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Publication Date

2018

DOI

10.3389/fmicb.2018.02363

Peer reviewed

Cell Autoaggregation, Biofilm Formation, and Plant Attachment in a *Sinorhizobium meliloti* *lpsB* Mutant

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Accepted 9 May 2018.

Bacterial surface molecules are crucial for the establishment of a successful rhizobia-legume symbiosis, and, in most bacteria, are also critical for adherence properties, surface colonization, and as a barrier for defense. Rhizobial mutants defective in the production of exopolysaccharides (EPSs), lipopolysaccharides (LPSs), or capsular polysaccharides are usually affected in symbiosis with their plant hosts. In the present study, we evaluated the role of the combined effects of LPS and EPS II in cell-to-cell and cell-to-surface interactions in *Sinorhizobium meliloti* by studying planktonic cell autoaggregation, biofilm formation, and symbiosis with the host plant *Medicago sativa*. The *lpsB* mutant, which has a defective core portion of LPS, exhibited a reduction in biofilm formation on abiotic surfaces as well as altered biofilm architecture compared with the wild-type Rm8530 strain. Atomic force microscopy and confocal laser microscopy revealed an increase in polar cell-to-cell interactions in the *lpsB* mutant, which might account for the biofilm deficiency. However, a certain level of biofilm development was observed in the *lpsB* strain compared with the EPS II-defective mutant strains. Autoaggregation experiments carried out with LPS and EPS mutant strains showed that both polysaccharides have an impact on the cell-to-cell adhesive interactions of planktonic bacteria. Although the *lpsB* mutation and the loss of EPS II production strongly stimulated early attachment to alfalfa roots, the number of nodules induced in *M. sativa* was not increased. Taken together, this work

demonstrates that *S. meliloti* interactions with biotic and abiotic surfaces depend on the interplay between LPS and EPS II.

Lipopolysaccharide (LPS) is one of the most important structural components of the outer membrane of gram-negative bacteria by contributing to structural properties and acting as a permeability barrier. LPS is formed by amphiphilic glycoconjugates of variable composition within and between species and consists of three portions, lipid A, core oligosaccharides, and O antigen (Lerouge and Vanderleyden 2002). Because of their position at the contact zone with the external environment, the LPS of many bacterial species is the main determinant of interaction with biotic or abiotic surfaces (Benito et al. 1997; Bouchet et al. 2003; Harvill et al. 2000; Lee et al. 2014; Lindhout et al. 2009; Nesper et al. 2001).

For the symbiotic bacteria of legume plants, collectively called rhizobia, LPS contributes to the establishment of the symbiotic relationship by suppressing host defenses, facilitating rhizobial entry into root hairs (Scheidle et al. 2005; Tellström et al. 2007), promoting infection thread formation (Dazzo et al. 1991; de Maagd et al. 1989), and, eventually, bacteroid differentiation (Campbell et al. 2002; Margaret et al. 2013; Stacey et al. 1991).

The *Medicago* symbiont *S. meliloti* produces a heterogeneous population of LPS, LPS-1, and LPS-2, based on electrophoretic profiles. It is believed that LPS-2 contains rough LPS (R-LPS), which lacks O-antigen, and that LPS-1 consists of smooth LPS, which includes the O-antigen (Sharypova et al. 2003). The *lpsB* gene codes for a type I glycosyltransferase involved in the synthesis of the LPS core. A *lpsB* mutant shows dramatic changes in the sugar composition of R-LPS, notably an increase in the relative amount of xylose and a disappearance of uronic acids as well as the absence of immunoreactivity with antibodies raised against wild-type (wt) LPS (Campbell et al. 2003; Kanipes et al. 2003; Lagares et al. 1992).

The strain Rm6963 (*lpsB* mutant in the Rm2011 background) is Fix⁺ in alfalfa but is less competitive for nodulation than the wt strain, showing a delayed onset of nodulation and a reduced ability to nodulate the primary root, which results in a higher number of nodules, albeit smaller in size, being formed on lateral roots (Lagares et al. 1992). Interestingly, this mutant is Fix⁻ with *M. truncatula* as its host and the nodules exhibit signs of a strong defense response and an abnormal pattern of infection (Niehaus et al. 1998). The *lpsB* mutation in the Rm1021 genetic background displays a Fix[±] phenotype with both *M. sativa* and *M. truncatula* hosts (Campbell et al. 2003).

Funding: Grants from the Secretaría de Ciencia y Técnica de la UNRC, Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) and Consejo Nacional de Investigaciones Científicas y Técnicas of the República Argentina (CONICET) supported this work. The Shanbrom Family Foundation supported biofilm research at the University of California-Los Angeles. Fernando Sorroche was supported by a fellowship from CONICET and also in part by a UNESCO American Society for Microbiology travel award. Pablo Bogino, Daniela M. Russo, Angeles Zorreguieta, Fiorela Nievas, Gustavo M. Morales, and Walter Giordano are career members of CONICET.

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*The e-Xtra logo stands for “electronic extra” and indicates that four supplementary figures and two supplementary tables are published online.

Because LPS is exposed to the external environment, it is therefore potentially active in terms of adhesive interactions with living and inert interfaces. Alterations in LPS lead to alterations in biofilm formation in several bacterial species, including *Escherichia coli* (Puttamreddy et al. 2010), *Pseudomonas aeruginosa* (Lau et al. 2009), *Xanthomonas axonopodis* (Li and Wang 2011a), *Klebsiella pneumoniae* (De Araujo et al. 2010), and *Porphyromonas gingivalis* (Yamaguchi et al. 2010).

It is likely that different polymer types mediate attachment depending on substrate chemistries. For example, polymers with nonpolar sites, such as LPS, may dominate in binding to hydrophobic surfaces, whereas polymers capable of hydrogen bonding or electrostatic interactions, such as polysaccharides, may govern binding to hydrophilic surfaces. Different polymer types may also act cooperatively in binding to a surface to stabilize the adhesive interaction. For example, a *Pseudomonas fluorescens* mutant lacking the O antigen of the LPS, which results in the consequent increased exposure of the lipid moiety of the LPS, displayed increased adhesion to hydrophobic substrates (Williams and Fletcher 1996). LPS is important in biofilm formation in rhizobia and the effects depend on the species, the particular mutation, or both. In fact, Lee et al. (2010) described a LPS mutant of *Bradyrhizobium japonicum* lacking the O antigen that showed increased formation of biofilms on a plastic support. In *Rhizobium leguminosarum* bv. *viciae* 3841, the participation of the lipid A component of the LPS in desiccation tolerance, biofilm formation, and motility has been reported (Vanderlinde et al. 2009), and mutants of *R. leguminosarum* bv. *viciae* A34 defective in the O chain or O-chain core moiety developed biofilms with an altered three-dimensional structure (Russo et al. 2015).

Table 1. *Sinorhizobium meliloti* strains used in this study

Strain	Relevant properties	Reference
Rm8530	SU47 <i>str21 expR101 (expR+)</i> , referred as the wild type in this work	Glazebrook and Walker 1989
Rm6963	Rm2011 <i>lpsB::Tn5</i>	Lagares et al. 1992
Rm8530 <i>expA</i>	<i>expA3::Tn5-233</i>	Sorroche et al. 2010
Rm8530 <i>lpsB</i>	Rm8530 <i>lpsB::Tn5</i>	Present work
Rm8530 <i>lpsB</i> pBBRLPSB	Complemented Rm8530 <i>lpsB</i>	Present work
Rm8530 <i>expA lpsB</i>	Rm8530 <i>expA::Tn5-233 lpsB::Tn5</i>	Present work

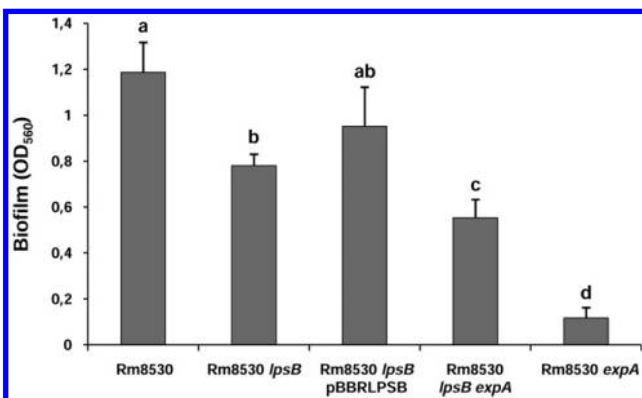


Fig. 1. Biofilm formation on microtiter plates after growth in tryptone yeast broth for 24 h, under shaking conditions (200 rpm). Low phosphate minimal glutamate medium (MGM) was used for the biofilm experiments because it facilitates EPSII production (Marketon and González 2002). Error bars represent standard deviation of the mean of at least four independent experiments with eight replicates each. Different letters indicate statistically significant differences, according to Fischer least significant difference test ($P \leq 0.05$).

Planktonic autoaggregation, which is based on adhesive interactions among bacteria (Rickard et al. 2003; Sorroche et al. 2012), provides information about bacteria-to-bacteria interactions that causes them to settle and sediment. Surface structures and extracellular polysaccharides, in combination with environmental signals, are critical for autoaggregation and biofilm development in most bacterial species (Fujishige et al. 2006; Schembri et al. 2001; Sorroche et al. 2010). For many rhizobacteria, including rhizobia, autoaggregation and biofilm formation are important for bacterial survival and plant colonization (Bogino et al. 2013).

In *S. meliloti*, autoaggregation, mucoid phenotype, and biofilm formation are three traits that were shown to depend on EPS II production (Rinaudi and González 2009; Sorroche et al. 2010). Moreover, a positive correlation was found between autoaggregation and biofilm formation in native *S. meliloti* strains, indicating that both phenotypes depend on the same physical adhesive forces (Sorroche et al. 2012). In this work, we explored the effect of a mutation in LPS in the presence and absence of EPSII in terms of cell-to-cell and cell-to-surface interactions as well as in symbiosis with the host plant.

RESULTS AND DISCUSSION

The *lpsB* mutation alters cell-to-cell interactions and biofilm formation in *S. meliloti*.

The *S. meliloti* strains used in this study are described in Table 1. Crystal violet staining of the attached bacterial populations in microtiter plate wells revealed an approximately 30% reduction in biofilm formation in the *lpsB* mutant as compared with the wt strain (Fig. 1), suggesting that LPS plays a role in bacterial attachment to plastic surfaces. We earlier demonstrated that EPS II provides an extracellular matrix to promote biofilm development on plastic and glass surfaces. Strains that did not produce EPS II, such as Rm8530 *expA* or Rm1021, were drastically affected in biofilm formation (Rinaudi and González 2009; Sorroche et al. 2012). Thus, we questioned whether EPS production was affected in the *lpsB* mutant. After 7 days of incubation on solid medium, the *lpsB* single mutant strain seemed to be slightly less mucoid than the wt strain (Fig. 2). Anthrone-mediated EPS quantification revealed a slight decrease in EPS production by the *lpsB* mutant (Supplementary Table S1). Moreover, swimming motility (Supplementary Figs. S1 and S2) and growth in the *lpsB* mutant background were strongly reduced (Supplementary Fig. S3; Supplementary Table S2). These additional phenotypes indicate that the *lpsB* mutation has pleiotropic effects and that the defect in growth might explain the reduced swimming behavior of this mutant.

The EPS II-deficient strain *expA* was strongly affected in biofilm formation, as previously shown by Rinaudi and González (2009), so we predicted that the double mutant *expA lpsB* would form an even more reduced biofilm. However, the *lpsB* mutation partially rescued the biofilm-deficient phenotype of *expA* (Fig. 1). Although *expA* and *expA lpsB* mutants did not show clear differences in mucoid appearance after 7 days of incubation on solid medium, culture supernatants of the latter contained more than twice the amount of anthrone-positive material as the single *expA* mutant and even more so in comparison with an *exoY expA* double. Taken together, the above results indicate that in the absence of EPS II, the *lpsB* mutation enhances attachment to plastic. A possible explanation is that a *lpsB* mutant has a more hydrophobic surface or that it produces another polysaccharide.

The reduced biofilm effect of the *lpsB* mutation observed in the presence of EPS II prompted us to explore biofilm architecture and cellular interactions in the wt strain and *lpsB* mutant

in greater detail, using confocal laser scanning microscopy (CLSM) (Fig. 3). We therefore restricted our observations to EPS II-producing strains, because it has been shown that EPS II *S. meliloti* strains develop a flat and unstructured biofilm (Rinaudi and González 2009). Green fluorescent protein (GFP)-tagged wt and *lpsB* mutant biofilms were grown in minimal glutamate medium (MGM) for 3 days inside glass chambers and were visualized by CLSM. As shown in Figure 3, biofilm formation was notably perturbed in the *lpsB* mutant as compared with wt strain, which developed a more compact and organized structure. A similar result was observed in plant-pathogenic *Xanthomonas* spp. (Petrocelli et al. 2012; Li and Wang 2011b) and *Xylella fastidiosa* (Clifford et al. 2013), in which LPS mutants developed less-structured biofilms.

Honeycomb-like structures are a type of highly ordered bacterial array in which the bacteria adhere to each other through lateral cell interactions, forming rows of identically oriented cells. Rhizobial biofilms with honeycomb-like structures have been previously reported in *R. leguminosarum* (Russo et al. 2006) and *S. meliloti* Rm8530 (Rinaudi and González 2009). In the latter, EPS II seems to be essential for the development of these bacterial arrangements. As expected, honeycomb-like structures were visualized in the Rm8530 strain (Fig. 3A). Interestingly, despite the fact that *lpsB* synthesizes EPS II, honeycomb-like arrays were not observed in mutant biofilms (Fig. 3B). Although lateral interactions between cells occurred in both wt and mutant rhizobia, polar interactions were seen almost exclusively in the *lpsB* mutant, leading to the formation of typical bacterial zigzag rows or bouquets, as observed by atomic force microscopy (AFM) and CLSM (Fig. 4A and B, respectively).

These observations indicate that *lpsB* has a role in determining the type of cell-to-cell interaction and, therefore, biofilm development. Microscopic observations suggest that an increase in cell-to-cell polar interactions in the *lpsB* mutant disrupts the development of a highly structured biofilm. Our results agree with those obtained in *Pseudomonas aeruginosa*, in which changes in LPS expression resulted in quantifiable cellular mechanical changes that were correlated with structural changes in bacterial biofilms (Lau et al. 2009). Conversely, in *Bradyrhizobium* spp., an altered LPS leads to increased biofilm development (Lee et al. 2010, 2014).

Taken together, our results indicate that a mutation affecting the LPS core impacts biofilm formation, possibly via an increase of polar interactions between the bacteria.

Cell-to-cell adhesive interactions in the planktonic phase are increased in the *lpsB* mutant.

Based on our previous results on biofilm formation of *S. meliloti*, we proceeded to study cell-to-cell interactions of planktonic bacteria. During bacterial growth in MGM, we noticed that the *lpsB* mutants were so strongly flocculated that it

proved almost impossible to disperse the rhizobia after vortexing, suggesting that the mutated LPS strongly increased cell-to-cell interaction (Supplementary Fig. S4). In addition, observations of 12 images (40 × 40 μm) obtained from AFM of three independent cultures of each strain allowed us to determine that, in the wt strain, 61% of the bacteria were solitary, whereas 39% of the bacterial cells were clustered. By contrast, a reciprocal relationship was seen in the *lpsB* mutant in that 34% of the bacteria were solitary and 66% of the cells were in groups. Formation of the flocs during growth in MGM, however, seemed not to be correlated with the quantity of anthrone-positive

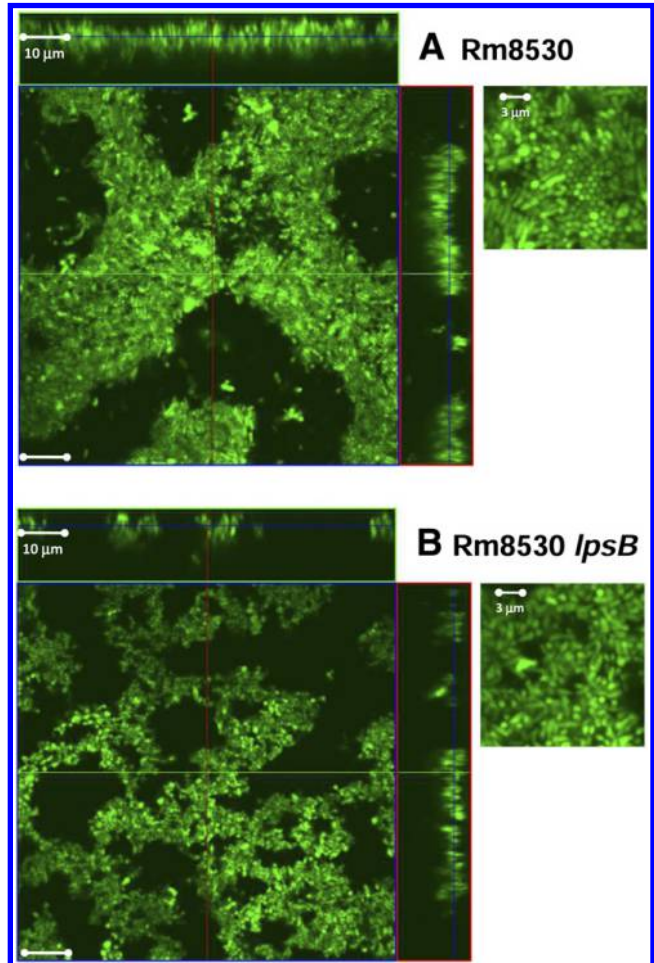


Fig. 3. A, Confocal laser scanning microscopy of green fluorescent protein-tagged Rm8530 and B, Rm8530 *lpsB* strains. Biofilm structure at 72 h. Higher magnification views on the right show differences in biofilm architecture between the two strains. Lateral cell interactions and honeycomb-like structures were visualized only in the Rm8530 strain.



Fig. 2. Bacterial growth on Luria Bertani (LB) plates. Mucoid (Rm8530 and Rm8530 *lpsB*) and nonmucoid (Rm8530 *expA* and Rm8530 *expA lpsB*) phenotypes were observed after 7 days of incubation at 28°C. The mucoid phenotype, present or absent, is more obvious on LB plates.

material in culture supernatants and was independent of EPS II, because both wt and *expA*-mutant cultures grew as dispersed cultures. In contrast, in tryptone yeast (TY) rich medium, all strains grew normally as suspensions, and autoaggregation was manifested only in EPS II-producing strains when they were left in static conditions after late exponential growth phase, as previously described in Sorroche et al. (2010).

To quantify autoaggregation, we used bacterial cultures grown in TY medium and proceeded as described below. In these conditions, the *lpsB* mutant displayed full autoaggregation, similar to the wt strain (Fig. 5). We hypothesized that the strong aggregative activity of EPS II masks the relatively milder adhesive effects of the mutated LPS, thus precluding the study of the specific contribution of the *lpsB* mutation in autoaggregation under these conditions. To test this, we assayed the autoaggregation of the *lpsB* mutant in the presence of the *expA* mutation. As shown in Figure 5, the *expA lpsB* double mutant showed increased autoaggregation as compared with the *expA* single mutant, indicating that a mutated LPS increases cell-to-cell interaction that leads to an augmented autoaggregation percentage in the absence of EPS II.

To corroborate this, we centrifuged wt and *lpsB* cultures, washed the pellets to eliminate the EPS II in the culture medium, resuspended the cells in fresh TY medium, and left them to autoaggregate in static conditions. Suspensions using washed

pellets of *expA* and *expA lpsB* were included as controls. Under these artificial EPS-free suspension conditions, the autoaggregation percentage of both *lpsB* mutants was significantly higher than the wt strain (Table 2), whereas autoaggregation of

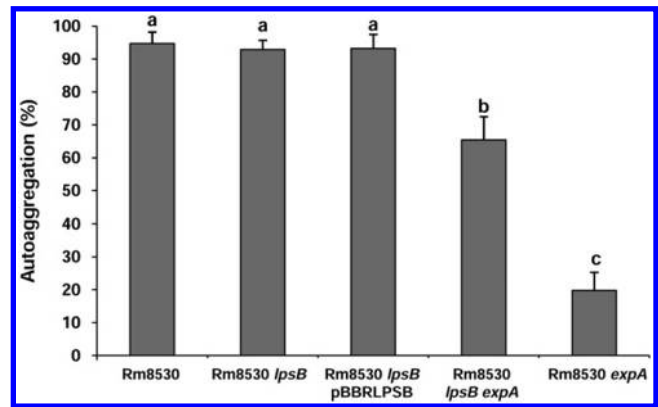


Fig. 5. Autoaggregation of the *Sinorhizobium meliloti* wt strain and the *expA* and *lpsB* mutants in tryptone yeast medium. Error bars represent standard deviation of the mean of at least three independent experiments with two replicates each. Different letters indicate statistically significant differences, according to Fischer least significant difference test ($P \leq 0.05$).

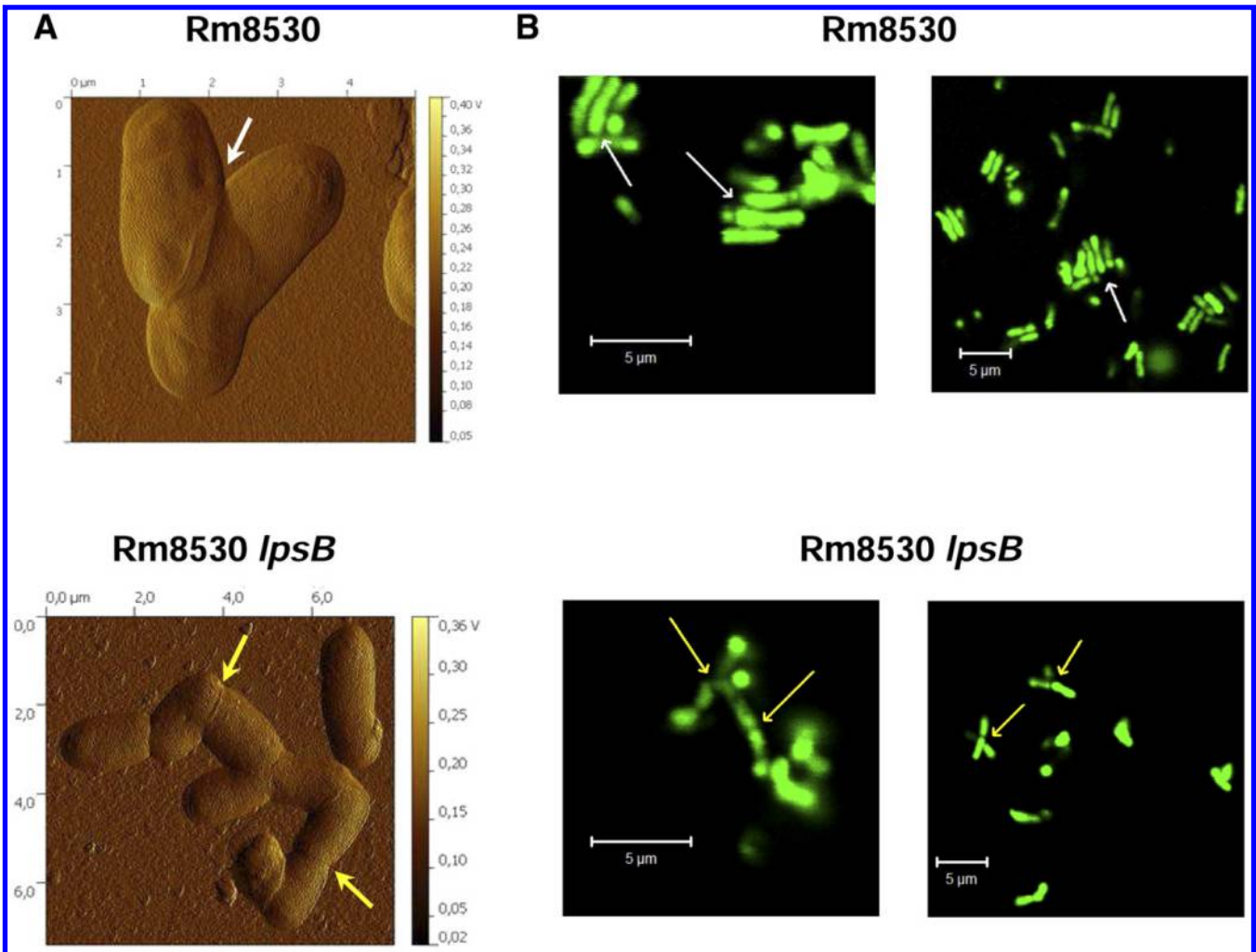


Fig. 4. Cell-to-cell interactions of Rm8530 and Rm8530 *lpsB* strains. **A**, Atomic force microscopy images of wild-type (wt) and mutant strains on PEI-modified glass ($5 \times 5 \mu\text{m}$ and $8 \times 8 \mu\text{m}$). **B**, GFPuv derivative strains after incubation in minimal glutamate medium for 24 h. Lateral bacterium-to-bacterium adhesive interactions of the Rm8530 wt strain are indicated with arrows in the upper images. Polar adhesive interactions of the Rm8530 *lpsB* strain are indicated with arrows in the lower images.

the *expA* mutant was similar to the wt strain. All washed pellets were also resuspended in cell-free EPS II-containing supernatants (from cultures of the wt strain) and full autoaggregation was observed in all artificial suspensions (Table 2).

Taken together, the above results suggest that the *lpsB* mutation generates a more adhesive LPS that stimulates bacterial autoaggregation independently of the production of extracellular polysaccharides. Such “stickiness” in a mutated LPS might be the result of an increased cell surface hydrophobicity. It has been previously reported that an altered LPS in *B. japonicum* increased cell surface hydrophobicity, which was related to cell aggregation (Lee et al. 2014).

A *lpsB* mutation stimulates bacterial adsorption to roots but does not improve symbiosis.

Rhizobial attachment to roots is a critical point in the establishment of a symbiotic interaction and is dependent on bacterial surface-associated components and root lectins (Gage 2004; Hirsch 1999; Rinaudi and Giordano 2010; Rodríguez-Navarro et al. 2007). We hypothesized that genes affecting adhesion to abiotic surfaces will also have an impact on rhizobial attachment to roots. Based on the different adhesive behaviors of *S. meliloti* mutants on inert surfaces, we quantified early adhesive interactions between rhizobial cells and alfalfa roots. In our experimental conditions, the wt strain showed the lowest percentage of root adhesion (0.04 ± 0.01). Mutations in LPS (Rm8530 *lpsB*) and EPS II (Rm8530 *expA*) significantly increased root attachment (0.67 ± 0.08 and 0.28 ± 0.1 , respectively), supporting the hypothesis that the rhizobial cell surface is changed, most likely by becoming stickier. Interestingly, the double mutant (Rm8530 *expA lpsB*) attached to alfalfa roots at a significantly higher percentage (1.13 ± 0.16) compared with the single mutants. For all *S. meliloti* mutants, the experiment was repeated three times.

Although rhizobial adsorption to roots was elevated in the treatments with the *expA* mutant compared with the wt strain, it was lower than that of the *lpsB* mutants. Previous results suggested that EPS II partially inhibits rhizobial adhesion to roots, possibly through a “shielding effect” (Sorroche et al. 2012). Even though we used highly diluted inocula in our adsorption assays (approximately 10^3 colony-forming units [CFU]/ml), we cannot rule out the possibility that weak adsorption of EPS II to the bacterial cell surface exerts an inhibitory effect through at

least three different mechanisms: i) EPS II causes rhizobia to aggregate, thus preventing them from adsorbing to roots, ii) EPS II does not exhibit affinity for the root surface, which results in a lack of adherence of EPS II-producing bacteria to roots, and iii) the presence of EPS II on the bacterial surface reduces free adhesives sites of the mutated LPS, thereby blocking cell adhesion to the root surface. Similar results were obtained for *R. rhizogenes*, in which a mutation in the *rkpK* gene that leads to the synthesis of R-LPS (LPS II) causes the formation of denser biofilms on both abiotic and root-tip surfaces and a strong autoaggregation phenotype. These data suggested that the lack of the O antigen in *rkpK* mutant LPS enhanced adherence among cells (Abarca-Grau et al. 2012).

Nodulation assays performed on *M. sativa* (Table 3) demonstrated that the higher degree of attachment to roots in *lpsB* mutants did not result in an increase of either nodule number or the number of nodulated plants 4 weeks postinoculation. These results indicate that rhizobial adsorption to roots does not correlate with the nodule number in *lpsB* mutants. It furthermore suggests a mechanism whereby the mutualistic interaction becomes restricted after the initial attachment phase of rhizobia to plant roots.

In conclusion, results obtained in the present study showed that the interplay between the *lpsB* mutation, leading to the synthesis of an altered LPS, and EPS II determines adhesion phenotypes. The mutation in LPS clearly influenced cell-to-cell physical interactions by increasing pole-to-pole attachment in both biofilms and planktonic cells. In addition, the mutants showed a greater tendency to autoaggregate independently of EPS II. However, the biofilm phenotype of *lpsB* mutant depended on EPS II in that a stimulation of biofilm formation was observed in the *expA lpsB* mutant as compared with the *expA* mutant (i.e., in absence of EPS II). On the other hand, if *lpsB* were mutated in an EPS II-producing background, a biofilm deficiency resulted instead. Mutations in LPS and EPS II improved rhizobial attachment to roots but did not stimulate nodulation. Our results also suggest that the coordination of LPS and EPS synthesis in the rhizosphere might have an impact on the colonization of host plants and possibly affect competition or cooperation with other bacteria.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

S. meliloti strains (Table 1) were grown in TY broth (Berliner 1974) on a rotary shaker (200 rpm) at 30°C, until reaching late exponential growth phase. For CLSM, the strains were subcultured (1:100) in MGM medium (Marketon and González 2002). When required, the final concentrations of antibiotics used were streptomycin, 500 µg/ml; neomycin, 200 µg/ml; and gentamicin, 40 µg/ml. For visualization of mucoid or nonmucoid phenotypes, *S. meliloti* strains were streaked onto plates containing Luria Bertani (LB) medium (Sambrook et al. 1989).

Phage transductions.

Transductions using phage ΦM12 were performed as described by Finan et al. (1984). The mutant allele *lpsB::Tn5* was transduced from the donor Rm6963 (Lagares et al. 1992) to

Table 2. Autoaggregation of artificial bacterial suspensions^a

Pellets	Resuspended in	
	TY medium	Supernatant from Rm8530
Rm8530	19.1 ± 4.3 b	95.4 ± 1.5 a
Rm8530 <i>lpsB</i>	69.4 ± 6.0 c	96.5 ± 1.5 a
Rm8530 <i>lpsB</i> pBBRLPSB	20.8 ± 7.0 b	94.2 ± 1.9 a
Rm8530 <i>expA lpsB</i>	65.0 ± 5.4 c	95.9 ± 1.9 a
Rm8530 <i>expA</i>	17.2 ± 5.9 b	93.5 ± 2.8 a

^aMean autoaggregation percentages and standard deviations of at least four independent experiments with two replicates each are shown. Different letters indicate statistically significant differences, according to Fischer least significant difference test ($P \leq 0.05$). TY = tryptone yeast.

Table 3. Nodulation assay^a

Plant responses	Rm8530	Rm8530 <i>lpsB</i>	Rm8530 <i>lpsB</i> pBBRLPSB	Rm8530 <i>expA lpsB</i>	Rm8530 <i>expA</i>	Noninoculated
Nodule number	2.7 ± 0.4	3.2 ± 0.4	3.1 ± 0.3	1.4 ± 0.2 *	3.0 ± 0.3	0
Nodulated plants (%)	100.0	90.0	89.0	71.0	90.9	0
Shoot fresh weight (mg)	64.7 ± 3.9	66.6 ± 4.0	69.0 ± 4.2	60.3 ± 3.9	69.0 ± 5.2	71.2 ± 3.6
Root fresh weight (mg)	37.2 ± 2.7 ab	39.6 ± 2.4 a	36.2 ± 3.0 a	31.7 ± 2.2 bc	29.4 ± 1.1 c	28.4 ± 1.3 c

^aMean and standard deviations of at least three independent experiments with five replicates each are shown. For each parameter, different letters or an asterisk indicate statistical differences among strains according to Fisher’s least significant difference test ($P \leq 0.05$).

recipient strains Rm8530 and Rm8530 *expA*. Cotransduction of the neomycin marker and the sodium dodecyl sulfate (SDS)-sensitivity phenotype associated with the *lpsB* mutation (Campbell et al. 2002) were verified in the transductant strains.

Complementation of the *lpsB* mutation.

The complete *lpsB* gene was amplified by polymerase chain reaction (PCR) using primers *lpsB*PROMFw (5'-TCTAGAAAG GAAGTCGGCGATTTCGAT-3'; *Xba*I restriction site is underlined) and *lpsB*Rev (5'-GAATTCTCAACGCATCAGGCTTTTCG TA-3'; *Eco*RI restriction site is underlined). The PCR product was cloned into pGEM-T Easy (Promega) and was checked by sequencing. A 1,220-bp fragment containing the complete *lpsB* was excised, using *Xba*I-*Eco*RI, and was ligated into pBBRMCS5 (Kovach et al. 1995) to generate pBBRLPSB. This plasmid was used to transform competent cells of *E. coli* S17. A biparental mating conjugation was carried out between a transformant clone and Rm8530 *lpsB* strain to obtain Rm8530 *lpsB* pBBRLPSB.

Sensitivity to hydrophobic compounds was evaluated according to the protocol described by Lagares et al. (1992). The addition of 0.1 g of SDS per liter to the LB medium clearly inhibited the growth of the Rm8530 *lpsB* mutant, whereas the wt and complemented strains grew normally. Similarly, the other phenotypes linked to the *lpsB* mutation described in this work were also complemented by plasmid pBBRLPSB.

Swimming assay.

Swimming motility was determined in plates containing diluted (1:10) TY medium with 0.3% agar. A bacterial suspension was inoculated by puncture in the center of the plate and incubation for 8 days at 28°C. Colony diameters were measured in centimeters.

Bacterial growth kinetics.

Bacterial growth was determined by measuring optical density at 600 nm (OD_{600}). *S. meliloti* strains were grown at 28°C in TY medium on a rotary shaker at 150 rpm. Cultures started with an initial OD_{600} of 0.001 (approximately 10^5 CFU/ml). The number of viable cells was determined by performing serial dilutions, plating on TY medium, and counting colonies after 48 h at 28°C. Results were expressed as the number of CFU per milliliter.

Biofilm formation assay.

Biofilm formation was determined macroscopically by a quantitative assay in 96-well microtiter dishes made of polyvinylchloride. Biofilms were stained with crystal violet (CV) based on the method of O'Toole and Kolter (1998), with modifications. Briefly, bacteria were grown in 2 ml of TY broth supplemented with the appropriate antibiotic and were incubated with agitation for 48 h at 28°C. The cultures were diluted with fresh medium to give an $OD_{600} = 0.1$. A suspension (100 μ l) was added to each well and was incubated with agitation for 24 h at 30°C. Bacterial growth was quantified by measuring the OD_{600} . Planktonic cells were gently aspirated with a pipette, and then, 180 μ l of a CV aqueous solution (0.1% wt/vol) was added, and staining proceeded for 15 min. Each CV-stained well was rinsed thoroughly and repeatedly with water, then scored for biofilm formation by the addition of 150 μ l of 95% ethanol. The OD_{560} of solubilized CV was measured in a MicroELISA auto reader (Series 700 microplate reader, Cambridge Technology). At the same time, negative controls were made using sterile TY medium.

EPS determination.

The anthrone-sulfuric acid and glucose standard method (Loewus 1952) was used to determine EPS in the supernatant of bacterial cultures grown in MGM medium. The experiment was

done three times and the EPS concentration was normalized to the dry weight of bacterial cells.

CLSM.

A confocal laser-scanning microscope (Carl Zeiss LSM510-Axiovert 100 M) was used to visualize *S. meliloti* biofilms during a three-day time course experiment, using chambered cover glass slides with a 1 μ m thick borosilicate glass base (Lab-Tek Nunc). Confocal images were acquired from bacterial cultures carrying the plasmid pRU1319, which expresses GFP (GFPuv) (Allaway et al. 2001). GFP-labeled bacterial cultures diluted 1:1,000 were grown in the chambers for 3 days at 30°C inside a humid petri dish, to prevent desiccation, as described by Russo et al. (2006). Images were processed using the Zeiss LSM Image Browser version 3.2.0.

AFM imaging.

For bacteria immobilization, glass slides were previously treated with branched polyethyleneimine (PEI) (molecular weight approximately 25,000) (Sigma-Aldrich). Briefly, glass slides were washed twice with 96% (vol/vol) ethanol and water (Elga Classic, resistivity >18 M Ω cm), were incubated overnight at 4°C with a 0.1% (wt/vol) aqueous PEI solution, and were rinsed twice with water. Fresh bacterial cultures at late exponential phase of growth on TY were washed twice with sterile saline solution and bacterial suspensions were adjusted to an OD_{600} of 0.25 nm. Bacteria were electrostatically immobilized by depositing 20 μ l of a bacterial suspension on the PEI-coated slides. Bacteria were allowed to adhere to the substrate for 30 min at 28°C. Afterward, the slides were washed twice with water to remove unattached cells. All imaging was performed in air using an Agilent Technologies model 5500 scanning probe microscope (Agilent Technologies, Inc.) working in acoustic AC mode. Rectangular aluminum-coated silicon cantilevers with force constants of 40 N/m and resonance frequency in the range of 300 to 350s kHz (MikroMasch) were employed for the characterizations. The experimental data were visualized and were analyzed utilizing Gwyddion 2.45 (a free SPM data analysis software).

Autoaggregation assay.

Bacteria were grown in 2 ml of TY broth supplemented with the appropriate antibiotics, were incubated for 24 h at 30°C, were diluted 1:100 in TY, and were further incubated for 48 h under the same conditions. Bacterial suspensions (5 ml) were then transferred to a glass tube (10 \times 70 mm) and were allowed to settle for 24 h at 4°C. A 0.2-ml aliquot of the upper portion of the suspension was transferred to a microtiter plate, and the OD_{600} was measured (OD_{final}). A control tube was vortexed for 30 s, and the OD_{600} was determined ($OD_{initial}$). The autoaggregation percentage was calculated as $100 \times [1 - (OD_{final}/OD_{initial})]$.

For autoaggregation experiments involving artificial bacterial suspensions (i.e., suspensions generated by resuspending the pellet of one strain with the cell-free supernatant of a different strain), the cultures were centrifuged at $4,200 \times g$ for 20 min. The supernatant and pellet were immediately used to reconstitute a different suspension, which was left in static conditions as described before.

Adsorption of rhizobia to alfalfa roots.

Early adsorption of bacteria to roots was done as previously described by Caetano-Anollés and Favelukes (1986). Adhesiveness (%) is defined as the percentage of bacteria in the inoculum that became adsorbed to roots.

Nodulation assays.

Seeds of the alfalfa cultivar Pampeana from INTA (Instituto Nacional de Tecnología Agropecuaria, Argentina) were

surface-sterilized 30 s by immersion in ethanol, then a 10-min immersion in hydrogen peroxide, and, finally, were washed in sterile distilled water. Sterilized seeds were aseptically germinated, were transferred to the petri plates containing solid (0.6% agar) Hoagland's medium, and were inoculated with a bacterial suspension containing 1×10^7 CFU/ml. Plants were grown in a chamber under controlled conditions (16 h of light and 8 h of dark, 28°C). The inoculated and uninoculated (control) plants were harvested 28 days later. The root systems were washed, nodules were counted, and the aerial plant parts and roots were weighed. All experiments were performed in triplicate.

Statistical analysis.

The autoaggregation assays were performed in quintuplicate. For the biofilm assays, each strain was plated in at least eight wells of each microtiter dish. The data were subjected to one-way analysis of variance, followed by comparison of multiple treatment levels with the control, using post hoc Fisher's least significant difference test. All statistical analyses were performed using Infostat version 1.0.

ACKNOWLEDGMENTS

The authors thank N. Fujishige and A. Angus (University of California-Los Angeles) for help with the experiments, G. Devescovi (International Centre for Genetic Engineering and Biotechnology) for helping in the methodology of complementation, and A. Lagares (Universidad Nacional de La Plata) for kindly providing strain Rm6963.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Gwyddion software: <http://gwyddion.net>