

## **Bacterial surface polysaccharides and their role in the rhizobia-legume association.**

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### **Introduction**

The establishment of a nitrogen-fixing symbiosis is an economically important plant phenomenon. Biological reduction of dinitrogen to ammonia is among the most effective fixation systems facilitating the plant growth in nitrogen starved soils without the requirement of massive inputs of fertilizers. This process occurs in legumes roots in structures called nodules. Nodule development is induced when legumes enters into association with gram-negative soil bacteria that belong to the *Rhizobiaceae* family, such as *Bradhyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Rhizobium* and *Azorhizobium*, all denoted with the general term, rhizobia. Rhizobia invade the nodule and differentiate into a state called bacteroid able to fix the atmospheric nitrogen under the appropriate conditions generated inside the nodule. The nodulation process is the result of a selective interaction between rhizobia and the legume family (*Leguminosae* or *Fabaceae*), which means that each legume is nodulated only by specific bacterial species. For example, *Medicago sativa* by *S. meliloti*, *Glycine max* by *B. japonicum* and *Lotus spp.* by *M. loti*. Some bacteria display broad host range such as the *Rhizobium* NGR234 that can nodulate more than 112 legume genera. On the other hand *M. loti* has a reduced host range allowing nodulation of different *Lotus* species (*L. corniculatus*, *L. japonicus*, *L. glaber* and *L. uliginosus*) and *Leucaena leucocephala*. Nodules are classified as determinate or indeterminate, depending on the legume that is nodulated. Indeterminate nodules are elongated and present a persistent meristem at their tip, which is infected by rhizobia residing in the nodule. This characteristic results in a gradient of development stages from the older tissue near the root to the growing meristem at the nodule tip. Determinate nodules lack a persistent meristem, are usually round, and don't show a gradient of development stages as is the case of indeterminate nodules. Examples of indeterminate nodule morphology are the ones formed on roots of *Medicago sativa* (alfalfa), *Medicago truncatula*, *Pisum sativum* (pea), *Trifolium* species (clovers) and *L. leucocephala*, meanwhile those formed on roots of *Lotus* species, *Glycine max* (soybean) and *Vicia faba* (bean) are determined.

Due to the importance of nodulation for agriculture, intensive researching is being carried on in this area in order to understand the molecular bases of this process. The new knowledge could be used to obtain more efficient nitrogen fixation process, modification of the host range or increased competitiveness that may influence its capacity to compete in the rhizosphere with other bacteria.

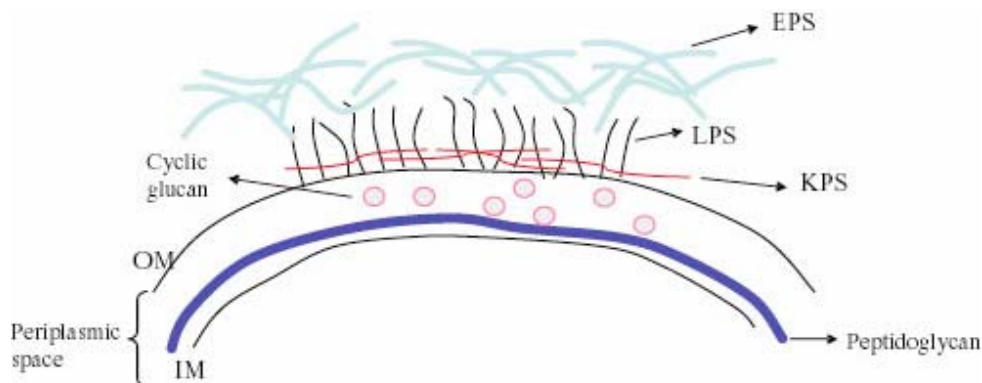
Rhizobium-legume associations take place through a sequence of events, starting with the exchange of signal molecules between the two symbiotic partners. Rhizobia are chemoattracted toward root legume proximity by some of the nutrients present in the root exudates: sugars, amino acids, some dicarboxylic acids and various aromatic compounds such as some flavonoids (Brenner and Winans, 2005). Flavonoids also are necessary to induce the bacterial production of the so called Nod factors (NF) which are lipochitooligosaccharides that have the capacity to induce the transcription of genes that control the morphological changes observed in the plant roots: deformation, branching and curling of root hairs, and cortical cells division which in turn gives rise to nodule primordium. Moreover in some legumes NF favors mature nodule development and pre-infection threads formation (Geurts and Bisseling, 2002). Simultaneously to these changes, bacterial adhesion to the hair root tip, colonization and entrance into the hair root through tubular structures called infection threads, occurs. The infection threads filled with bacteria grow toward the base of the root hair, branch and penetrate into the nodule cells. Upon released from the infection threads, bacteria became internalized in legume nodule cells through an endocytosis-like process (Gage, 2004). Rhizobia remain surrounded by the plant cell plasma membrane and differentiate into nitrogen-fixing bacteroids. Microsymbiont and host derived plasmatic membrane (or peribacteroid membrane) form the symbiosome. In indeterminate nodules each symbiosome contains one bacteroid (Brewin, 1998). In determinate nodules, one symbiosome contains several bacteroids (Cremola *et al.*, 2000).

Together with Nod factors, other bacterial components have been involved in bacterial adhesion, formation and extension of the infection thread, releasing of bacteria into the nodule cells and differentiation into bacteroids. Between these components, polysaccharides have a relevant role. The present review will describe the importance of rhizobial polysaccharides in the establishing of symbiosis in general, and we will focus on the results obtained in our laboratory in the *Lotus glaber-M. loti* system in particular.

## **Bacterial polysaccharides**

### ***Structure, characteristics and function in the free-living bacteria***

Rhizobia synthesize different classes of polysaccharides: exopolysaccharide (EPS), capsular polysaccharides (KPS), lipopolysaccharides (LPS) and the cyclic glucan. Some of them are secreted to the media, others are exposed on the surface or present in the periplasmic space (Figure 1). EPS is a heteropolysaccharide formed by a repetitive unit constituted by hexose residues such as glucose, galactose, mannose rhamnose, glucuronic acid and galacturonic acid with piruvyl, acetyl, succinyl and hydroxybutanoil substitutions. EPSs are abundant extracellular products secreted to the surrounding media and accumulated on the cell surface. In free-living bacteria it acts as a physical barrier against external agents and it is also involved in the attachment to surfaces (Spaink, 2000). The KPS is a class of exopolysaccharide that remains attached to the bacterial surface. It has a role in the protection against desiccation and it confers resistance to bacteriophages (Frayse *et al.*, 2003). Its structure is strain specific meanwhile the EPS is generally common to all the



**Figure 1.** Schematic representation of bacterial surface polysaccharides.  
OM: outer membrane, IM: internal membrane.

strains of the same specie. KPS also differentiate from the EPS in that it contains 3-deoxy-d-manno-2-octulosonic acid (KDO) (Forsberg and Reuhs, 1997). LPS is a polysaccharide anchored in the bacterial outer membrane and is formed by three different regions, the lipid A, the core and the O-antigen. Lipid A is the part of the molecule involved in the membrane anchoring. The core moiety is a polysaccharide region attached to the lipid A by KDO. The third region consists of a chain of repeating units called O-antigen formed principally by deoxy and/or dideoxy-sugar residues. LPS, in free-living bacteria, is involved in membrane stabilization and it also acts as a barrier against antibacterial compounds such as the cationic peptides. Composition and structure analysis revealed that LPSs are also strain specific (Lerouge and Vanderleyden, 2001). For *M. loti* only the LPS composition of two strains were reported, that of the strain NZP2213 which O-antigen is formed principally by 2-O-acetyl-6-deoxy-L-talose (Russa *et al.*, 1995) and that of *M. loti* Ayac 1 BII, an autochthonous strain from Argentina, which O-antigen is composed mainly by rhamnose (D'Antuono *et al.*, 2005). Finally, cyclic glucans are cyclic molecules formed by glucose that could be or not substituted with anionic groups. They are accumulated principally in the periplasmic space and they were found in bacteria belonging to the group of the  $\alpha$ -proteobacteria. *M. loti*, *S. meliloti* and the pathogenics *Agrobacterium tumefaciens* and *Brucella* spp. present a  $\beta$  (1-2) glucan type (Lepek *et al.*, 1990; Breedvald and Miller, 1998; Briones *et al.*, 2001), meanwhile *B. japonicum* presents a  $\beta$  (1-3)  $\beta$  (1-6) glucan type (Gore and Miller, 1992). Many observations are consistent with the hypothesis that cyclic glucans are involved in protection against hypoosmotic conditions (Spaink, 2000). More details about the different rhizobia polysaccharide structures could be found in the reviews of Spaink (2000) and Fraysse *et al.* (2003).

### ***Polysaccharides function during the nodulation process***

#### *Exopolysaccharides*

EPS appears to be essential for the successful invasion of indeterminate nodules (Pellock *et al.*, 2000). Two different EPS were described for *S. meliloti*, the succinoglycan (EPS I) and the galactoglycan (EPS II). Each of them can be found in the form of high or low molecular mass molecule depending on the number of repetitive units. The low molecular mass EPS I,

EPS II and KPS of *S. meliloti* were described to be required for the extension of the infection thread although the three does not have the same efficiency in promoting this process (Frayse *et al.*, 2003). Addition of the low molecular mass EPS to an *exo*<sup>-</sup> mutant reverts the non-infective phenotype. This may indicate that this polysaccharide acts as a signaling molecule (Gonzalez *et al.*, 1996). Interestingly EPS does not have the same relevance in the formation of determinate nodules (Borthakur *et al.*, 1986). This was elegantly demonstrated studying the nodulation induced by *M. loti* *exo*<sup>-</sup> mutants on *Lotus uliginosus* (determinate nodules) and on *Leucaena leucocephala* (indeterminate nodules). Meanwhile *M. loti* EPS-altered mutants were ineffective on the indeterminate nodulating host, they maintain their fully effectiveness on a determinate one (Hotter and Scott, 1991). However, since the structure and classes of *M. loti* EPS were not studied in detail, it is not possible to rule out the presence of other bacterial structures able to fulfill the EPS function in the *exo*<sup>-</sup> studied mutants. Moreover, there are results that suggest that EPS have also some role in the formation of determinate nodules. Nodules of plants inoculated with a mixture of *exo*<sup>-</sup> mutant and wild type strains of *B. japonicum* were principally occupied with the wild type strain (Parniske *et al.*, 1993).

One of the functions attributed to the EPS is the organization of root hair cytoskeleton influencing in this way the infection thread extension (Pellock *et al.*, 2000). Other authors suggested a function as negative modulator of the plant defense response (Frayse *et al.*, 2003). This conclusion was obtained from experiments made both with determinate and indeterminate systems (Niehaus *et al.*, 1993; Niehaus *et al.*, 1997; Parniske *et al.*, 1994).

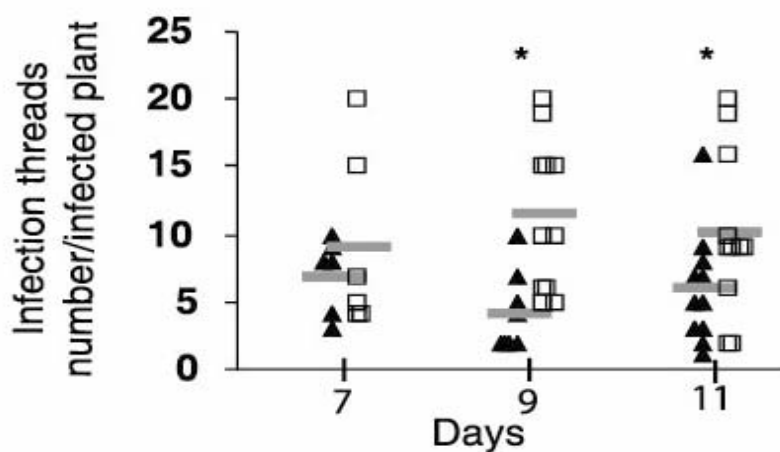
#### Lipopolysaccharides

LPS was involved in several developmental stages of the nodulation process. In root adhesion of *R. leguminosarum*-clover, *R. leguminosarum* bv. *trifolii* LPS was found to bind specifically to a lectin, accumulated in the tips of the root hair, called Trifoliin A (Dazzo and Brill, 1979). However, this effect cannot be generalized to other legume-rhizobia associations. In the case of *M. loti* selective binding of LPS molecules does not appears to be the determinant of the specificity of the interaction since a *R. leguminosarum* strain with the information necessary to express the *M. loti* Nod factors can nodulate Lotus in spite of the differences between the LPS of both bacteria (Pacios Bras *et al.*, 2000). Contrary to what happens with EPS, LPS molecules are involved principally in the formation of determinate nodules, especially during the initiation and elongation of the infection thread (Lerouge and Vanderleyden, 2001; Noel *et al.*, 2000). 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; Noel and Duelli, 2000; Noel *et al.*, 2000; Perotto *et al.*, 1994). In the development of indeterminate nodules it was found for *M. truncatula*, but not for *M. sativa*, that *S. meliloti* LPS is involved in the stage of colonization of plant nodule cells and in the differentiation into bacteroid (Niehaus *et al.*, 1998). However, a different role of LPS in determinate and indeterminate nodule development is not clear. For *M. sativa* (indeterminate nodules) it was described that *S. meliloti* *lps*<sup>-</sup> mutant induce the formation of normal nodules but this mutant is less competitive that the wild type strain (Lagares *et al.*, 1992). A similar result was obtained in our laboratory in the case of the association *M. loti*-*Lotus glaber* (determinate nodules) (D'Antuono *et al.*, 2005). *M. loti* *lps*β2 and *lps*β1 mutant strains, affected in the synthesis of the O-antigen, presented a

normal nodulation development but were out-competed when were co-inoculated on *Lotus* plants with the wild-type strain (Table 1). As other bacterial strains affected in the LPS synthesis, *M. loti lps<sup>-</sup>* shows an increased sensitivity to cationic peptides such as Polimixyn B and Melitin than the wild type strain. One of the suggested functions for LPS during the nodulation process is the protection against possible cationic peptides generated by the plant as part of its defense response. When the three strains studied are comparing, a correlation between lower competitiveness and higher sensitivity to the antimicrobial compounds is observed. Contrary to the results observed for other interactions that lead to the formation of determinate nodules, in the case of the *M. loti-Lotus glaber* system, normal infection threads are formed. Moreover, a significant higher number of infection threads were formed in plants inoculated with the *lpsβ2* mutant (Figure 2). Taken together all these data suggest that the event that affect competitiveness in the case of the *M. loti lpsβ2* mutant is down-stream the infection thread formation and may be the consequence of the absence of a protection barrier against the plant antibacterial compounds. Data obtained by different groups suggest that among the LPS functions several result of its relation with the plant defense response. LPS could act either as protection barrier or as a molecule that masks bacterial compounds that induce the host defense response and it may also act as suppressor of this response. Accordingly, not only the determinate or indeterminate legume phenotype should be relevant for the influence of the LPS in the nodulation process but also the class and intensity of the defense response generated in a particular legume.

**Table 1.** Comparison of competitiveness index and sensitivity to cationic peptides of the different *M. loti* LPS-affected mutants. Bacteria cells were incubated for one hour at room temperature with 20 mg/ml of PmB (polimixin B). Bacterial survivance was tittered by plating a series of dilutions on Minimal medium-Agar plates. For Competitiveness index determination, plants were inoculated with a mixture of an equal number of wild-type and mutant strains. After 3 weeks, bacteria were recovered form the nodules and plated. CI= colony forming units (CFU) of mutant recovered from the nodule/CFU of total bacteria recovered from the nodule. Data were extracted from D'Antuono *et al.* (2005).

Strains	Competitiveness index (CI)	% of survival after treatment with PmB
<i>M. loti lpsβ2</i>	10 <sup>-4</sup>	10-30
<i>M. loti lpsβ1</i>	10 <sup>-3</sup>	70
<i>M. loti wt</i>	—	99

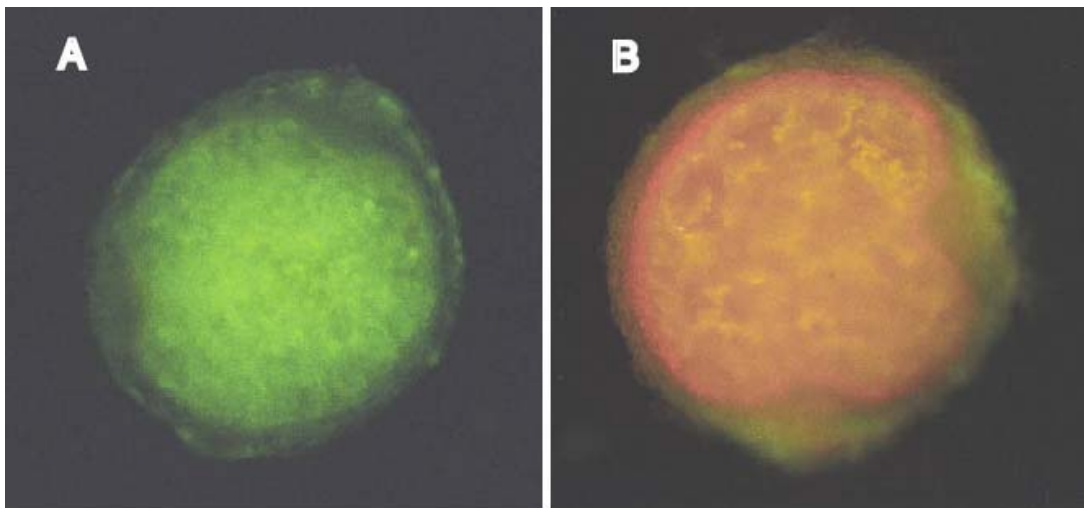


**Figure 2.** Comparison between the number of infection threads formed by the wild-type (▲) and by the *lpsβ2* mutant (◻) at different postinoculation times. Infection threads were visualized using for inoculation bacteria expressing green fluorescent protein. Mean number of infection threads is marked with a horizontal line. Statistically significant differences ( $P < 0.05$ ) are marked with asterisk.

An important characteristic of the LPS is its variation in response to external signals. There were found changes in the O-antigen structure when rhizobia were subjected to seed or root compounds (Noel *et al.*, 2004). Also, during the differentiation of the bacterium into bacteroid, LPS undergoes changes in its structure detected using LPS-specific monoclonal antibodies (Kannenberg *et al.*, 1998). A general observation is that the LPS changes during symbiosis lead to an increasing of surface hydrophobicity that influence the interaction between bacterial and plant cell membranes and could be relevant for the sincronic division bacteoid / symbiosome (Vedam *et al.*, 2004; Kannenberg and Carlson, 2001). Rhizobia as several other bacteria belonging to the  $\alpha$ -2 subclass of proteobacteria group, have as a distinctive feature of its lipid A, the presence of a long fatty acid chain, the 27-hydroxyoctacosanoic acid (27-OH-C28:0) (Bhat *et al.*, 1991). It was hypothesized that this molecule increases the stability of the bacterial membrane (Vedam *et al.*, 2003). During symbiosis a doubling of 27-OH-C28:0 amounts in the lipid-A molecule was reported (Kanneberg and Carlson, 2001) and this could be responsible in part of the increased surface hydrophobicity. The fact that inside the nodules a different LPS is present could explain why, in occasions, mutation in genes involved in the free-living bacterial LPS synthesis does not affect the nodulation process. Analyzing *M. loti* LPS by polyacrilamide gels electrophoresis, we observed that the pattern of bands of the LPS obtained from bacteria isolated from nodules was different to that extracted from free-living bacteria (unpublished results). Also we found that the mutation in the *lpsβ2* gene that has only effect on the bacterial competitiveness does not alter the pattern of bands of the bacteroid LPS (unpublished results).

### Cyclic glucans

Both in determinate and indeterminate nodule formation the absence of cyclic glucan synthesis affect the invasion capacity of the bacteria. As was mentioned above, *S. meliloti* and *M. loti* synthesize a  $\beta$  (1-2) cyclic glucan. Mutants affected in the synthesis of this polysaccharide induce the formation of empty nodules and does not induce the formation of the infection threads (Geremía *et al.*, 1987; D'Antuono *et al.*, 2005; Figure 3). It was described in *M. loti*, in *Agrobacterium tumefaciens* and in *Brucella abortus* that the absence of this polysaccharide in the periplamic space causes a pleiotropic phenotype at the level of the bacterial envelope. Mutants are no motile and more sensitive to detergents (D'Antuono *et al.*, 2005; Briones *et al.*, 2001). In the absence of the cyclic glucan, problems in the flagellum ensemble (Swart *et al.*, 1994) and reduction in the accumulation of proteins, which are components of a transport system (Banta *et al.*, 1998), occurs. The mechanism by which the glucan stabilizes the membrane and its components could be related with its osmoprotective function (Miller *et al.*, 1986) and with its capacity of protecting the membrane against the disruptive action of calcium (D'Antuono *et al.*, 2005). The pleiotropic effect on the bacterial envelope could be the cause of the early interruption of the symbiotic process at the initiation of infection thread formation and bacteria invasion. However it could not be ruled out that the  $\beta$ (1-2) cyclic glucan has itself some other function in the process.  $\beta$ (1-3)  $\beta$ (1-6) cyclic glucan of *B. japonicum* was involved in the suppression of the host defense response (Bhagwat *et al.*, 1999; Mithofer *et al.*, 2002). Recently it was hypothesized that  $\beta$ (1-2) cyclic glucan might be a virulence factor in *Brucella abortus* because it was demonstrated that this molecule interacts with the cholesterol of the host membrane lipid rafts, perturbing then the intracellular trafficking to the advantage of the pathogen (Arellano-Reynoso *et al.*, 2005).



**Figure 3.** Fluorescence light microscopy of cross section of nodules induced on *Lotus* roots inoculated with **A**, *M. loti* wild-type strain and **B**, *M. loti* *cgs* mutant strain. Bacteria expressing green fluorescent protein were used. Modified from D'Antuono *et al.* (2005) *Molecular Plant-Microbe Interactions*, volume 18, with permission.

## Conclusions and perspectives

Different bacterial polysaccharides have been found as relevant in the process that leads to the formation of an effective nodule. The results obtained by several laboratories suggest that they have principally influence on two aspects of the process: the bacterial relation with the defense response that could be generated in the plant host and the intimate interaction that occur between bacterial and plant cell membranes. The molecular mechanisms, by which each polysaccharide acts, remain to be determined.

In the last years, a great advance in the knowledge of the legumes genomic organization and gene expression was achieved. The use of bacterial mutants affected in different steps of the nodulation process helps to the identification of those plant genes which expression characterizes each step (Mitra and Long, 2004). Some polysaccharide-affected mutants were used with this purpose. Recently, *Lotus japonicus* became a model legume for the study the molecular process that leads to the formation of determinate nodules. About 112,000 *Lotus* EST sequences are now deposited in the public GenBank database. Technologies for high-throughput measurement of *Lotus* gene transcript levels during symbiosis began to be applied (Colebatch *et al.*, 2002; Colebatch *et al.*, 2004; Kouchi *et al.*, 2004). Inoculation assays with *M. loti* *cgs* and *lpsβ2* mutant strains, affected in two different steps of the nodule development, are being carried on in order to study the transcriptional *Lotus* gene response during the symbiosis event.

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