Nodule Development Induced by *Mesorhizobium loti* Mutant Strains Affected in Polysaccharide Synthesis

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The role of *Mesorhizobium loti* surface polysaccharides on the nodulation process is not yet fully understood. In this article, we describe the nodulation phenotype of mutants affected in the synthesis of lipopolysaccharide (LPS) and $\beta(1,2)$ cyclic glucan. *M. loti* *lps* β 2 mutant produces LPS with reduced amount of O-antigen, whereas *M. loti* *lps* β 1 mutant produces LPS totally devoid of O-antigen. Both genes are clustered in the chromosome. Based on amino acid sequence homology, LPS sugar composition, and enzymatic activity, we concluded that *lps* β 2 codes for an enzyme involved in the transformation of dTDP-glucose into dTDP-rhamnose, the sugar donor of rhamnose for the synthesis of O-antigen. On the other hand, *lps* β 1 codes for a glucosyl-transferase involved in the biosynthesis of the O-antigen. Although LPS mutants elicited normal nodules, both show reduced competitiveness compared with the wild type. *M. loti* $\beta(1-2)$ cyclic glucan synthase (*cgs*) mutant induces white, empty, ineffective pseudonodules in *Lotus tenuis*. *Cgs* mutant induces normal root hair curling but is unable to induce the formation of infection threads. *M. loti* *cgs* mutant was more sensitive to deoxycholate and displayed motility impairment compared with the wild-type strain. This pleiotropic effect depends on calcium concentration and temperature.

Additional keywords: epimerase/dehydratase family, glucosyl-transferases, LPS and $\beta(1-2)$ cyclic glucan mutants.

Rhizobia specifically interact with legume roots, inducing structures called nodules where atmospheric nitrogen fixation occurs. Nodule development is triggered by signals secreted by the bacteria that result in root hair colonization, initiation of hair curling, and root cell division to form the nodule primordium. Simultaneously, the bacteria are entrapped in the curled root hair, and then proliferate and infect the root hair through a tube-like structure made of plant cell wall called “infection threads.” The infection threads filled with bacteria grow toward the base of the root hair where rhizobia are released into the plant cells of the nodule primordium by endocytosis of the plasma membrane. Once inside the root, bacteria divide and

differentiate into nitrogen-fixing bacteroids (Brewin 1998; Geurts and Bisseling 2002; Patriarca et al. 2002; Udvardi and Day 1997).

Several bacterial polysaccharides (glucans, exopolysaccharides, lipopolysaccharides, and capsular polysaccharides) have been shown to play a role during the nodulation process. *Sinorhizobium meliloti* *ndvB* mutants, affected in the synthesis of $\beta(1-2)$ cyclic glucan, produce pseudonodules on alfalfa (Geremía et al. 1987). *Bradyrhizobium japonicum* *ndvB* mutants unable to produce $\beta(1-3)$ $\beta(1-6)$ cyclic glucan also form ineffective nodules on soybean plants (Dunlap et al. 1996). Contradictory results were obtained with *Rhizobium fredii* *ndvB* mutants that do not produce $\beta(1-2)$ cyclic glucan. On soybean cv. Williams, the mutants form pseudonodules (Baghawat et al. 1992) and, on soybean cvs. Peking and Mc Call, they form active, nitrogen-fixing nodules (Iñón de Iannino et al. 1996).

Nodules induced by rhizobia are of two general kinds, determinate and indeterminate. Determinate development leads to spherical nodules (lotus, soybean, and bean), whereas indeterminate development leads to elongated and cylindrical nodules (alfalfa, pea, and clover). The role of exopolysaccharide (EPS) and lipopolysaccharide (LPS) differs according to the type of development. Exopolysaccharide appeared to be essential for the successful invasion of indeterminate nodules (Pellock et al. 2000), but does not play a role in the formation of determinate nodules (Borthakur et al. 1986; Hotter and Scott 1991). In *S. meliloti*, capsular polysaccharides (KPS) can replace EPS in the infection process (Reuhs et al. 1995). Accumulating evidence suggests that an essential function of LPS is the nodule infection process. *S. meliloti* *lps* mutants display altered nodulation competitiveness (Lagares et al. 1992) and inability to establish a normal chronic intracellular infection required for the symbiosis in alfalfa (Campbell et al. 2002). O-antigen-deficient and core-altered LPS mutants of *R. leguminosarum*, *R. etli*, and *B. japonicum* exhibit defective infection thread formation and induce aberrant nodules (Carlson et al. 1995; Cava et al. 1989; Noel and Duelli 2000; Noel et al. 2000; Perotto et al. 1994; Stacey et al. 1991). It seems that LPS is essential at least to start the formation of the infection threads of determinate nodules (Lerouge and Vanderleyden 2001; Noel et al. 2000). In indeterminate nodules, there is evidence showing that LPS is necessary for the release of the bacteria from the infection thread into the host cells (Campbell et al. 2002; Kijne 1992; Lerouge and Vanderleyden 2001). *Mesorhizobium loti* forms determinate nodules on *Lotus tenuis*, *L. corniculatus*, and *L. japonicus*. In recent years, *L. japonicus* became a model legume for the study the molecular process that leads to the formation

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of determinate nodules (Colebatch et al. 2002; Stracke et al. 2002; Webb et al. 2000). The complete genome sequence of a strain identified as *M. loti* strain MAFF303099 has been obtained recently (Kaneko et al. 2000a and b). Recently, strain MAFF303099 has been reassigned as *M. huakuii* biovar *loti* (Turner et al. 2002). Despite these advances, little is known of the role of *M. loti* polysaccharides in the symbiotic process. Hotter and Scott (1991) communicated that *M. loti* exopolysaccharide has no role in *L. corniculatus* nodulation.

Previously, we have described an *M. loti* *pgm* mutant that is unable to form cyclic glucan and EPS, accumulates an altered form of LPS, and does not nodulate *L. tenuis* (Lepek et al. 2002). Here, we describe the nodulation phenotype of *M. loti* LPS and β (1-2) cyclic glucan mutants. We found that mutants affected in the synthesis of the O-antigen LPS presented a normal nodulation development but were out-competed when co-inoculated on *Lotus* plants with the wild-type strain. In contrast, the mutation in the periplasmic β (1-2) cyclic glucan synthesis causes a total impairment of the developing nodule invasion.

RESULTS

Identification of *Mesorhizobium* LPS-biosynthetic genes and construction of null mutants.

Analysis of the *M. huakuii* MAFF303099 genome revealed the presence of two chromosomal genes coding for proteins similar to *R. etli* Lps β 2 and Lps β 1; they were named *Mesorhizobium* lps β 2 and lps β 1, respectively.

The *Mesorhizobium* lps β 2 gene codes for a predicted protein of 662 amino acids (aa) similar to the WbcP, WbpM, CapD, LpsB, WbiI family of proteins. These proteins are required for the biosynthesis of surface polysaccharides in numerous bacterial species and may function as nucleotide diphosphate sugar epimerases or dehydratases. Over its entire length, *Mesorhizobium* Lps β 2 displays a 24% similarity to the putative dTDP-glucose 4,6-dehydratase coded by the *R. etli* lps β 2 gene involved in LPS synthesis (García-de los Santos and Brom 1997). Also, it presents homology to a putative dTDP-glucose 4,6 dehydratase of *R. leguminosarum* (CAE00204; 33% identity over 558 aa), a nucleotide sugar epimerase/dehydratase WbpM of *Pseu-*

domonas syringae (NP791581; 31% sequence identity over 595 aa) and an epimerase/dehydratase of *Xanthomonas campestris* (NP637545; 35% sequence identity over 503 aa).

Mesorhizobium lps β 1 codes for a predicted protein (184 aa) 35% identical, over its entire length, to a putative galactosyltransferase codified by *R. etli* lps β 1, also involved in LPS synthesis (García-de los Santos and Brom 1997), and displays significant homology with other glycosyltransferases such as a putative UDP-glucose lipid carrier transferase of *Salmonella typhimurium* (NP461048; 40% sequence identity over 183 aa).

As in *R. etli*, *Mesorhizobium* lps β 1 and lps β 2 genes are contiguous, but lps β 2 is upstream lps β 1.

Based on the genome sequence of strain MAFF303099, we designed primers which were used to clone *M. loti* lps β 1 and *M. loti* lps β 2 genes from *M. loti* Ayac 1 BII strain (Table 1). We confirmed by DNA sequencing that, in *M. loti* Ayac 1 BII, the genetic order and organization of these two genes was the same as in the *M. huakuii* MAFF303099 strain. *M. loti* lps β 1 and *M. loti* lps β 2 mutant strains were obtained by introducing a gentamicin cassette, without a terminator sequence, interrupting both open reading frames in the *M. loti* Ayac 1 BII strain.

Characterization of LPS in *M. loti* lps β 1 and lps β 2 mutants.

Polyacrylamide gel electrophoresis analysis. LPS from the *M. loti* wild-type strain, as well as from lps β 1 and lps β 2 mutants, was obtained, subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and stained with silver reagent. Wild-type LPS was resolved into slow (S-LPS) and fast (R-LPS) migrating species (Fig. 1A, lane 1). S-LPS represents smooth LPS molecules that migrated as a ladder-banding pattern, typical of molecules formed by repeating O-antigen oligosaccharide units with different degree of polymerization. In some rhizobia the ladder pattern is also characteristic of KPS molecules. However for visualization of KPS, treatment with Alcian Blue before silver stain is required (Reuhs et al. 1998). In our case, visualization of the slower-migrating species with silver stain was possible without a previous Alcian Blue treatment. R-LPS represents rough LPS containing LPS core molecules lacking the O-antigen polysac-

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics ^a	Reference or source ^b
Strains		
<i>Escherichia coli</i>		
DH5 α -F'IQ	Host for cloning	Sambrook et al. 1989
<i>Mesorhizobium loti</i>		
Ayac 1 BII	Wild type, Nod ⁺	Lepek. et al. 1990
<i>M. loti</i> cgs	cgs::Gm ^r	This study
<i>M. loti</i> lps β 1	lps β 1::Gm ^r	This study
<i>M. loti</i> lps β 2	lps β 2::Gm ^r	This study
Plasmids		
pBC SK (+)	pUC derivative, F1 replication origin, Cm ^r	Stratagene
pBCS	pBC SK(+) devoid of the SalI site from the MCS	This study
pBCS-cgs	1.2-kb <i>Eco</i> RI fragment containing the 5' end of cgs gene cloned into pGEMT-Easy	This study
pBCS-cgs::Gm	pBCS-cgs containing a Gm resistance cassette without transcriptional terminator	This study
pCD523	pLAFR1-Region of <i>Agrobacterium tumefaciens</i> chromosome containing <i>chv</i> genes	Douglas et al. 1985
pGEMT-Easy	pGEM-5Zf(+) derivative, F1 replication origin, Amp ^r	Promega Corp.
pGEMT-Easy-lps β 1	750-bp PCR fragment containing the <i>M. loti</i> lps β 1 gene cloned into pGEMT-Easy	This study
pGEMT-Easy-lps β 2	1500-bp PCR fragment containing the <i>M. loti</i> lps β 2 gene cloned into pGEMT-Easy	This study
pGEMT-Easy-lps β 1::Gm	pGEMT-Easy-lps β 1 containing a Gm resistance cassette without transcriptional terminator	This study
pGEMT-Easy-lps β 2::Gm	pGEMT-Easy-lps β 2 containing a Gm resistance cassette without transcriptional terminator	This study
pTB93F	ptrp-GFP-S65T in pMB393, Sp ^r Cm ^r	Gage et al. 1996
PBBR1MCS-4	Broad-host-range cloning vector (Amp ^r)	Kovach et al. 1995
pLPSB1	Fragment containing the lps β 2-lps β 1 intergenic region and the lps β 1 codifying sequence cloned in PBBR1MCS-4.	This study

^a Gm = gentamicin, Cm = chloramphenicol, and Amp = ampicillin, PCR = polymerase chain reaction.

^b Stratagene = Cloning Systems, La Jolla, CA, U.S.A. and Promega Corp. = Promega Corporation, Madison, WI, U.S.A.

charide. The *lpsβ1* mutant accumulated R-LPS and completely lacked S-LPS (Fig. 1A, lane 2). An SDS-PAGE LPS profile of *lpsβ2* mutant also showed an accumulation of R-LPS and a faint ladder-like structure migrating in a position similar to wild-type S-LPS (Fig. 1A, lane 3). LPS of *M. loti lpsβ1* complemented with plasmid pLPSB1 (Table 1) recovered the wild-type LPS phenotype (data not shown). This indicates that mutation in the *lpsβ1* gene has no additional effect on the *lpsβ2* gene activity. On the other hand, LPS of *M. loti lpsβ2* complemented with plasmid pLPSB1 did not change (data not shown). This indicates that the altered LPS phenotype of *lpsβ2* mutant strain was not due to a polar effect on *lpsβ1* gene.

LPS sugar composition of *M. loti* wild-type, *lpsβ2*, and *lpsβ1* mutants. To gain further insight into the nature of the mentioned changes, a three-step separation method was used to purify LPS from wild-type and mutant cells. In each case, the aqueous phase of the hot phenol-water extraction of the cells was subjected to Sephadex G-150 chromatography. Those fractions containing wild-type or mutant S-LPS and R-LPS were pooled individually and subjected to purification through polymyxin-affinity chromatography (Kannenberg and Carlson 2001; Reuhs et al. 1998). The S-LPS and R-LPS SDS-PAGE profile after purification by polymyxin columns is shown in Figure 1B. After mild acid hydrolysis, the water-insoluble fraction (lipid A) was removed by ultracentrifugation. Supernatants were lyophilized and the sugar composition of the wild-type and mutant fractions was determined.

Neutral sugar analysis (Table 2) showed that wild-type R-LPS contains galactose and glucose and that S-LPS contains rhamnose and glucose with a ratio 7 to 1. Neutral sugar analysis also revealed that the difference between the wild-type and the *lpsβ2* mutant S-LPS was a severe decrease in the rhamnose/glucose relationship. In contrast, neutral sugar composition of the *lpsβ1* mutant R-LPS showed a similar glucose/galactose ratio when compared with the wild-type R-LPS.

Acidic sugar composition (Table 3) analysis revealed that wild-type S-LPS contains KDO (2-keto-3-deoxyoctulosonic acid), glucuronic acid (GlcA), galacturonic acid (GalA), and two not yet identified compounds named X (retention time, 6.8 min) and Y (retention time, 9.0 min) that eluted between KDO

and GalA. Compounds Y and GlcA should be part of the O-antigen LPS because they were not detected in the wild-type R-LPS. In the *lpsβ2* mutant, these two sugars were recovered in the R-LPS fraction. Due to the small amount of *lpsβ2* S-LPS, it was not possible to determine its acidic sugar composition. Acidic sugar composition of *lpsβ1* R-LPS was similar to that of the wild-type strain. The relative glucose/KDO value was higher in the *lpsβ2* than in wild-type R-LPS, whereas the galactose/KDO ratio was similar for the two fractions (data not shown). This could explain the difference in the glucose/galactose ratio between the wild-type and *lpsβ2* R-LPS fractions. It is possible that, even after the purification process, the *lpsβ2* R-LPS fraction remained contaminated with other polysaccharides containing glucose.

Enzymatic activities affected in *lpsβ2* and *lpsβ1* mutant strains.

The *lpsβ2* gene codifies for a protein with homology to the epimerase/dehydratase family of proteins. Enzymes belonging to this family are involved in deoxysugars biosynthetic pathways (Wang and Gabriel, 1969). Numerous deoxysugars are part of bacterial LPS. *M. loti* wild-type O-antigen is composed mainly of the deoxysugar rhamnose. Total cell extracts of wild-type and *lpsβ2* mutant strains were used to assay the enzymatic synthesis of dTDP-rhamnose (as described below). The formation of dTDP-rhamnose was 8- to 10-fold lower with *lpsβ2* mutant extracts than with the wild-type strain (Fig. 2), thus indicating that, in the mutant, the synthesis of dTDP-rhamnose is severely reduced. The inability to synthesize a normal amount of the corresponding sugar-nucleotide donor may explain the reduction of rhamnose content in *M. loti lpsβ2* R-LPS (Table 2).

Glycosyltransferase activity was assayed as described below. Incorporation of radioactive sugar from UDP-[¹⁴C]glucose to R-LPS was similar with wild-type and *lpsβ2* mutant enzymatic preparations and severely reduced with *lpsβ1* extracts (Fig. 3A). SDS-PAGE analysis confirmed that fractions corresponding to the radioactivity peak contained the *lpsβ1* R-LPS used

Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of *Mesorhizobium loti* wild-type and lipopolysaccharide (LPS) mutant strains. **A**, Silver stain of crude cell extracts. Cell pellets of 1.5 ml of culture at an optical density at 600 nm of 1.2 were homogenized with 100 μ l of proteinase K lysis buffer as described (Hitchcock et al. 1983), diluted 100 times with the lysis buffer and 10 μ l was submitted to SDS-PAGE. Lane 1, *M. loti* wild-type strain; lane 2, *M. loti lpsβ2* mutant strain; lane 3, *M. loti lpsβ1* mutant strain. **B**, Silver stain of purified *M. loti* wild-type slow (S-LPS) and fast (R-LPS). The aqueous phase of a hot water-phenol extraction was subjected to Sephadex G-150 chromatography. Fractions corresponding to S-LPS and R-LPS were pooled separately and purified by polymyxin affinity chromatography. Lane 1, purified *M. loti* S-LPS; lane 2, purified *M. loti* R-LPS; lane 3, unpurified *M. loti* LPS.

Table 2. Neutral sugar composition

Sugar	Molar ratio found in strain: ^a				
	Wild type		<i>lpsβ2</i> Mutant		<i>lpsβ1</i> Mutant
	R-LPS	S-LPS	R-LPS	S-LPS	R-LPS
Glucose	1.0	1.0	1.0	1.0	1.0
Rhamnose	TR	7.0	TR	0.5	TR
Galactose	0.9	ND	0.14	ND	0.7

^a Entries are molar ratios of the sugars normalized to 1.0 glucose. R-LPS and S-LPS = rapid and slow lipopolysaccharide migrating species, respectively; TR = traces; ND = not detected.

Table 3. Acidic sugar composition

Compound ^b	Mass ratio found in strain ^a				
	Wild-type		Mutant		
	R-LPS	S-LPS	<i>lpsβ2</i> R-LPS	<i>lpsβ1</i> R-LPS	
KDO	1.0	1.0	1.0	1.0	
GalA	1.2	0.5	1.4	1.9	
GlcA	ND	0.8	0.3	0.2	
X	0.6	0.2	1.8	0.4	
Y	ND	2.4	0.7	ND	

^a Entries are mass ratios of the sugars normalized to 1.0 KDO. ND = not detected.

^b GalA = galacturonic acid, GlcA = glucuronic acid, X = unknown compound that elute with a retention time of 6.8 min, and Y = unknown compound that elute with a retention time of 9.0 min.

as acceptor (Fig. 3C). No significant radioactive incorporation was observed when the acceptor substrate was omitted in the reaction mixture (Fig. 3A). *M. loti* *lps* β 2 mutant displayed wild-type glycosyltransferase activity, thus confirming nonpolarity of the *lps* β 2 mutation on *lps* β 1 gene. Thin-layer chromatography analysis of the product after hydrolysis indicated that the incorporated radioactive sugar was glucose (data not shown). Low and similar radioactive sugar incorporation was detected when both the wild-type and the *lps* β 1 mutant strain were incubated with UDP-[¹⁴C]galactose (Fig. 3B).

Cationic peptide sensitivity.

Alterations in LPS structure often result in increased cationic peptide sensitivity (Campbell et al. 2002; Lerouge and Vanderleyden 2001). The bactericidal effect of polymyxin B (PmB) on wild-type, *lps* β 1, and *lps* β 2 mutant strains was determined. Exposure to PmB at 20 μ g/ml for 1 h had no effect on the wild-type strain (99% of the cells were recovered). In contrast, only 10 to 30% of the *lps* β 2 and 70% of the *lps* β 1 mutant cells were recovered after the treatment. Cationic pep-

tides produce morphological and permeability changes of the bacterial outer membrane (Freer et al. 1996). In the presence of PmB, the outer membranes of *lps* β 2 and *lps* β 1 mutant strains were more permeable to *N*-phenyl-1 naphthylamine (NPN) than the wild type (Fig. 4A). Similar results on membrane permeability were obtained using the cationic peptide melitin (Fig. 4B).

Nodulation analysis with *lps* mutants.

Nodulation experiments were carried out on *L. tenuis* plants. Nodulation effectiveness, determined as the number of pink nodules per plant and height and greenish feature of plants, was identical in the wild-type and the *lps* β 1 and *lps* β 2 mutants. Observation under light microscopy revealed that the nodules of plants inoculated with all the strains appeared 7 to 8 days after inoculation. The number of nodules per plant was 2 to 3

Fig. 2. dTDP-rhamnose synthesis. Enzymatic transformation of dTDP-[¹⁴C]glucose into dTDP-[¹⁴C]rhamnose in the presence of NADPH was carried out as described (Groninger et al. 2002). The reaction was stopped with the addition of water. After protein precipitation and desalting, dTDP-nucleotides were hydrolyzed by mild acid and were subjected to descending paper chromatography. **A**, Control reaction: reaction mixture was incubated without the addition of enzyme preparation. **B**, Products obtained with *Mesorhizobium loti* wild-type enzyme preparation. **C**, Products obtained with *M. loti* *lps* β 2 mutant enzyme preparation. Glc = glucose; Rha = rhamnose.

Fig. 3. Glycosyltransferase activity. Enzymatic activity was carried out, as described (Piekarowicz and Stein 2002) using LpsB, R-LPS as acceptor substrate. Incorporation of **A**, UDP-[¹⁴C]glucose and **B**, UDP-[¹⁴C]galactose was assayed using enzyme preparations obtained from *Mesorhizobium loti* wild-type strain (●), *M. loti* *lps* β 1 mutant strain (■), and *M. loti* *lps* β 2 mutant strain (○). In the control experiment, the addition of the acceptor substrate was omitted (▲). **C**, Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of the fractions corresponding to the radioactivity peak.

at the third week and 8 to 10 at the fifth week postinoculation. Infection threads were visualized under a fluorescence microscope (Fig. 5A through C). The number of infection threads formed by the wild-type and by the *lpsβ2* mutant at different postinoculation times revealed significantly higher values in plants inoculated with the mutant (data not shown). Occasionally, regions of the roots inoculated with the *lps* mutant contained a high density of infection threads (Fig. 5C). Nodule occupancy was determined by visualization under a fluorescence microscope of cross sections of nodules inoculated with green fluorescent protein (GFP)-labeled strains. No significant differences were detected between the wild type and the mutants (Fig. 5D and F). Occasionally, in both wild-type and *lpsβ* mutants, regions of nodules were empty. No phenotype reversion inside the nodules was detected by either polymerase chain reaction (PCR) or culturing bacteria isolated from the nodules on media with the appropriate antibiotic.

Nodulation competitiveness assays.

Nodule competitiveness was determined as described below. Plants were inoculated with a mixture of an equal number of wild-type and mutant strains. After 3 weeks, bacteria were recovered from the nodules and plated. The competitiveness index (CI) was calculated as the following ratio: CFU of mutant recovered from the nodule per CFU of total bacteria recovered from the nodule. The CI of *lpsβ1* and *lpsβ2* mutants was 10^{-3} and 10^{-4} , respectively, thus indicating that both mutants have a severe reduction in competitiveness when compared with the wild type.

Characterization of the *M. loti* cyclic β(1,2)-glucan synthase mutant.

The *M. loti* cyclic glucan synthase gene (*cgs*) is 56% identical to *S. meliloti* *ndvB* and 53% to *Agrobacterium tumefaciens*

chvB. An *M. loti* *cgs* mutant did not accumulate cyclic β(1,2) glucan (Fig. 6).

It has been described that *Agrobacterium chvB* mutant displayed some pleiotropic phenotypes, such as absence of motility in tryptone yeast (TY)/Ca²⁺ medium (Swart et al. 1994). *M. loti* wild-type strain was motile in TY medium when Ca²⁺ was added in a range between 0.5 to 7 mM. No motility was observed when Ca²⁺ was omitted in the medium (Table 4). The requirement of calcium for the correct assembly of flagella was described in *S. meliloti* (Robinson et al. 1992). The effect of Ca²⁺ on the motility of the *cgs* mutant depends not only on Ca²⁺ concentration but also on temperature. Between 0.5 to 2.0 mM Ca²⁺, the *cgs* mutant was less motile than the wild-type strain at any temperature (Table 4). However, at higher concentrations of Ca²⁺ (between 5 and 7 mM), the motility of the mutant was more affected at 28 than at 18°C (Table 4). The effect of temperature on the motility of the *cgs* mutant at 5 mM Ca²⁺ is shown in Figure 7.

The *Brucella abortus* *cgs* mutant displays increased sensitivity to surfactant compounds (Briones et al. 2001). An altered sensitivity to detergents usually is an indicator of a change in the bacterial cell envelope. Sensitivity of the *M. loti* wild-type and mutant strains to deoxycholate (DOC) was assayed at different calcium concentrations. The *cgs* mutant strain was unable to grow at 28°C in the presence of 1 mM DOC and calcium above 2 mM (Table 5). This indicates that calcium concentration affects the effect of *cgs* mutation on the bacterial cell envelope. This effect was reversed at 18°C.

Symbiotic phenotype of the *M. loti* *cgs* mutant.

The *M. loti* *cgs* mutant induced the formation of round white nodules in *L. tenuis* plants. Light microscopic examination of roots inoculated with *M. loti* *cgs* mutant revealed normal hair-curling induction and nodule primordium formation (Fig. 5G and H). In order to examine the infection process, the *M. loti* *cgs* mutant strain was labeled with plasmid pTB93F expressing GFP and observed under a light fluorescence microscope. Very few infection threads were observed (only 4 of 37 plants examined). When 4-week-old nodules elicited by the *cgs* mutant were examined by fluorescence microscopy, a high percentage (95%) showed no fluorescence (Fig. 5E). *M. loti* *cgs* mutant complemented with cosmid pCD523 containing *A. tumefaciens* cyclic glucan synthetase recovered the synthesis of cyclic glucan (Fig. 6) and the wild-type nodulation phenotype, thus indicating that the absence of cyclic glucan is responsible for the mutant phenotype.

DISCUSSION

In this study, we disrupted two chromosome-contiguous *M. loti* loci, *lpsβ1* and *lpsβ2*, and determined their participation in the LPS synthesis and the nodulation phenotype of the mutants. We also analyzed the role of β(1-2) cyclic glucan on nodule development and occupancy in plants that form determinate nodules.

The nodulation process of *lpsβ2* and *lpsβ1* mutants was not affected despite their effect on LPS structure. Both nodule number and macroscopic appearance were similar to those obtained with the wild-type strain. These results contradict others in which the O-antigen was shown to be required for the nodulation process in plants that form determinate nodules (Carlson et al. 1987; Cava et al. 1989; Kannenberg et al. 1998; Noel and Duelli 2000; Stacey et al. 1991). Moreover, an *R. etli* mutant that synthesizes reduced amounts of O-antigen containing LPS was described to be symbiotically defective (Noel et al. 2000). Despite the fact that the symbiotic process is not affected for *lpsβ1* and *lpsβ2* mutants, nodulation competition assays re-

Fig. 4. Fluorometry assay of bacteria treated with cationic peptides. N-phenyl-1 naphthylamine (NPN) permeability was assayed in the presence of **A**, polymyxin B and **B**, melitin on *Mesorhizobium loti* *lpsβ2* mutant strain (1), *M. loti* *lpsβ1* mutant strain (2), and *M. loti* wild-type strain (3). Dark and white arrows indicate the time of addition of NPN and of the cationic peptide, respectively.

vealed that they were out-competed when co-inoculated with the wild-type strain in *Lotus* plants. Our experiments revealed a reduction in the competitiveness index of three and four orders of magnitude for *lpsβ1* and *lpsβ2*, respectively. Competitiveness is a very important property under normal field conditions, in which the bacteria in the rhizosphere have to compete with other bacteria to invade the plants; thus, it is ex-

pected that, in nature, the presence of a complete S-LPS may be subjected to positive selective pressure. The ability to form normal and effective nodules but with reduced competitiveness also was observed in some LPS mutants of *S. meliloti* that form indeterminate nodules on alfalfa (Campbell et al. 2003). We found that *M. loti* mutants, especially *lpsβ2*, showed increased sensitivity to PmB and melitin when compared with

Fig. 5. Light and fluorescence light microscopy of *Lotus tenuis* roots inoculated with different *Mesorhizobium loti* strains. Infection thread visualization on roots inoculated with **A**, *M. loti* wild type strain and **B** and **C**, *M. loti* *lpsβ2* mutant strain using bacteria expressing green fluorescent protein (GFP). Cross section of nodules observed on roots inoculated with **D**, *M. loti* *lpsβ2* mutant strain, **E**, *M. loti* *cgs* mutant strain, and **F**, *M. loti* wild-type strain using bacteria expressing GFP. **G**, Root hair observed in roots inoculated with the *M. loti* *cgs* mutant strain (deformed and curled root hair are indicated with arrows). **H**, Nodule primordium observed in roots inoculated with *M. loti* *cgs* mutant strain.

the wild-type strain. These results are in agreement with those obtained in other bacteria in which alterations of the LPS structure often result in changes in cationic peptide sensitivity (Campbell et al. 2003). Cationic peptides are part of the innate defense response of many organisms, including plants; therefore, alterations in cationic peptide sensitivities might reflect a susceptibility to the plant defenses against bacteria. It is probable that the susceptibility of *lpsβ1* and *lpsβ2* mutants to the innate plant defense response also is affected and that, as a consequence, competitiveness also is reduced. The *lpsβ2* mutant was more sensitive to cationic peptides and out-competed better by the wild-type strain than the *lpsβ1* mutant strain. These results suggest a correlation between cationic peptide sensitivity and competitiveness. It was described that *R. tropici lpiA* mutant displayed a sevenfold decrease in relative competitiveness compared with the wild-type strain (Vinuesa et al. 2003). LpiA belongs to a family of integral membrane proteins structurally and phylogenetically related to the membrane proteins MprF and FmtC from low G+C gram positives. The later proteins are involved in resistance to cationic antimicrobial peptides (Peschel

et al. 2001). Vinuesa and associates (2003) speculate about the possibility that the loss of competitiveness in the *lpiA* mutant was due to an increased sensitivity to plant defense peptides. It is interesting to remark that a significantly higher number of infection threads was observed with the *lpsβ2* mutant than with the wild-type strain at initial times of nodulation. This suggests that the nodulation step that may affect competitiveness is downstream of the infection thread formation.

Neutral sugar composition analysis of wild-type LPS indicated that rhamnose and glucose are present in the O-antigen with a ratio of 7:1. The trace amount of S-LPS present in the *lpsβ2* mutant contains rhamnose and glucose with a ratio of 0.5:1.0, thus indicating that the presence of rhamnose is severely affected in the mutant LPS. This result suggests that the synthesis of dTDP-rhamnose, the sugar nucleotide donor (Allard et al. 2001), or the LPS-rhamnosyl-transferase may be affected in the mutant. The pathway of dTDP-rhamnose synthesis from dTDP-glucose consists of three steps in which one dehydratase, one epimerase, and one reductase are involved (Wang and Gabriel 1969). The deduced amino acid sequence of *lpsβ2* revealed homology to proteins of the epimerase/dehydratase protein family. The enzymatic transformation of dTDP-glucose into dTDP-rhamnose was 8- to 10-fold lower in the *lpsβ2* mutant than in the wild type; thus, we concluded that the *lpsβ2* gene is involved in the synthesis of dTDP-rhamnose.

The synthesis of dTDP-rhamnose was not completely abolished in the mutant and rhamnose still was detected in the mutant S-LPS. Two interpretations are possible for this result: i)

Fig. 6. Cyclic glucan analysis by thin-layer chromatography. Bacterial cells were extracted with 70% ethanol and subjected to thin-layer chromatography as described previously (Briones et al., 2001). **A**, *Mesorhizobium loti* wild-type strain, **B**, *M. loti cgs* mutant strain, and **C**, *M. loti cgs* mutant strain complemented with plasmid pCD523. Neutral glucan (*) and anionic glucan (**).

Fig. 7. Motility of wild-type and *cgs* mutant strains. Motility was analyzed in 0.2% TY-agar plates with 5 mM Ca^{2+} . At 28°C, observation was carried out at day 6, when the wild type had a diameter of approximately 3 cm. At 18°C, observation was carried out at day 10, when the wild type halo had a diameter of approximately 2.8 cm.

Table 4. Comparison of wild-type and *cgs* mutant strain motility^a

Strain	TY media with Ca^{2+}					
	0 mM	0.5 mM	1 mM	2 mM	5 mM	7 mM
Wild type at 28 and 18°C	–	++	++	++	++	++
<i>Cgs</i> mutant at 28°C	–	+	+	+	–	–
<i>Cgs</i> mutant at 18°C	–	+	+	+	+	+

^a Assays were carried out on tryptone yeast (TY) media containing the indicated concentration of calcium. Comparison was made when wild-type halo reached a diameter of approximately 2.5 cm. This occurred on day 5 at 28°C and on day 9 at 18°C. Bacterial growth is indicated as – = nongrowth to ++ = maximal growth.

Table 5. Wild-type and *cgs* mutant sensitivity to deoxycholate (DOC)^a

Strain	Media with 1 mM DOC and Ca^{2+}					
	0 mM	0.2 mM	0.5 mM	1 mM	2 mM	5 mM
Wild type at 28 and 18°C	–	±	+	++	++	++
<i>Cgs</i> mutant at 28°C	±	+	++	+	–	–
<i>Cgs</i> mutant at 18°C	±	+	++	++	++	++

^a Assay was carried out in plates containing tryptone yeast (TY) medium with 1 mM DOC and the indicated calcium concentrations. Bacterial culture (10 µl) at an optical density of 0.2 was spotted on the plate and bacterial growth was observed at different days of growth. Bacterial growth is indicated as – = nongrowth to ++ = maximal growth.

the *lpsβ2* disrupted gene still produces a protein with residual enzymatic activity or ii) there are other functional genes in the *M. loti* genome that may be involved in the biosynthesis of dTDP-rhamnose. We favor the latter interpretation because the analysis of the *M. loti* genome reveals the existence of a cluster of genes (mlr 7550, 7551, and 7552), homologues to *rmlA*, *rmlC*, and *rmlB*, that are involved in the synthesis of dTDP-rhamnose in *Salmonella* spp. (Graninger et al. 1999).

The deduced amino acid sequence of the *lpsβ1* gene showed homology to galactosyl and glucosyltransferases. Neutral and acidic sugar composition analysis showed that *lpsβ1* and wild-type R-LPS are similar. This result and the total absence of *lpsβ1* LPS molecules containing O-antigen indicated that the putative glycosyltransferase encoded by *lpsβ1* is involved in the synthesis of the O-antigen. The enzymatic activity assay indicated that, with wild-type preparation, a specific glucose incorporation to the added acceptor occurred using UDP-glucose as nucleotide-sugar donor. The *Lpsβ1* mutant strain was affected in this activity. This result, together with the knowledge that glucose is an O-antigen component, indicates that *Lpsβ1* codified for a glucosyltransferase.

The gene organization of *lpsβ1* and *lpsβ2* in different genomes (*B. abortus*, *B. melitensis*, *B. suis*, *R. etli*, *S. meliloti*, *B. japonicum*, *R. leguminosarum*, *R. palustris*, and *B. pseudomallei*) revealed that, whatever the orientation and order of transcription in all these genomes, *lpsβ1* and *lpsβ2* are always adjacent. This analysis also revealed that these genes are always located in a region containing genes involved in LPS biosynthesis. We concluded that this gene cluster is functionally and evolutionary relevant and conserved in a variety of species.

Russa and associates (1995) determined the composition of the *M. loti* LPS O-chain in strain NZP2213. It consists of a β-(1,3)-linked 2-O-acetyl-6-deoxy-L-talose homopolymer (Russa et al. 1995). We did not detect L-talose in the O-antigen of the *M. loti* strain Ayac 1 BII; therefore, it seems that the composition of the O-antigen varies among strains.

Cyclic glucan is required for normal nodule development in different rhizobia/legume associations (Baghawat et al. 1999; Geremía et al. 1987). In *S. meliloti* that form indeterminate nodules on alfalfa, *ndvB* mutants affected in the synthesis of the β(1-2) cyclic glucan did not form infection threads and induced the formation of empty pseudonodules (Geremía et al. 1987). In *B. japonicum* that form determinate nodules on soybean, mutants in the synthesis of the β(1-3) β(1-6) cyclic glucan form defective nodules, but the overall ultrastructure was similar to nodules formed by the wild-type strain and bacteroids were visualized in part of the infected cells (Dunlap et al. 1996).

No role has yet been assigned to cyclic glucan in the nodulation of *L. tenuis* by *M. loti*. As do other fast-growing rhizobia, *M. loti* synthesizes β(1-2) cyclic glucan (Lepek et al. 1990). In this report, we showed that β(1-2) cyclic glucan is required for successful symbiosis between *M. loti* and *L. tenuis*. Fluorescence microscopy examination of the pseudonodules induced by *M. loti cgs* mutant on *L. tenuis* revealed that they were devoid of bacteria and that the formation of infection threads was severely impaired. These results are similar to those obtained in *S. meliloti*-alfalfa interaction (Geremía et al. 1987). Then, the absence of β(1-2) cyclic glucan synthesis affects the symbiotic process in a similar way, independently from the determinate or indeterminate character of the formed nodule.

The precise role of cyclic glucan during legume symbiosis is under discussion. It has been suggested that these molecules could be involved in an osmoprotective function, the transport of hydrophobic molecules such as legume-derived flavonoids or Nod factors, or a specific suppression of a plant defense response (Bhagwat et al. 1999; Spaink 2000). Plant flavonoids

induce the bacterial production of Nod factors provoking the establishment of root hair deformation and curling and formation of nodule primordium (Geurts and Bisseling 2002). The *M. loti cgs* mutant was able to induce root hair curling and nodule primordium formation; therefore, the initial signaling process that involves flavonoid *nod* gene induction, Nod factors synthesis, and secretion seems not to be affected.

Regulated synthesis and export of glucan to the periplasmic space is believed to provide a mechanism by which the cell can minimize the osmotic gradient across the inner membrane (Miller et al. 1986). Several reports describe deficiencies in cell envelope of mutants affected in β(1-2) glucan synthesis and efforts are being made to determine the mechanism of action of this polysaccharide (Breedveld and Miller 1994; Briones et al. 2001; Swart et al. 1994). It has been described that *A. tumefaciens chvB* mutants are nonmotile due to defective assembly of flagella (Swart et al. 1994). Also, the *M. loti cgs* mutant was affected in its motility. A more drastic effect on motility occurred with the *M. loti cgs* mutant strain at high Ca²⁺ concentration. The *M. loti cgs* mutant sensitivity to DOC also was assayed. *Brucella cgs* mutant was described to be more sensitive to surfactants, indicating changes in its envelope. The *M. loti cgs* mutant also was affected in its sensitivity to DOC in the presence of Ca²⁺ above a certain concentration. The effect of Ca²⁺ on mutant motility and DOC sensitivity enforce the idea that there is a relationship between the effect of mutation in *cgs* and the presence of calcium. It has been described that, in the *A. tumefaciens chvB* mutant strain, a pleiotropic phenotype that correlates with changes in the cell envelope occurs in medium containing 7 mM CaCl₂ (Swart et al. 1994). It is known that calcium produces membrane disruption; thus, these results have suggested that β(1-2) glucan could participate in membrane stabilization against the disrupting effect of calcium (Swart et al. 1994). Reversion of the phenotype of *M. loti cgs* mutant at high calcium concentration was observed lowering the incubation temperature. The influence of the temperature on the phenotype of β(1-2) glucan synthase mutants has been reported previously (Banta et al. 1998; Bash and Matthyse 2002). The *A. tumefaciens chvB* mutant strain displayed decreased accumulation of VirB10 protein when cells were grown at 28°C but not at 19°C. Incubation of the *chvB* mutant at 19°C partially restored the virulence (Banta et al. 1998). Taking into account the changes in the levels of calcium during the early stages of the nodulation process (Erhardt et al. 1996), it can be concluded that the mutant sensitivity to this cation may be important during the symbiosis. It remains to be determined which components relevant for the symbiosis are affected by the absence of the glucan.

MATERIALS AND METHODS

Bacterial strains and growth media.

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown at 37°C in Luria-Bertani media broth. *M. loti* strains were grown at 28°C in AB minimal medium (Douglas et al. 1985) supplemented with sucrose (0.5% wt/vol). When necessary, antibiotics were added to final concentrations of: gentamicin (Gm) at 30 µg/ml, ampicillin (Amp) at 100 µg/ml, and chloramphenicol (Cm) at 20 µg/ml.

Cloning and gene disruption.

In order to mutagenize the *M. loti lpsβ2* gene, an internal fragment of the gene was amplified from *M. loti* Ayac 1 BII DNA with primers 5'-TGGCGCTTAGTCTCGTCTC-3' and 5'-GATCATGGTTTCGGCGACTT-3' and ligated to pGEMT-Easy. The resulting plasmid was named pGEMT-*lpsβ2*. A gentamicin cassette, devoid of transcriptional terminator sequence

(Ugalde et al. 2000), was introduced into a unique *Nru*I site. Plasmid pGEMT-*lps* β 2::Gm was electroporated into *M. loti* Ayac 1 BII wild-type strain. Double recombination events (Gm^r, Amp^s) were selected and confirmed by PCR with the same primers used to amplify the gene. A DNA fragment of 1,500 bp from the wild type and 2,200 bp from the double recombinant was amplified. A mutant named *M. loti lps* β 2 was selected for further studies. A PCR product of 750 bp containing the *lps* β 1 gene was amplified using primers 5'-GTTGGGCAAGCAGGTTAG-3' and 5'-GTATCCGAAGGTTGGCTG-3' from *M. loti* Ayac 1 BII, and ligated to pGEMT-Easy to generate pGEMT-*lps* β 1. The resulting plasmid was digested with *Nru*I and ligated to the Gm nonpolar cassette (Ugalde et al. 2000) to generate the recombinant plasmid pGEMT-*lps* β 1::Gm. Plasmid pGEMT-*lps* β 1::Gm was electroporated into *M. loti* Ayac 1 BII wild-type strain. Double recombination events (Gm^r Amp^s) were selected and confirmed by PCR with the same primers used to amplify the gene. A DNA fragment of 750 bp from the wild type and 1,450 bp from the double recombinant was amplified. A mutant named *M. loti lps* β 1 was selected for further studies. For the construction of the recombinant pLPSB1 (Table 1), pGEMT-*lps* β 1 was digested with *Eco*RI and subcloned into pBBR1MCS-4 (Table 1) digested with the same enzyme. The vector replicates in *Rhizobium* spp. and confers resistance to Amp. The *Eco*RI fragment contained the *lps* β 2-*lps* β 1 intergenic region and the *lps* β 1 codifying sequence. Recombinant plasmid pLPSB1 was introduced into *M. loti lps* β 1 and *M. loti lps* β 2 mutant strains by conjugation and cells were plated on selective medium containing Gm and Amp.

PCR was carried out with primers 5'-GCTGCATGAGCTTGACCTTT-3' and 5'-CCGGCGGCAGATTGTGGT-3' to amplify a 1.2-kb DNA fragment containing the amino terminal fragment of *cgs* gene from *M. loti* Ayac 1 BII wild-type strain. PCR products were cloned into pGEMT-Easy to generate plasmid pGEMT-*cgs*. This plasmid then was digested with *Eco*RI and the 1.2-kb fragment was ligated into pBCS (Table 1). The resulting recombinant plasmid was named pBCS-*cgs*. To obtain a nonpolar mutation of *cgs*, a Gm cassette devoid of transcriptional terminator sequence (Ugalde et al. 2000) was ligated into the unique *Sal*I site. The resulting plasmid was named pBCS-*cgs*::Gm and was electroporated into *M. loti* wild-type strain. Gm-resistant colonies were isolated and a double crossover event was obtained by testing sensitivity to chloramphenicol and PCR analysis. A mutant named *M. loti cgs* was selected for further studies. *M. loti cgs* mutant was complemented in triparental mating with cosmid pCD523 (Tc^r) that contained the *A. tumefaciens chvB* gene.

All the oligonucleotides for gene amplification by PCR were designed based on the complete genome sequence of *M. huakuii* strain MAFF303099 (Kaneko et al. 2000a and b). *Lps* β 2, *lps* β 1, and *cgs* genes correspond to the genes denoted as mlr7568, mlr7570, and mlr8325, respectively, in the above-mentioned database.

LPS extraction, purification, and sugar composition.

Hot phenol-water cell extraction was carried out as described elsewhere (Westphal and Jann 1965). Most LPS of *M. loti* strain Ayac 1 BII was extracted in the water phase. LPS fractions were purified by Sephadex G-150 chromatography in the presence of DOC as described previously (Gudlavalleti and Forsberg 2003). Fractions were pooled and dialyzed against water. In order to purify these LPS-containing fractions from other contaminant polysaccharides, Polymyxin-agarose affinity column chromatography was performed. Columns containing 1 ml of bed volume of Detoxi-Gel (Pierce) were used as described (Kannenberg and Carlson

2001). After dialysis against water, acetic acid was added to 1% and the solution heated at 100°C for 3 h to hydrolyze the glycosidic bond between the polysaccharide and the lipid A. The lipid A was removed by ultracentrifugation and the carbohydrate was lyophilized and then resuspended in a small volume. Total hydrolysis was performed with 2 N trifluoroacetic acid at 120°C during 2 h and samples were subjected to composition analysis. A DX-300 Dionex BioLC system with a pulse amperometric detector and a CarboPack PA-10 column equipped with a PA-10 precolumn was used. The following conditions were employed: for neutral sugar analysis, an isocratic elution with 7% solution A (NaOH 200 mM) and 93% solution B (water); for flow, 2-deoxyglucose at 1 ml/min was used as internal standard; for acidic sugars analysis, an isocratic elution with 25% solution A (NaOH 200 mM), 65% solution B (water), and 10% solution C (NaAcO 1M); and for flow, sialic acid at 1 ml/min was used as an internal standard.

LPS extraction and SDS-PAGE analysis.

Bacterial cultures were harvested at an optical density at 600 nm (OD₆₀₀) of 1.2. Samples were processed as previously described by Hitchcock and Brown (1983). Briefly, bacteria were homogenized with lysing buffer and treated with proteinase K. Tricine-gel polyacrylamide gels were run as described by Schagger and von Jagow (1987). Gels were silver stained as was described by Tsai and Frisch (1982).

Determination of dTDP-Rhamnose biosynthesis activity.

M. loti cell extracts were obtained as described (Lepek et al. 2002) in 50 mM Tris-HCl buffer, pH 7.7, also containing 5 mM MgCl₂, 500 mM EDTA, 0.5 mM DTT, and 5% glycerol, and stored at -20°C. The enzymatic assay described by Graninger and associates (2002), with slight modifications, was used. Reaction mixture containing 300,000 counts per minute of dTDP-[¹⁴C]glucose (prepared by S. Raffo), 0.1 μ mol NAD⁺, 0.3 mg of protein extract, and an appropriate volume of 50 mM Tris-HCl buffer (pH 7.7) to complete 50 μ l, was incubated for 1 hour at 28°C. Then, 0.5 μ mol of NADPH was added to each reaction mixture and the mixture was incubated for an additional 1 h. Water (450 μ l) was added and proteins were removed by precipitation with an equal volume of absolute ethanol. Samples were spotted onto Whatman 1 paper and desalted by elution with absolute ethanol. dTDP-nucleotides were water eluted from the paper and were hydrolyzed with 0.01 N HCl at 100°C for 10 min. HCl was removed by evaporation under an air stream and the hydrolysate was subjected to descending paper chromatography developed with butanol/pyridine/water (6:4:3). Radioactivity was detected with a scanner. Nonradioactive rhamnose and glucose were used as internal standards and detected by the alkaline-silver method (Trevelyan et al. 1950).

Determination of glycosyltransferase activity.

M. loti cell extracts were obtained as described previously (Lepek et al. 2002). The glycosyltransferase assay was performed as described by Piekarowicz and Stein (2002). Briefly, total extract from *M. loti* wild type, *M. loti lps* β 1, and *M. loti lps* β 2 were incubated with *lps* β 1 R-LPS in the presence of UDP-[¹⁴C]glucose (310 μ Ci/ μ mol) or UDP-[¹⁴C]galactose (300 μ Ci/ μ mol). For the separation of unincorporated radioactive material from labeled LPS, the reaction mixtures were loaded onto a 3-ml Sephadex G-100 column. One-third of the fractions were used for scintillation counting and those fractions containing labeled LPS were vacuum dried, hydrolyzed, and analyzed by thin-layer chromatography as was described previously (Almirón-Roig et al. 2000).

Cyclic β (1-2) glucan determination.

Isolation and detection of β (1-2) cyclic glucan was carried out by thin-layer chromatography as described previously (Briones et al. 2001).

Motility and DOC and cationic peptide sensitivity assays.

The bactericidal effect of PmB was tested as described elsewhere (Campbell et al. 2002). *M. loti* strains were grown in AB minimal medium to an OD₆₀₀ of 1.0. Bacteria cells were incubated for 1 h at room temperature with PmB at 20 μ g/ml. After the incubation period, bacterial survival was titrated by plating a series of dilutions on AB-sucrose agar plates. The percentage of surviving bacteria was calculated according to CFU recovered. PmB assay was performed in duplicate. PmB and melitin effect on outer membrane permeability was assayed by fluorometry using the fluorescent probe NPN as described previously (Freer et al. 1996), with some modifications. Briefly, exponentially growing *M. loti* cells were harvested and suspended in 10 mM sodium phosphate saline buffer (pH 7.4) to an absorbance at 550 nm of 0.5. NPN was added to a final concentration of 10 μ M into polystyrene cuvetts containing 1 ml of cell suspension. The permeating agents were added at the following final concentrations: 50 U of PmB and melitin at 10 μ g/ml. Fluorescence was monitored at 28°C with an AMINCO-BOWMAN Luminescence Spectrophotometer with excitation and emission wavelengths set to 350 and 420 nm, respectively. The results were expressed in relative fluorescence units. Motility was determined in TY 0.2% agar plates containing different Ca²⁺ concentrations. Strains were inoculated by puncture and were incubated at 28 or 18°C, and the halo of growth was visualized at different days postpuncture. For DOC sensitivity assay, bacterial culture at late logarithmic phase growth was centrifuged and cells resuspended at a final OD of 0.2 in TY medium. The suspension (10 μ l) was spotted into plates of TY containing 1 mM DOC and different Ca²⁺ concentrations. Bacterial growth was examined after incubation at 28 or 18°C.

Nodulation assay and fluorescence microscopy experiments.

Seed were surface sterilized and pregerminated. Three-day-old seedlings were planted (20 plants per jar) in autoclaved modified Leonard jars filled with vermiculite and Fahraeus N-free solution (Fahraeus 1957) and inoculated with *M. loti* strains at an OD₆₀₀ of 0.6 (10 μ l/plant). Number of nodules per plant was determined at four weeks postinoculation.

pTB93F plasmid (Table 1) was introduced into *M. loti* wild-type and mutant strains and, at different times post-inoculation, the occurrence of infection thread formation was evaluated by using fluorescence microscopy. Difference between the infection thread mean number of experimental groups was analyzed using the Student *t* test. Nodules were harvested 20 days after inoculation and the infection state of nodule cells was evaluated by using fluorescence microscopy. Fluorescence microscopy experiments were made using a Nikon ECLIPSE E600 EPI-FL microscope, and images were captured with a Nikon Single-Lens Reflex Camera. The filter set for fluorescence microscopy consisted of a 450- to 490-nm band-pass excitation filter and a 515-nm barrier filter.

Isolation of bacteria from nodule.

Nodules were treated for 5 min with 0.2% HgCl₂ in 0.5% HCl, washed five times with sterile water, and crushed in AB medium. This suspension was used either for recovery of viable bacteria or for PCR reactions.

Competition assay.

For competition assay, mutant and wild-type strains were mixed together in a 1:1 ratio and inoculated on *L. tenuis* plants as described previously. At three weeks postinoculation, six nodules of each experiment were harvested, sterilized, and crushed in AB medium as described previously. To determine the relative proportions of the mutant strain with respect to the wild-type strain, serial dilutions were plated on AB sucrose 0.5% minimal medium with or without Gm.

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