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The O-chain core region of the lipopolysaccharide is required for cellular cohesion and compaction of *in vitro* and root biofilms developed by *Rhizobium leguminosarum*

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SUMMARY

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.

INTRODUCTION

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 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). In *S. meliloti* strain 1021, attachment to polystyrene and growth as a biofilm depends on the environmental conditions (11) and biotic and abiotic surfaces colonization is affected by succinoglycan production (12). Establishment of a three dimensional biofilm structure and autoaggregation depend on the production of another exopolysaccharide, 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/CPS of *R. leguminosarum*, supporting a role of Rap(s) in the development of the biofilm matrix (17). Recently,

overexpression of several Rap(s) 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 calcium influences surface roughness and the hydrophilic character that will ultimately affect 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 common 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 it 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 either absent or, when present, they 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-gluco-hexuronic acid (Glc3NAmA, 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. Besides, the outermost part of the LPS influences adhesion properties on both abiotic and root surfaces.

EXPERIMENTAL PROCEDURES

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

125 medium (41) containing mannitol (0.2%, wt/vol) as carbon source. *Escherichia coli*
126 cultures were grown at 37°C in LB medium (42). Bacterial growth was monitored at
127 600 nm using an Amersham Pharmacia spectrophotometer. Plasmids were mobilized
128 into *Rhizobium* by triparental mating using a helper strain of *E. coli*. Cellulose
129 production was detected using Y-mannitol minimal medium agar plates containing
130 0.001% (w/v) Congo red. *R. leguminosarum* strains were inoculated using a toothpick
131 and cultured for three days at 28^{o}C. Red or pink colonies are indicative of cellulose
132 production in *Rhizobium leguminosarum* (19, 43). Swimming motility was assayed (10)
133 by inoculating bacteria from cultures (OD_{600nm} adjusted to 1.0) on Y-mannitol minimal
134 medium containing 0.3% agar and measuring the colony diameters after 4 days growth.
135 Statistical analysis was done using GraphPad Prism 5 software.
136
137 **Tn*5* mutagenesis and screening of the LPS mutants**
138 A suicide plasmid pJB4JI containing Tn*5* was conjugated from *E. coli* into *R.*
139 *leguminosarum.* bv. *viciae* A34 by filter mating as described (44). The *lpcA* mutant was
140 identified by screening for colonies with a rough morphology on TY agar. To determine
141 the Tn*5* insertion site, an EcoRI fragment containing the transposon from genomic DNA
142 of the mutant was cloned in pBluescript. A BamHI fragment was subcloned in
143 pBluescript and PCR amplified using primers from the end of IS50
144 (TTCCGTTCAGGACGCTA) and the T7 (GTAATACGACTCACTATAGGGC) site
145 from pBluescript. The PCR product was sequenced to identify the transposon insertion
146 point. The *lpcB* and *lpsD* mutant derivatives of strain 3841 were isolated by gene-
147 specific PCR amplification using pools of Tn*5* mutants as previously described (19) and
148 the insertion sites confirmed by DNA sequencing using products amplified by Tn5 and
149 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 oligonucleotide 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 (bold): *lpcAB*for: CA**GGATCC**TCTAAGTTCACGTTCCGATTC and *lpcAB*rev: AG**GGATCC**GCCACGTAGCGTCAACTCAAAG. A PCR product of 2074 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 (bold): *lpsD*for **CAAGATCT**GAAGGTTCGACACGCCCATATTG and *lpsD*rev **CAAGATCT**CGAGCCAATACGGCTACCTCAG. A PCR product of 1600 bp, including the coding and regulatory sequences of *lpsD* (pRL90053), was cloned into pGEMTeasy (pGEMTeasy Cloning kit -Promega) to generate pFC219. The 1600 bp BglII fragment from pFC219 was subcloned in the BamHI site of the pLAFR3 cosmid to make pFC224.

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 1:1000 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 microtitre plates, rhizobia were

inoculated as above and cultured in 96 well flat bottom polystyrene (PE)-culture plates (Greiner, CellStar #655180) for three days without shaking. Unbound bacteria were removed by gently washing with 0.9% NaCl and attachment was quantified by staining with 0.1% crystal violet (46). To analyzed the biofilm structures, bacteria carrying the plasmid pRU1319, which expresses the green fluorescent protein (GFPuv) (47) were cultured for three days at 28 ^{o}C in 5 ml of TY medium. After centrifugation, bacteria were washed and suspended in Y-mannitol medium at 1:1000 dilution; 0,5 ml of this bacterial suspension was cultured statically in chambered glass-coverslides (Nunc, Lab Tek #155411) at 28^{o}C (9). Observation of biofilm formation in a four day time course experiment was done as previously described using Plan-Apochromat 100X/1.40 oil or C-Apochromat 40X/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 CSLM image-stacks taken from five independent experiments are shown. To quantify the biofilm structures developed in the chambers, at least five 40X-image stacks taken from three independent experiments were analyzed by COMSTAT software (48). Movies enclosed in supplementary files are representative image stacks of biofilms developed after four days in chambered coverslides from A34 and *lpcA* strains observed from the base to the top using a Plan-Apochromat 100X/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 wet cells) was suspended in sterile milliQ water and phenol (1:1) at 70 °C as described (28). After mixing with LAEMLI´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 immunoblots 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, UK) were used to detect the signals using a Storm 840 Imager (Amersham Pharmacia Biotech) following manufacturer´s instructions.

Quantification of EPS and CPS production

To obtain the EPS and CPS, rhizobia were cultured for five days at 28 °C in 100 ml Y-mannitol minimum medium and centrifuged at 8000 x *g* for 1 hour at 4 °C (53). The supernatants were re-centrifuged to remove remaining cells and then the EPS was precipitated with two 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 PBS pH 7.4 containing 1 mM $MgSO_4$ and centrifuged at 10000 x *g* for 15 minutes at 4 °C. The cells were suspended in PBS containing 1 mM MgSO4 and 0.5 M NaCl and stirred vigorously for 1 hour 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). Mean and standard error of replicated samples of EPS and CPS polysaccharides from two independent experiments are shown.

Autoaggregation assay

To monitor differences in auto-aggregation, each rhizobial strain from a TY medium-starter culture of four days was diluted to 1:100 in 5 ml of Y-mannitol or TY medium (inoculum OD_{600nm}= 0.01) and shaken (200 rpm) at 28°C. After five days, the cultures were mixed vigorously for 15 seconds and the suspensions were left standing to start the assay. At regular time intervals, a 150 µl-sample was taken at 0.5 cm from the liquid surface and the OD_{600nm} quantified in a microtiter plate in a Multimode Detector DTX880 Beckman Coulter 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, ten days post-germination *Pisum sativum* variety Frisson (pea) plantlets were dissected and roots sectioned in 1 cm-segments. Root sections were placed on a Fahräeus chamber containing 0.5 ml of 0.3% Fahräeus Plant Medium (FP)-agar and incubated for 45 minutes in 20 ml of GFP-tagged bacterial suspension ($OD_{600\ nm}$ 0.06) in darkness at room temperature (56). The pea roots sections were observed by scanning different focal planes of the root-surface using a C-Apochromat 40X/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 to 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 to 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_{600nm}=0.06) were used to inoculate each plantlet grown in FP and incubated at 22°C in a plant growth chamber (16 h light/ 8 h darkness). After five days, the entire plant was removed and the roots were washed twice in FP liquid medium under 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 colony-forming units (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. CSLM-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. v*iciae* 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 the genome sequenced strain 3841. The gene mutated in B772 is 99% similar to *lpcA* from *Rhizobium leguminosarum* bv. *phaseoli*

8002 (X94963.1) and is 90% similar to RL3440 from *R. l.* 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 co-transcribed 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 Tn*5* 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_PB0001) and LpsB2 (RHE_PB0002) from *R. etli* CFN42 are implicated in O-chain synthesis and 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 immunoblot using MAC 114 or MAC 57 antibodies, we

confirmed that the O-antigen is absent in the LPS fraction of the *lpcA*, *lpcB* and *lpsD* mutants (Fig. 1B). Silver-periodate staining and immunoblot analysis showed that *lpcA* and *lpcB* cloned in pFC222 complemented the LPS pattern of both the *lpcA* and *lpcB* mutants (Fig. 1B) and *lpsD* cloned in 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 (Fig. S1). 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 only differ in their degree of non-carbohydrate substitutions (53, 63). A defective LPS could affect the interaction of CPS with the cell surface. We observed a reduction of 30-40% 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 (Fig. S2), 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 (Fig. S3). The high carbon/nitrogen ratio of the Y-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 three 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 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* showed thicker rings of biofilms (Fig. S4) than WT strains while pFC222 (*lpcA* and *lpcB*) or pFC224 (*lpsD*) complemented the mutants to normal (not shown).

A possible interpretation of these results is that absence of the outermost part of the LPS makes rhizobia more proficient to attach to hydrophilic surfaces but less capable to attach to hydrophobic surfaces. Other 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 GFP-labeled *lpcA* mutant grown for one 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 two or three days, the *lpcA* mutant formed unusual nets of bacteria connected mostly through their cell poles and after four days, loose and ramified structures were observed in contrast with the typical compact honeycomb-like structure developed by the WT (Fig. 4, Movies S1 and S2). The movies 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 one day, the *lpcB* and *lpsD* mutants also showed the formation of premature microcolonies, with most bacteria interacting through their poles and, 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 with 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, CSLM images were analyzed with the *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 40X/1.2 W objective (Fig. S5). In addition, the mutants showed reduction of both the roughness coefficient (Ra) and the surface to volume ratio in comparison to the isogenic WTs and 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 (38-48%) in the same layer. In the WT biofilms, bacterial coverage showed a pronounced reduction to 3-5% 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 interactions in the LPS-mutants leads to ramified and abnormal microcolony structures, which in turn results 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 pseudo-nodules induced by the *lpcA, lpcB, lpsD* mutants (data not shown). This indicates that nitrogen fixation was not taking place

401 fitting with the observation that the plants showed signs of nitrogen deficiency (data not
402 shown).

403 Initial attachment to pea roots was evaluated after 45 minutes of incubation of 1
404 cm-root sections with rhizobia in FP medium using Fahräeus chambers (19, 56). CSLM
405 visualization of root sections showed that both A34 and 3841 initially attached to the
406 root epidermis as single bacterium or groups of 2-3 bacteria (Fig. S6). In contrast, the
407 *lpcA*, *lpcB* and *lpsD* mutants were seen associated to the epidermal root surface as star-
408 like microcolonies (Fig. S6). This is probably related with the premature formation of
409 abnormal microcolonies in the mutants. Projections of Z-stack images from different
410 scanned root sections showed that these microcolonies were attached to the surface by a
411 limited number of bacteria. In line with this observation, the proportion of bacteria that
412 attach directly to the epidermis surface relative to total rhizobia counted in the same
413 image (attachment index: AI) was lower in all the mutants in comparison with the
414 isogenic WT strain (Fig. S7). We examined the total bacteria associated to the root
415 surface, i.e., observed in all focal planes, per square centimeter of root section using
416 Zeiss Image Browser software. Comparable amounts of WTs and mutant bacteria per
417 square centimeter of root section scanned were observed (Fig. S7). Therefore, although
418 the mutants deficient in the outermost part of the LPS were initially able to colonize the
419 root epidermis, anchoring of individual bacteria to the root surface seemed to be
420 impaired.

421 Biofilms associated to root surfaces at a later stage were examined five days
422 after inoculation of whole plantlets with the different strains. The WT strains developed
423 compact and robust patch-like bacterial aggregates mostly distributed on the epidermis
424 of the pea roots, whereas bacterial aggregates of the *lpcA*, *lpcB* and *lpsD* mutants were
425 scattered on the root epidermis and, in general, they 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 where 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 (Fig. S7), 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 to 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 here, 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 is 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.

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 together with the core oligosaccharide are involved in a tight attachment of the CPS on the cell surface (26, 67). We observed a 30-40% 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/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 other 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 located molecules and cell-cell interactions.

476 Impaired attachment and biofilm formation have been reported for O-antigen- or
477 core-oligosaccharide-deficient mutants in other species, such as, *Xanthomonas citri* ssp.
478 *citri* (37), *Pseudomonas fluorescens* SBW25 (36) and *E. coli* (39). In laboratory and
479 clinical isolates of *E. coli*, several lines of evidence, using time-lapse microscopy,
480 pointed to a model in which electrostatic interactions between the poly-N-
481 acetylglucosamine (PNAG) polysaccharide and the LPS, are critical for PNAG-induced
482 biofilm formation (39). *Pseudomonas aeruginosa* LPS mutants that lack or display
483 truncated core or O-antigen oligosaccharides had enhanced biofilms on abiotic surfaces
484 and/or host surfaces in comparison to the parental strain (38). *P. aeruginosa* biofilm
485 interactions assayed by microbead force spectroscopy and atomic force microscopy
486 revealed that, in contrast to what we observed with rhizobial mutants, cell adhesion and
487 cohesion (cell-to-cell adherence) were enhanced in mutants with core and O-antigen
488 defects (70). Furthermore, an O-antigen-deficient mutant of *Bradyrhizobium japonicum*
489 showed an enhanced biofilm formation on a polyvinyl chloride (PVC) surface
490 apparently due to a cell surface more hydrophobic than that of the wild-type strain (71).
491 Similarly, lack of the O-antigen in a mutant of *Rhizobium rhizogenes* enhanced
492 adherence among cells, allowing higher bacterial numbers within the biofilms formed
493 on either an abiotic or the root tip surface (72). These observations all support the
494 hypothesis that the exposed moiety of the LPS is important to develop biofilms.
495 Differential phenotypes suggest that the overall effect of a mutation in a LPS
496 biosynthetic gene depends on the interplay between the hydrophobic nature of both, the
497 surface and the O-antigen core region and the other extracellular factors involved in
498 biofilm formation.

499 The LPS-defective mutants of *R. leguminosarum* were affected in the nodulation
500 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, 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 and the glucomannan-cellulose induced attachment of *Rhizobium* to host surfaces.

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Legends to figures

Figure 1. (A) Diagram of the *R. leguminosarum* strain 3841 *loci* organization involved in LPS biosynthesis. The location of each gene in 3841 chromosome (or pRL9 plasmid) is indicated by numbers relating to the genome sequence. The positions of the Tn*5* insertions in mutants are shown by inverted black (A950 and A951) or white arrows (B772). The lower bold lines indicate the amplified products used to generate the complementation plasmids. *δ-lps locus: cpaA* encodes a LPS-associated cation exporter; *lpcB* encodes a CMP-Kdo transferase; *lpcA* encodes a galactosyl transferase. pRL9-borne *β locus*: *lpsB2* encodes a hypothetical O-antigen biosynthesis related protein; *lpsB1* a putative galactosyl transferase protein; *lpsD* a putative O-antigen ligase. **(B)** 12% SDS PAGE-Silver periodate oxidation (left) or immunoblot (right) analysis of the LPS extracted from A34, 3841, *lpcA*, *lpcB*, *lpsD* and complemented strains. O-chains were detected by immunoblots using the specific monoclonal antibodies MAC 114 or MAC57, which recognize the O-LPS from A34 or 3841 strains, respectively. LPS I and LPS II components of the LPS are indicated.

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

Figure 3. Rhizobial adhesion to a hydrophobic abiotic surface. *R. leguminosarum* A34 or 3841 derivative strains were grown in polystyrene multiwell plates in static Y-

551 mannitol minimal medium for 3 days at 28 °C and bacterial attachment was quantified
552 by crystal violet (CV) staining. Horizontal values correspond to average of six replicate
553 samples in at least two different experiments. (***) p<0.0001 One way ANOVA was
554 performed using Graphpad Prism 5 software.

555 **Figure 4. Cellular interactions and biofilms formed by *R. leguminosarum* A34**
556 **derivative strains.** CLSM images are horizontal (X-axis) projections of optical sections
557 showing bacterial attachment at day 1 and the biofilms formed at day 4 in chambered
558 coverglass slides (1000 X magnification) by A34, the isogenic LPS-mutant *lpcA* and the
559 complemented *lpcA* pFC222 strains. The inserted images are zooms (3X). Sized bars
560 indicate 2 µm.

561 **Figure 5. Cellular interactions and biofilms formed by *R. leguminosarum* 3841**
562 **derivative strains.** CLSM images showing bacterial attachment at day 1 and biofilms
563 formed at day 4 in chambered coverglass slides by 3841, the isogenic LPS-mutants *lpcB*
564 and *lpsD* and the complemented *lpcB* pFC222 and *lpsD* pFC224 strains after one and
565 four days (1000X magnification). The inserted images are zooms (3X). Sized bars
566 indicate 2 µm.

567 **Figure 6. Rhizobial biofilm formation on pea roots. (A)** Five-days-old GFP-labelled
568 biofilm formed by the WT strains and the LPS derivative mutants. Note the compact
569 microcolony-patches formed by the A34 and 3841 WT strains and the ramified or star-
570 like microcolonies scattered on the root epidermis developed by the mutants. 6X-zoom-
571 images (right) show the detail of a root-attached bacterial aggregate. Magnifications:
572 400X (left) or 2400X (right). **(B)** CSLM images of bacterial aggregates associated to
573 root hairs**.** White arrows indicate bacterial clumps associated to root hairs developed by
574 the LPS mutants. Magnification: 400X. CSLM images are horizontal (X axis)

projections of representative images of five independent experiments. Sized bars indicate 10 µm.

579 **Table 1. Strains and Plasmids used in this work**.

Strain/ Plasmid	Description	Source or Reference
3841	*R. leguminosarum* bv. *viciae* 3841 (Str^R)	(76)
A34	*R. leguminosarum* bv. *viciae* 8401 /pRL1JI (Str^R)	(77)
A950	Mutant of 3841, *lpsD*::Tn*5*	This work
A951	Mutant of 3841, *lpcB*::Tn*5*	This work
B772	Mutant of A34, *lpcA*:: Tn*5*	This work
pRU1319	Plasmid pOT1 carrying green fluorescent protein (GFPuv)	(47)
pJB4JI	pPH1JI derivative plasmid carrying Mu and Tn*5*.	(44)
pGEM-T easy	Cloning vector for PCR products	Promega
pLAFR3	Broad host range cosmid cloning vector	(78)
pFC222	pLAFR3 cosmid carrying the *lpcA* and *lpcB* genes and the upstream regulatory sequences from 3841	This work
pFC224	pLAFR3 cosmid carrying the *lpsD* gene and the upstream regulatory sequences from 3841	This work

580

581

582 **Table 2. EPS and CPS production**

Strain	EPS (mg GlcA equivalents/100 ml)	CPS (µg GlcA equivalents/100 ml)
A34	17± 2	127 ± 4
lpcA	17 ± 7	73 ± 23 (-42%)
3841	27 ± 6	113 ± 6
lpcB	22 ± 8	69 ± 5 (-39%)
lpsD	23 ± 3	75 ± 5 (-34%)

583

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

586

587 **Table 3. COMSTAT analysis of four-day biofilms**

588

Parameter		A34	*lpcA*	3841	*lpcB*	*lpsD*
Average thickness (µm)		28 ± 6	103 ±12 (**)	31 ± 6	103 ± 16 (*)	110 ± 18 (*)
Roughness coefficient		0,41 ± 0,06	0,23 ± 0,02 (*)	0,66 ± 0,03	0,45 ± 0,06	0,31 ± 0,03 (*)
Surface to volume ratio ($\mu m^2/\mu m^3$)		0,12 ± 0,01	0,05 ± 0,00 (*)	0,08 ± 0,00	0,04 ± 0,00	0,04 ± 0,01
Percentage of the area covered by bacteria in each layer (%)	**Layer 1**	26,9 ± 3,2	7,8 ± 5,4 (*)	26,8 ± 5,7	7,5 ± 1,5 (*)	4,0 ± 2,0 (*)
	Layer 15	88,9 ± 0,2	48,7 ± 5,9 (*)	88,8 ± 7,9	39,6 ± 6,9 (*)	38,2 ± 2,6 (**)
	Layer 50	3,9 ± 0,7	23,8 ± 9,0 (***)	5,9 ± 2,6	40,0 ± 3,8 (*)	12,7 ± 1,7
	Layer 150	_	3,0 ± 2,7	_	1,3 ± 0,7	3,0 ± 1,3

589

590 Values are means of data from at least 5 independent experiments. Parameters were
591 calculated using COMSTAT and statistical analysis by Graphpad Prism 5 software (One
592 way ANOVA (*) $p<0.05$; (**) $p<0,01$; (***) $p<0,001$).
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