leguminosarum* A34 or 3841 derivative strains were grown in polystyrene multiwell plates in static Y-mannitol minimal medium for 3 days at 28°C, and bacterial attachment was quantified by crystal violet staining. Horizontal values correspond to average of six replicate samples in at least two different experiments. ***, $P < 0.0001$. One-way analysis of variance was performed using GraphPad Prism 5 software.

through their cell poles; after 4 days, loose and ramified structures were observed, in contrast with the typical compact honeycomb-like structure developed by the WT (Fig. 4; see also Videos S1 and S2 in the supplemental material). The videos show the bacterial distribution in the multiple layers from the base to the top of the structure. As expected, pFC222 restored lateral cellular interactions and the typical biofilm in the *lpcA* mutant (Fig. 4). After 1 day, the *lpcB* and *lpsD* mutants also showed the formation of premature microcolonies, with most bacteria interacting through their poles; after 4 days, biofilm structures with branched chains of rhizobia were observed (Fig. 5). Complementation with pFC222 or pFC224 restored lateral interactions and the formation of a compact biofilm (Fig. 5). Formation of premature (and abnormal) microcolonies in the mutants could be related to the augmented autoaggregation observed in the mutants in comparison with the WT strains (Fig. 2).

To provide quantitative measurements of the three-dimensional biofilm structures, CLSM images were analyzed with COMSTAT software (48). The *lpcA*, *lpcB*, and *lpsD* mutants produced 3-fold-thicker biofilm structures than the WTs (Table 3). The pronounced increment of the thickness was also evident by vertical (Z axis) projection of several CSLM images stacks obtained with a C-Apochromat 40×/1.2 W objective (see Fig. S5 in the supplemental material). In addition, the mutants showed reduction of both the roughness coefficient (Ra) and the surface-to-volume

FIG 4 Cellular interactions and biofilms formed by *R. leguminosarum* A34 derivative strains. CLSM images are horizontal (x-axis) projections of optical sections showing bacterial attachment at day 1 and the biofilms formed at day 4 in chambered glass cover slides ($\times 1,000$ magnification) by A34, the isogenic *lpcA* LPS mutant, and the complemented *lpcA* pFC222 strains. The insets are zooms ($3\times$). Size bars indicate 2 μm .

ratio in comparison to the isogenic WTs, reflecting a tendency to form structures with impaired profiles (Table 3). Importantly, the bacterial distribution in the multiple layers of the biofilm developed by the LPS mutants was altered by means of the proportion of area covered by bacteria in each layer (Table 3). The surface colonization and the overall bacterial density in the layers near the substratum (layer 1 and layer 15) were significantly reduced in the *lpcA*, *lpcB*, and *lpsD* mutants compared with those of the WTs (Table 3). In both A34 and 3841 WT strains, maximum coverage of the surface (of around 89%) was observed at an intermediate layer (layer 15), while the mutants occupied a lower proportion of the area (38 to 48%) in the same layer. In the WT biofilms, bacterial coverage showed a pronounced reduction to 3 to 5% at layer 50, while in the mutants, a similar reduction was observed at layer 150 (Table 3). Therefore, it seems that preponderance of polar interactions between cells and reduction of tight lateral interac-

FIG 5 Cellular interactions and biofilms formed by *R. leguminosarum* 3841 derivative strains. CLSM images show bacterial attachment at day 1 and biofilms formed at day 4 in chambered glass cover slides by 3841, the isogenic *lpcB* and *lpsD* LPS mutants, and the complemented *lpcB* pFC222 and *lpsD* pFC224 strains after 1 and 4 days ($\times 1,000$ magnification). The insets are zooms ($3\times$). Size bars indicate 2 μm .

tions in the LPS mutants leads to ramified and abnormal microcolony structures, which, in turn, result in thicker biofilms.

Attachment to pea roots. As seen with other rhizobial LPS mutants (21, 52), the establishment of symbiosis between the *lpcA*, *lpcB*, and *lpsD* mutants and the host legume was impaired with the mutants developing white nodules; using GFP-labeled rhizobia, we confirmed the absence of bacteria inside the pseudonodules induced by the *lpcA*, *lpcB*, and *lpsD* mutants (data not shown). This indicates that nitrogen fixation was not taking place, fitting with the observation that the plants showed signs of nitrogen deficiency (data not shown).

Initial attachment to pea roots was evaluated after 45 min of incubation of 1-cm root sections with rhizobia in FP medium using Fahræus chambers (19, 56). CLSM visualization of root sections showed that both A34 and 3841 initially attached to the root epidermis as single bacteria or groups of 2 or 3 bacteria (see Fig. S6 in the supplemental material). In contrast, the *lpcA*, *lpcB*, and *lpsD* mutants were seen associated with the epidermal root surface as star-like microcolonies (see Fig. S6). This is probably related to the premature formation of abnormal microcolonies in the mutants. Projections of z-stack images from different scanned root sections showed that these microcolonies were attached to the surface by a limited number of bacteria. In line with this observation, the proportion of bacteria that attach directly to the epidermis surface relative to total rhizobia counted in the same image (attachment index [AI]) was lower in all the mutants than in the isogenic WT strain (see Fig. S7). We examined the total bacteria associated with the root surface, i.e., observed in all focal planes, per square centimeter of root section using Zeiss Image Browser software. Comparable amounts of WT and mutant bacteria per square centimeter of root section scanned were observed (see Fig. S7). Therefore, although the mutants deficient in the outermost part of the LPS were initially able to colonize the root epidermis, anchoring of individual bacteria to the root surface seemed to be impaired.

Biofilms associated with root surfaces at a later stage were examined 5 days after inoculation of whole plantlets with the different strains. The WT strains developed compact and robust patch-like bacterial aggregates mostly distributed on the epidermis of the pea roots, whereas bacterial aggregates of the *lpcA*, *lpcB*, and *lpsD* mutants were scattered on the root epidermis, and in general, the mutants were seen as star-like bacterial aggregates or small ramified structures (Fig. 6A). The LPS mutant strains developed root-hair-associated clumps of bacteria that persisted even after the washing steps (Fig. 6B). This pattern of colonization was less frequent in the WT strains, for which bacteria were observed as small groups interacting with the hair root surface. The quantification of root-associated bacteria as CFU per gram of root tissue showed similar values for the WTs and the LPS mutants (see Fig. S7 in the supplemental material), suggesting that differences between the parental and mutant strains in the biofilm patterns observed on root epidermis and root hairs somehow compensate total bacterial counts.

DISCUSSION

The exposed O antigen of *R. leguminosarum* is built up of deoxyhexoses and methylated deoxyhexoses, which confer hydrophobic character on the cellular surface (25, 26). Strains such as the *lpcA*, *lpcB*, and *lpsD* mutants that lack the O antigen but express lipid A attached to a complete or truncated core are expected to expose the most hydrophilic portion of the core (nearest to the lipid bilayer surface) (21). Thus, the bacterial surface would become more hydrophilic, and as observed in this study, this would be predicted to make the mutants less proficient to bind hydrophobic surfaces. Absence of the outermost part of the LPS also affected cell-cell cohesion. Analysis of the biofilm structures using the COMSTAT program confirmed that the degree of microcolony and biofilm compaction was strongly reduced in the LPS mutants. The simplest interpretation for these observations is that the surface-exposed moiety of the LPS, i.e., the O-chain core region itself, plays a direct role in cell-cell interactions between bacteria.

TABLE 3 COMSTAT analysis of 4-day biofilms

Parameter	Value for strain ^a				
	A34	<i>lpcA</i> mutant	3841	<i>lpcB</i> mutant	<i>lpsD</i> mutant
Average thickness (μm)	28 $\pm$ 6	103 $\pm$ 12**	31 $\pm$ 6	103 $\pm$ 16*	110 $\pm$ 18*
Roughness coefficient	0.41 $\pm$ 0.06	0.23 $\pm$ 0.02*	0.66 $\pm$ 0.03	0.45 $\pm$ 0.06	0.31 $\pm$ 0.03*
Surface-to-vol ratio ($\mu\text{m}^2/\mu\text{m}^3$)	0.12 $\pm$ 0.01	0.05 $\pm$ 0.00*	0.08 $\pm$ 0.00	0.04 $\pm$ 0.00	0.04 $\pm$ 0.01
% of the area covered by bacteria					
in each layer					
Layer 1	26.9 $\pm$ 3.2	7.8 $\pm$ 5.4*	26.8 $\pm$ 5.7	7.5 $\pm$ 1.5*	4.0 $\pm$ 2.0*
Layer 15	88.9 $\pm$ 0.2	48.7 $\pm$ 5.9*	88.8 $\pm$ 7.9	39.6 $\pm$ 6.9*	38.2 $\pm$ 2.6**
Layer 50	3.9 $\pm$ 0.7	23.8 $\pm$ 9.0***	5.9 $\pm$ 2.6	40.0 $\pm$ 3.8*	12.7 $\pm$ 1.7
Layer 150		3.0 $\pm$ 2.7		1.3 $\pm$ 0.7	3.0 $\pm$ 1.3

^a Values are means of data from at least 5 independent experiments. Parameters were calculated using COMSTAT and statistical analysis by GraphPad Prism 5 software (one-way analysis of variance). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

The possibility exists that the exposed portion of the LPS is required for the correct localization or assembly of other surface structures involved in attachment to abiotic or biotic surfaces and cell-cell interactions. It has been suggested that the O antigen and the core oligosaccharide are involved in a tight attachment of the CPS on the cell surface (26, 67). We observed a 30 to 40% reduction in the CPS fraction extracted from the LPS-defective mutants that might be in part responsible for the altered biofilm phenotypes of these strains. However, the reduction in the CPS of the LPS mutants cannot account for the severe phenotype and the aberrant cell-to-cell interactions displayed by the LPS mutants. In fact, the biofilm phenotype of EPS- and CPS-defective mutants differs from that of the LPS mutants analyzed in this work, since they were completely unable to form microcolonies and polarly attached cells were not observed (9). Therefore, it seems that aberrant interactions between bacteria are caused mainly by the defect in the O-chain core region of the LPS.

Although lateral interactions between bacteria were impaired in the LPS mutants, chains of cells attached mostly through their poles were formed. Hence, the question arises as to what molecules are responsible for these polar interactions. One possibility is that in the wild-type strains, the LPS structure exposed on the cell surface is not identical all around the cell. In this case, defective O-antigen core structures in the mutants could somehow affect to a greater extent side-to-side interactions. Alternatively, the LPS portion exposed on the surface of the wild-type strains could mask or interfere with another surface and polarly localized component and the absence of the O-antigen structure in the LPS mutants may lead to the exposure of this polar component that mediates aberrant (and strong) cell-cell interactions. Several surface-associated factors have been shown to display polar localization. The RapA lectins of *R. leguminosarum* have affinity for the EPS and CPS and are polarly localized on the cell surface (17, 68), and the glucomannan polysaccharide is also located at one pole on the bacteria (69). Further studies will be required to understand the interplay between the LPS, polar molecules, and cell-cell interactions.

Impaired attachment and biofilm formation have been reported for O-antigen- or core-oligosaccharide-deficient mutants in other species, such as *Xanthomonas citri* subsp. *citri* (37), *Pseudomonas fluorescens* SBW25 (36), and *E. coli* (39). In laboratory and clinical isolates of *E. coli*, several lines of evidence, obtained using time-lapse microscopy, pointed to a model in which elec-

trostatic interactions between the poly-*N*-acetylglucosamine (PNAG) polysaccharide and the LPS are critical for PNAG-induced biofilm formation (39). *Pseudomonas aeruginosa* LPS mutants that lack or display truncated core or O-antigen oligosaccharides had enhanced biofilms on abiotic surfaces and/or host surfaces in comparison to the parental strain (38). *P. aeruginosa* biofilm interactions assayed by microbead force spectroscopy and atomic-force microscopy revealed that in contrast to what we observed with rhizobial mutants, cell adhesion and cohesion (cell-to-cell adherence) were enhanced in mutants with core and O-antigen defects (70). Furthermore, an O-antigen-deficient mutant of *Bradyrhizobium japonicum* showed an enhanced biofilm formation on a polyvinyl chloride (PVC) surface apparently due to a cell surface more hydrophobic than that of the wild-type strain (71). Similarly, lack of the O-antigen in a mutant of *Rhizobium rhizogenes* enhanced adherence among cells, allowing higher bacterial numbers within the biofilms formed on either an abiotic or the root tip surface (72). These observations all support the hypothesis that the exposed moiety of the LPS is important to develop biofilms. Differential phenotypes suggest that the overall effect of a mutation in a LPS biosynthetic gene depends on the interplay between the hydrophobic nature of both the surface and the O-antigen core region and the other extracellular factors involved in biofilm formation.

The LPS-defective mutants of *R. leguminosarum* were affected in the nodulation process, since the developed nodules were white and free of bacteria. Impaired nodulation phenotypes were also reported for other LPS mutants of *R. leguminosarum* (27, 52), *R. etli* (64, 65), and *S. meliloti* (73). We showed that mutants that lack the surface-exposed portion of the LPS are altered in both the initial attachment to the root epidermis and the formation of compact root-associated bacterial aggregates at later stages. Interestingly, the LPS mutants showed a tendency to develop bacterial clumps around the root hairs, while this pattern was barely observed in the parental strains. Therefore, absence of the surface-exposed moiety of the LPS may affect root colonization and eventually root hair invasion. But other factors were shown to be required to colonize the root surface. As mentioned previously, glucomannan is required for initial and polar bacterial binding along the root hair surface (19, 69), and induction of cellulose synthesis is responsible for cap formation on the hair root surface (19, 74, 75). It will be interesting to perform further studies to evaluate the relation between the O-chain core region of the LPS

FIG 6 Rhizobial biofilm formation on pea roots. (A) Five-day-old GFP-labeled biofilm formed by the WT strains and the LPS derivative mutants. Note the compact microcolony patches formed by the A34 and 3841 WT strains and the ramified or star-like microcolonies scattered on the root epidermis developed by the mutants. 6 $\times$ -zoom images (right) show the detail of a root-attached bacterial aggregate. Magnifications: $\times 400$ (left) and $\times 2,400$ (right). (B) CLSM images of bacterial aggregates associated to root hairs. White arrows indicate bacterial clumps associated to root hairs developed by the LPS mutants. Magnification: $\times 400$. CLSM images are horizontal (x-axis) projections of representative images of five independent experiments. Size bars indicate 10 μm .

and the glucomannan-cellulose-induced attachment of *Rhizobium* to host surfaces.

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