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Molecular Microbiology (2006) **59**(6), 1704–1713
doi:10.1111/j.1365-2958.2006.05057.x
First published online 30 January 2006

A novel polar surface polysaccharide from *Rhizobium leguminosarum* binds host plant lectin

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Summary

Rhizobium **bacteria produce different surface polysaccharides which are either secreted in the growth medium or contribute to a capsule surrounding the cell. Here, we describe isolation and partial characterization of a novel high molecular weight surface polysaccharide from a strain of *Rhizobium leguminosarum* that nodulates *Pisum sativum* (pea) and *Vicia sativa* (vetch) roots. Carbohydrate analysis showed that the polysaccharide consists for 95% of mannose and glucose, with minor amounts of galactose and rhamnose. Lectin precipitation analysis revealed high binding affinity of pea and vetch lectin for this polysaccharide, in contrast to the other known capsular and extracellular polysaccharides of this strain. Expression of the polysaccharide was independent of the presence of a Sym plasmid or the *nod* gene inducer naringenin. Incubation of *R. leguminosarum* with labelled pea lectin showed that this polysaccharide is exclusively localized on one of the poles of the bacterial cell. Vetch roots incubated with rhizobia and labelled pea lectin revealed that this bacterial pole is involved in attachment to the root surface. A mutant strain deficient in the production of this polysaccharide was impaired in attachment and root hair infection under slightly acidic conditions, in contrast to the situation at slightly alkaline conditions. Our data are consistent with the hypothesis that rhizobia can use (at least) two mechanisms for docking at the root surface, with use of a lectin–glycan mechanism under slightly acidic conditions.**

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Introduction

Under nitrogen-limited conditions, the Gram-negative soil bacterium *Rhizobium leguminosarum* can establish a symbiotic interaction with certain leguminous plants such as *Trifolium* (clover), *Pisum* (pea), *Vicia* (vetch) and *Phaseolus* (bean). This host plant-specific interaction results in development of root nodules in which the *Rhizobium* bacteria differentiate into nitrogen fixing bacteroids (for a review, see Kijne, 1992). Root nodule formation is triggered by rhizobial signal molecules, called LCOs (lipochitin oligosaccharides) or Nod factors. Biosynthesis of Nod factors is encoded by *nod* genes. With *R. leguminosarum*, these genes are usually located on a so-called Sym(biosis) plasmid. Host plant specificity of nodulation is primarily determined by induction of *nod* gene expression and by production of specific Nod factors.

Infection of plant root and nodule tissue by *R. leguminosarum* requires attachment of the bacteria to host plant root hairs. Attachment to plant cells is a complex process in which multiple factors are involved (Matthysse and Kijne, 1998). For root hairs, two main steps have been distinguished, primary and secondary attachment. Primary attachment has been described to be mediated via bacterial adhesins (Smit *et al*., 1992) or by plant-produced lectins (Dazzo *et al*., 1984). It is followed by a secondary attachment step which can consist of anchoring by bacterial cellulose fibrils (Dazzo *et al*., 1984). This secondary form of attachment has been shown not to be essential for successful invasion of the host root (Smit *et al*., 1987), but likely facilitates infection of fast growing root hairs (Laus *et al*., 2005).

Lectins are proteins with at least one non-catalytic domain that can reversibly bind specific carbohydrate structures. Lectin molecules of a special family, the Candy lectins (Laus and Kijne, 2004), are abundant in legume seeds. In addition, ligand and immunolocalization have shown the presence of Candy lectins on tips of emerging root hairs (Díaz *et al*., 1995). In *Pisum sativum* (garden pea), root hair lectin and seed lectin represent the same protein, encoded by one functional gene (Hoedemaeker *et al*., 1994). In the seventies, Bohlool and Schmidt (1974) were the first to report evidence that Candy lectins may allow specific attachment of homologous rhizobia to host plant roots. Lectins present at the tip of a root hair were suggested to recognize and bind specific carbohydrate

structures in the polysaccharide capsule surrounding the bacteria. Now Nod factor production and recognition have been discovered as primary determinants of host plant specificity in the *Rhizobium*–legume interaction, sugar binding specificity of the lectins produced by the host plant and the presence of a corresponding carbohydrate epitope in the bacterial capsule may still play an additional role in recognition of some homologous rhizobial strains.

We have studied the interaction between surface polysaccharides of *R. leguminosarum* strain RBL5523 that nodulates pea (*Pisum sativum*) and vetch (*Vicia sativa*), and pea/vetch lectin. Pea (*P. sativum*) lectin (PSL) and vetch (*V. sativa*) lectin (VSL) are well-characterized and almost identical proteins. Both lectins possess high binding affinity for glucose/mannose-related saccharides (Van Wauwe *et al*., 1975; Gebauer *et al*., 1979; 1981). The sugar binding site of PSL has been studied in detail, both by X-ray crystallography and mutational analysis (Van Eijsden *et al*., 1992; Rini *et al*., 1993). In order to bind in the carbohydrate binding site of PSL, the C4 and C6 hydroxyls of the binding sugar must be unsubstituted (Van der Schaal *et al*., 1984). The rhizobial surface is characterized by a variety of polysaccharides such as lipopolysaccharide (LPS) and acidic capsular polysaccharides (CPS), and some of these may contain lectin binding epitopes. PSL binding polysaccharide fractions from *R. leguminosarum* have been reported in literature, but up to now purification was insufficient, lectin preparations were poorly characterized and specific mutant bacteria deficient in production of lectin binding polysaccharides have not been characterized (Wolpert and Albersheim, 1976; Planqué and Kijne, 1977; Kamberger, 1979; Hrabak *et al*., 1981). We describe isolation and partial characterization of a novel polar capsular glucomannan of *R. leguminosarum* with high binding affinity for both PSL and VSL, as well as the infection behaviour of a glucomannan-deficient mutant bacterium.

Results

Polysaccharide purification

At the start of this study, four surface polysaccharides of *R. leguminosarum* RBL5523 bacteria had been (partially) characterized: cellulose microfibrils (Smit *et al*., 1987), a secreted extracellular polysaccharide (EPS), an EPS-like CPS, and LPS (Laus *et al*., 2004). Here, we report three additional surface polysaccharides of this strain: a low molecular weight neutral polysaccharide (LMW NP), a high molecular weight neutral polysaccharide (HMW NP) and a gel-forming polysaccharide (GPS).

Isolation of LPS from log-phase RBL5523 bacteria by using the hot water-phenol method appeared to yield a crude surface polysaccharide preparation, containing LPS, CPS and neutral polysaccharides (see *Experimental procedures*). The neutral polysaccharide fraction was subjected to Sephadex G50 size exclusion chromatography. A polysaccharide fraction of about 3 kDa in size was found, called LMW NP. However, the majority of the neutral polysaccharide fraction eluted in the void volume of the column, indicating a size larger than 40 kDa. Attempted fractionation by size exclusion chromatography using Sephacryl S-400HR showed this fraction to elute in the void volume again, indicating a size larger than 2×10^6 Da. Other polysaccharide peaks were absent, and this elution pattern did not change when fractionation took place in the presence of 0.1% sodium dodecyl sulphate to inhibit aggregation. This fraction was therefore referred to as HMW NP. Furthermore, a GPS was isolated from RBL5523 bacteria with use of the method of Breedveld *et al*. (1990). GPS is a poorly water-soluble neutral polysaccharide, produced under stationary growth conditions (Zevenhuizen and van Neerven, 1983).

RBL5523 bacteria secrete a large amount of acidic EPS molecules in the culture medium. Removal of EPS from the culture medium by ethanol precipitation followed by ion exchange chromatography revealed a remaining amount of LMW NP. Both HMW NP and GPS could not be detected in the culture medium.

Polysaccharide characterization

The composition of EPS/CPS and LPS from strain RBL5523 has been reported recently (Laus *et al*., 2004). Carbohydrate composition analysis of LMW NP showed the exclusive presence of glucose (data not shown). Permethylation studies only revealed 1,2-linkages, which strongly suggests that LMW NP represents the well-known rhizobial cyclic β-1,2-glucan (Zevenhuizen *et al*., 1990). β-1,2-glucan is known to be a cell-associated as well as an extracellular polysaccharide, and plays a role in osmoadaptation (Miller *et al*., 1986). GPS from other rhizobial strains, purified in an identical way, has been characterized before and consists of repeating units of galactose, mannose and glucose in a 4:1:1 ratio (Zevenhuizen and van Neerven, 1983).

Analysis of HMW NP isolated from the capsule of RBL5523 bacteria revealed the presence of a relatively large amount of glucose and mannose (Table 1). In addition, minor amounts of galactose and rhamnose were identified. Permethylation studies identified different linkages within the HMW glucomannan. Terminal mannose and, to some extent, glucose were identified, in addition to 1,2-mannose, 1,4-glucose and 1,3,4-linked glucose as other major components (Table 2). The small amounts of galactose and rhamnose were not identified by permethylation analysis. Additional analysis will be required for establishment of the structure of this glucomannan.

Table 1. Carbohydrate composition of the HMW NP.[a]

Glycosyl residue	RBL5523	RBL5833
Rhamnose	0.6	1.7
Mannose	55.5	55.8
Galactose	3.6	2.1
Glucose	40.3	40.4

a. HMW NP was isolated from wild-type *R. leguminosarum* RBL5523 and the EPS-deficient mutant strain RBL5833, and data are presented in mole per cent of total carbohydrate. Error ± 5%.

EPS, CPS and LPS are possible contaminants of other rhizobial surface polysaccharides, including glucomannan. Glucose and galactose are constituents of EPS/CPS from RBL5523, and glucose, mannose and galactose form part of its LPS (Laus *et al*., 2004). As a test for EPS/CPS-contamination, we studied two EPS/CPS-deficient mutants of RBL5523, i.e. *R. leguminosarum* strains RBL5808 and RBL5833 (Laus *et al*., 2004). Both strains appeared to produce glucomannan, in an amount similar to that of wild-type bacteria. Glucomannan from RBL5833 was analysed, showing the same composition as wild-type glucomannan (Table 1). Also in view of the absence of the characteristic EPS/CPS-constituent glucuronic acid in glucomannan, we conclude that wild-type glucomannan is not contaminated with EPS/CPS. Contamination of glucomannan with LPS is improbable as well, as indicated by the absence of quinovosamine (Laus *et al*., 2004).

In order to test whether the small amount of galactose present in glucomannan is due to contamination, we studied a third mutant of RBL5523, the *exoB*-mutant strain RBL5811. This pleiotropic mutant produces a strongly reduced amount of EPS/CPS, is O-antigen- and GPS-deficient and does not nodulate host plant roots (Canter Cremers *et al*., 1990). Due to a Tn5-insertion in the *exoB* gene, RBL5811 bacteria are deficient in the production of UDP-galactose (Canter Cremers *et al*., 1990). As galactose serves as a link between the LPS core and the O-antigen chain (Laus *et al*., 2004), absence of galactose explains absence of O-antigen. Likewise, absence of galactose interferes with production of the galactose-containing EPS/CPS and GPS. Glucomannan appeared to be absent in the crude surface polysaccharide preparation of this mutant as well as in its growth medium. This indicates that galactose is a genuine constituent of glucomannan. A similar test for the presence of rhamnose is not available.

Table 2. Permethylation linkage analysis of the HMW NP.[a]

Permethylated carbohydrate	RBL5523	RBL5833
1-Mannose	29	32
1-Glucose	15	14
1,2-Mannose	24	23
1,4-Glucose	24	25
1,3,4-Glucose	7	6

a. HMW NP was isolated from wild-type *R. leguminosarum* RBL5523 and the EPS-deficient mutant strain RBL5833, and data are presented in mole per cent of total carbohydrate. Error ± 5%.

Lectin interaction

Glucomannan, EPS, CPS, LPS, β-1,2-glucan, and GPS were dialysed, lyophilized and tested for the ability to precipitate PSL and VSL (Table 3). Only glucomannan of both wild-type and EPS-deficient bacteria showed the ability to specifically precipitate a standard amount of 150 μg PSL, down to 16 ng ml^{-1} of polysaccharide. PSL precipitation was completely abolished in the presence of 15 mM 3-*O*-methyl-glucopyranose (a strong PSL hapten; Van der Schaal *et al*., 1984), whereas it was not affected in the presence of 15 mM galactose (not a PSL hapten). Identical results were obtained with 150 μg VSL (data not shown). Albeit β-1,2-glucan showed a limited ability to precipitate 150 μg PSL at a polysaccharide concentration of 0.8 mg ml^{-1}, this interaction could not be abolished by 3-*O*-methyl-glucopyranose. Precipitation may be due to physico–chemical interactions, other than sugar binding. EPS, CPS and LPS did not show PSL precipitation in concentrations up to 0.8 mg ml^{-1} polysaccharide. GPS could be solubilized up to 0.1 μg ml^{-1}, without showing an interaction with PSL in a precipitation test. Due to its insolubility, cellulose could not be tested in the lectin precipitation assay. However, its well-established β-1,4-glucan structure precludes PSL/VSL binding.

From these results, we conclude that glucomannan is the only PSL/VSL binding surface polysaccharide produced by *R. leguminosarum* RBL5523. In particular the presence of terminal mannose and glucose as well as 1,2-linked mannose explains the specific interaction of glucomannan with PSL and VSL.

Table 3. Pea lectin binding activity of polysaccharides from wild-type *R. leguminosarum* RBL5523 bacteria.

Polysaccharide	OD_{405}
HMW NP	0.404[a]
HMW NP + 15 mM galactose	0.386
HMW NP + 15 mM 3-*O*-methyl-glucopyranose	0.062
CPS	0.057[b]
EPS	0.057[b]
LMW NP	0.070
LPS	0.059[c]
GPS	0.055[a]
150 μg PSL (control)	0.056
Yeast mannan (control)	0.369

a. Not present in strain RBL5811.
b. Not present in the EPS-deficient strains RBL5808 and RBL5833.
c. Only present in the rough form in strain RBL5811.
In a standard precipitation assay, 150 μg PSL and 50 ng ml^{-1} polysaccharide were combined. Turbidity was measured at an OD value of 405 nm.

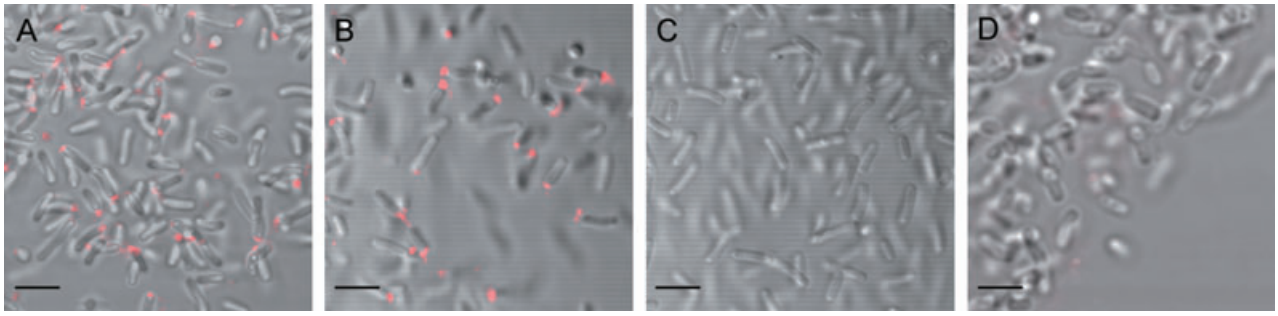

Fig. 1. Binding of rh-PSL to *R. leguminosarum* cells.
A. Incubation of wild-type RBL5523 bacteria with rh-PSL.
B. Incubation of wild-type RBL5523 bacteria with rh-PSL in the presence of 20 mM galactose.
C. Incubation of wild-type RBL5523 bacteria with rh-PSL in the presence of 20 mM 3-*O*-methyl-glucopyranose.
D. Incubation of glucomannan-deficient RBL5811 bacteria with rh-PSL. Bar represents 2 μm.

Polysaccharide localization

Because glucomannan represents the only PSL binding polysaccharide on the surface of RBL5523 bacteria, it can be localized with the use of labelled PSL. Rhizobia were cultured in B^- minimal medium, washed and incubated with rhodamine-labelled PSL (rh-PSL). As PSL is poorly soluble at an acidic pH, localization experiments were performed at pH 7.2. Microscopical analysis of the bacteria showed that rh-PSL molecules bound to one of the poles of the bacteria. However, a number of bacteria did not show any binding of rh-PSL. In the presence of 20 mM galactose, rh-PSL could still bind to the bacterial pole, whereas incubation in the presence of 20 mM 3-*O*-methyl-glucopyranose completely inhibited rh-PSL binding (Fig. 1). Polar binding of PSL was found with bacteria in exponential up to late stationary growth phases.

Strain RBL5523 is derived from the clover nodule *Rhizobium* RCR5, in which the pRtr5a biovar *trifolii* Sym plasmid (responsible for the ability to nodulate clover) has been replaced by the biovar *viciae* pRL1JI Sym plasmid (responsible for the ability to nodulate pea and vetch) (Table 4). Both the original parent strain RCR5 and strain LPR5045 that is cured from its Sym plasmid showed an identical ability to bind rh-PSL to one of the bacterial cell poles. In order to test whether expression of nodulation (*nod*) genes influenced rh-PSL binding, RBL5523 cultures were incubated with the *nod* gene inducer naringenin (1 μM or 10 μM). This treatment did not alter rh-PSL binding to the bacterial cell pole (data not shown).

Incubation of the *exoB* mutant strain RBL5811 with rh-PSL showed that these bacteria lacked polar rhodamine fluorescence, consistent with the absence of glucomannan (Fig. 1). Introduction of a 2.6 kb genomic fragment, containing the *exoB* gene, on plasmid pMP4697, restored glucomannan production as well as rh-PSL binding to the pole of RBL5811 bacteria (data not shown).

Attachment studies

We tested whether the bacterial pole, able to bind rh-PSL, is involved in attachment to host plant root hairs. For this

Table 4. Strains and plasmids used in this study.

Strain	Characteristics	Reference
RCR5	*R. leguminosarum* bv. *trifolii, rif*	RCR[a]
LPR5045	*R. leguminosarum* RCR5 cured of Sym-plasmid, *rif*	Hooykaas *et al.* (1982)
RBL5523	*R. leguminosarum* RCR5 Sym-plasmid replaced by pRL1JI *Spc3*::Tn *1831, rif str*	Priem and Wijffelman (1984)
RBL5808	*R. leguminosarum* RBL5523 *exo5*::Tn5, *rif str*	Laus *et al.* (2004)
RBL5811	*R. leguminosarum* RBL5523 *exoB52*::Tn5, *rif str*	Canter Cremers *et al.* (1990)
RBL5833	*R. leguminosarum* RBL5523 *pssD111*::Tn5, *rif str*	Van Workum *et al.* (1997)
RBL5760	*R. leguminosarum* RBL5523 *celE*::Tn5, *rif str*	Smit *et al.* (1987)
pXLGD4	*hemA-lacZ, Tc*	Leong *et al.* (1985)
pBBR-MCS5	*gm*, *mob*$^+$, *LacZα*	Kovach *et al.* (1995)
pMP2603	7.1 kb genomic fragment of *R. leguminosarum* RBL5523 containing *exoB* in pMP92, *Tc*	Canter Cremers *et al.* (1990)
pMP4697	a 2.6 kb HinDIII/EcoRI subclone of pMP2603 containing *exoB* in pBBR-MCS5, *gm*	This study
pMP4655	P*lac-egfp, Tc*	Bloemberg *et al.* (2000)

a. Rothamsted Collection of Rhizobium, Harpenden.

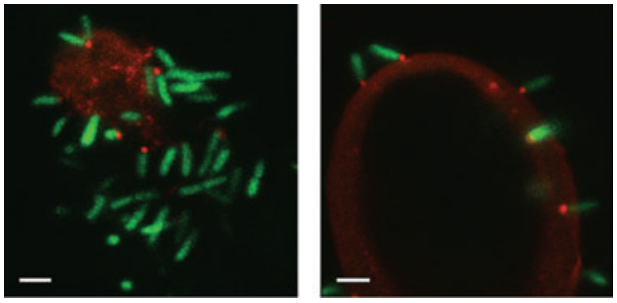

Fig. 2. Two *V. sativa* root hairs with attached cellulose-deficient RBL5760 bacteria (green) expressing GFP from pMP4655 followed by incubation with rh-PSL. Lectin binding is visible as red spots on the tip of most bacteria. Bar represents 3 μm.

purpose, we used the cellulose-deficient mutant RBL5760 (Smit *et al*., 1987). These mutant bacteria do not show the pronounced aggregation as observed with the wild-type strain RBL5523, and are more suitable for the investigation of single cell attachment. *V. sativa* roots were incubated with RBL5760 bacteria followed by incubation with rh-PSL. RBL5760 bacteria showed the presence of fluorescent cell poles indicating that glucomannan is also produced by these bacteria. Bacteria attached randomly on the root surface without a clear preference for the root hair tip. Significantly, the rh-PSL binding bacterial cell pole appeared to be the pole which is involved in attachment to the root hair cell surface (Fig. 2). Addition of rh-PSL to the root hairs in the presence of galactose did not affect bacterial rh-PSL binding. In contrast, fluorescent bacterial poles were not observed when rh-PSL was added in the presence of 3-*O*-methyl-glucopyranose. Interestingly, in both cases the bacteria remained attached to the root surface. Furthermore, attachment could not be prevented in the presence of 3-*O*-methyl-glucopyranose (data not shown). We also observed precipitation of rh-PSL to the root hair surface resulting in a high background for rhodamine fluorescence (Fig. 2). These results strongly suggest that the glucomannan pole is involved in rhizobial attachment to host plant root hairs. However, from the inability of 3-*O*-methyl-glucopyranose to prevent attachment or to detach bacteria from the root surface, we must conclude that an additional attachment mechanism other than lectin binding is active. This observation is consistent with data from Kijne *et al*. (1988) showing that presence of a lectin hapten does not inhibit attachment of single rhizobial cells to host root hair tips under the test conditions.

Nodule formation

In order to test whether the absence of glucomannan influences attachment efficiency, we compared the abilities of wild-type RBL5523 bacteria and the glucomannan-deficient RBL5811 mutant bacteria to infect and nodulate *V. sativa* roots in Jensen medium buffered at pH 7.2. Inoculation of *V. sativa* roots with wild-type bacteria resulted in an average of 5.8 nodules per root. As expected, *V. sativa* roots inoculated with RBL5811 bacteria did not show nodule formation on any of the roots (see also Canter Cremers *et al*., 1990). Expression of LacZ from plasmid pXLGD4 was used to monitor the infection process of RBL5811 bacteria. An average of 25.3 ± 11.4 infection sites per root was identified. Approximately 10% of these infection sites showed induction of formation of infection threads, which aborted in either the root hair or in the first cortical cell layers of the root. Since infection site formation requires efficient attachment, we conclude that in Jensen medium at pH 7.2 glucomannan is not required for efficient attachment and nodulation of RBL5523 rhizobia.

Because attachment characteristics of rhizobia are pH-dependent (Matthysse and Kijne, 1998), we also tested infection and nodulation of wild-type RBL5523 bacteria and RBL5811 mutant bacteria at pH 5.6. Inoculation of *V. sativa* roots resulted in an average of 7.6 nodules per root with wild-type bacteria, and zero nodules with RBL5811 bacteria. However, in contrast to the wild-type situation, microscopic examination of the roots stained for LacZ activity showed that under these conditions RBL5811 bacteria induce the formation of only very few infection sites (on average about 0.5 per root) in *Vicia* root hairs, indicative of poor attachment. Possibly, glucomannan is involved in efficient attachment of RBL5523 rhizobia at slightly acidic conditions.

Discussion

Seven different surface polysaccharides of *R. leguminosarum* strain RBL5523 have now been characterized (Zevenhuizen and van Neerven, 1983; Smit *et al*., 1987; Breedveld *et al*., 1990; Laus and Kijne, 2004; Laus *et al*., 2004). One of these, a novel polar glucomannan, appeared to be the only PSL/VSL binding surface polysaccharide of strain RBL5523. We do not exclude the possibility that other surface polysaccharides are produced under different culture conditions. Glucomannan may well be involved in bacterial attachment to a plant root surface on which PSL or VSL is exposed (for example, Díaz *et al*., 1995). Presence of terminally linked mannose and glucose as well as 1,2-linked mannose explains its nature as a PSL and VSL ligand. PSL and VSL are dimeric hydrophobic proteins with two sugar binding sites, well-equipped for cell-to-cell binding. The structure of the glucomannan is subject of further studies. Other surface polysaccharides produced by RBL5523 bacteria, EPS, CPS, LPS, GPS and β-1,2-glucan did not specifically pre-

cipitate PSL and VSL. With the exception of GPS and β-1,2-glucan, this can be deduced from the absence of mannose/glucose epitopes with unsubstituted C4 and C6 hydroxyls. GPS and β-1,2-glucan show putative lectin binding residues, but steric hindrance may prevent binding into the sugar binding site of the protein. Cellulose fibrils of *R. leguminosarum* which consist of 1,4-linked glucose can not bind PSL, with the exception of the non-reducing terminal residue.

The lectin recognition hypothesis predicts that presence of a specific lectin binding surface ligand is limited to homologous rhizobia. However, the glucomannan appeared also to be produced by the original clover-nodulating RCR5 parent strain, containing a biovar *trifolii* Sym plasmid instead of the biovar *viciae* pRL1JI Sym plasmid, and by a Sym plasmid-cured strain, LPR5045. This demonstrates that its formation is likely encoded by the bacterial chromosome and not by *nod* genes. Thus, production of a lectin binding polysaccharide is not necessarily restricted to a certain rhizobial biovar. Obviously, a clover symbiote can produce a 'pea- and vetch-specific' polysaccharide. Clover lectin has not yet been fully characterized, and it is not known whether clover lectin is a Candy lectin. Inhibition studies with clover lectin preparations showed that quinovosamine is an effective haptenic sugar (Hrabak *et al*., 1981). Interestingly, quinovosamine has been shown to be a component of the O-antigen chain of *R. leguminosarum* strain RBL5523 LPS (Laus *et al*., 2004). Possibly, strain RBL5523 produces two different lectin ligands, one specific for clover roots and another specific for pea/vetch roots. Production of compatible Nod factors will determine the host plant for nodulation.

Glucomannan could not be identified in both CPS and EPS fractions of mutant strain RBL5811. Consistently, incubation of these bacteria with rh-PSL did not yield the presence of fluorescent bacterial cell poles. Introduction of a genomic fragment containing the *exoB* gene restored glucomannan (and LPS O-antigen) production (data not shown). Probably, the small amount of galactose as shown by sugar composition analysis is a genuine part of the glucomannan. Galactose might serve as an initiation residue for its production.

The PSL binding bacterial cell pole was shown to bind to the surface of *V. sativa* root hairs. Incubation in the presence of 3-*O*-methyl-glucopyranose prevented binding of rh-PSL to the bacteria but not attachment to the plant root. Many if not all rhizobia show rhicadhesin-mediated attachment (Smit *et al*., 1986; Dardanelli *et al*., 2003). Swart (1994) has shown that rhicadhesin-mediated binding of *R. leguminosarum* to plant roots is a pH-dependent process and occurs primarily at a pH value above 6.5 (Matthysse and Kijne, 1998). In our present experiments, attachment of *R. leguminosarum* to plant roots followed by incubation with rh-PSL was performed at a pH of 7.2 to maximize rh-PSL solubility. Under these conditions, the bacteria are likely attached to the plant root via rhicadhesin, not to be detached in the presence of 3-*O*-methyl-glucopyranose. Correspondingly, this PSL/VSL hapten will not prevent rhicadhesin-mediated attachment. Similar adhesins have been shown to be localized unipolarly on the rhizobial cell (Ausmees *et al*., 2001). This suggests that both adhesins and glucomannan are expressed on the same bacterial cell pole, with adhesins as dominant attachment factors at pH 7.2.

Nodulation of *V. sativa* roots by wild-type RBL5523 bacteria resulted in successful nodulation at both pH 5.6 and pH 7.2. Swart (1994) showed that rhicadhesin detaches from the bacterial cell envelope at a pH below 6.5, due to release of calcium from the bacterial surface. As a result, the bacteria require under acidic conditions another attachment mechanism, to enable root hair infection and subsequent nodulation. This can be lectin-mediated docking. Inoculation of *V. sativa* roots with the glucomannan-deficient strain RBL5811 in pH 5.6-buffered medium resulted in poor root hair curling and formation of very few infection sites, consistent with a defect in root hair attachment. Strain RBL5811 normally produces Nod factors. Root hair attachment and infection site formation were restored when inoculation took place in pH 7.2-buffered medium, to allow rhicadhesin-mediated attachment. Induction of infection site formation by RBL5811 at pH 7.2 suggests that this strain is essentially able to attach to root hairs despite its O-antigen and GPS deficiency. However, infection threads formed were abortive due to production of a reduced amount of (galactose-deficient) EPS (Canter Cremers *et al*., 1990). Because the glucomannan-producing strains RBL5523, RBL5808 and RBL5833 each attach to host root hairs and induce formation of infection sites at pH 5.6, we adopt the working hypothesis that vetch root hair attachment is mediated by the glucomannan at slightly acidic conditions.

Taking previous and recent data together, we propose that *R. leguminosarum* RBL5523 can use at least two mechanisms for primary attachment to pea/vetch root hairs (i) rhicadhesin-mediated attachment, and (ii) a lectin-glucomannan-mediated attachment. We hypothesize that growth of the plant root in a slightly alkaline environment results in enhanced lectin solubility which will cause diffusion of the lectin molecule from the root hair tip into the rhizosphere. Under these circumstances, the bacteria require rhicadhesin-mediated attachment for root infection. At an acidic pH, lectin is poorly soluble and is retained at the root hair surface, enabling lectin-glucomannan-mediated attachment, whereas rhicadhesin is released from the rhizobial surface. For the test of this hypothesis, both a non-pleiotropic glucomannan-deficient mutant and a rhicadhesin-deficient mutant of strain

RBL5523 will be required as well as detailed attachment experiments with a range of pH values.

Experimental procedures

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 4. *R. leguminosarum* was routinely maintained on YMB plates (Hooykaas *et al*., 1977) containing the appropriate antibiotics. Polysaccharides were isolated from 1 l cultures grown in B^- minimal medium (Van Brussel *et al*., 1977) until an OD_{660} of 0.75 or as otherwise indicated. B^- minimal medium has a high carbon/nitrogen ratio, yielding copious polysaccharide production by rhizobia. Plasmid pMP4697 has been constructed by transfer of a 2.6 Kb EcoRI/HinDIII fragment of pMP2603 containing *exoB* to pBBR-MCS5 (Kovach *et al*., 1995).

Polysaccharide isolation

Bacterial cells were washed three times with an excess of 0.9% sodium chloride solution to remove extracellular polysaccharide. The bacterial cell pellet was lyophilized and surface polysaccharides were isolated by hot water-phenol extraction according to Westphal and Jann (1965). The water phase was dialysed extensively against H_2O to remove residual phenol using dialysis tubing with a molecular weight cut-off of 12–14 kDa (Medicell Int., London). This crude polysaccharide solution was brought to 50 mM Tris HCl, pH 7.0, and 10 mM $MgSO_4$, and 450 units RNase A and 667 units DNase I (Sigma) were added per equivalent of 1 l bacterial culture. Contaminating DNA and RNA were removed enzymatically by overnight incubation at 4°C followed by dialysis, as described above. The crude polysaccharide solution was brought to 100 mM NH_4HCO_3, pH 8.0, and 0.9% NaCl, and was applied to a 10 ml polymyxin B-agarose (Detoxi-Gel, Pierce) column and incubated overnight to facilitate LPS binding. Polysaccharides of non-lipid origin were removed from the column with binding buffer, dialysed and lyophilized. LPS was eluted from the column with a solution of 1% deoxycholic acid in 100 mM NH_4HCO_3, pH 8.0. Deoxycholic acid was removed from the LPS samples by dialysis against 4 mM Tris HCl, pH 9.1, 0.25% NaCl and 10% ethanol, followed by dialysis against H_2O (Reuhs *et al*., 1993). The LPS samples were lyophilized and stored at room temperature until further use. Approximately 200 μg of the CPS obtained from the flow through after polymyxin affinity chromatography were applied to a DEAE Sephadex A25 (Amersham) column (2.5×50 cm) equilibrated with 25 mM Tris HCl, pH 7.5. Acidic CPS were eluted from the column with 1 M NaCl. Presence of carbohydrates in the different fractions was determined by the orcinol-sulphuric acid method (Monsigny *et al*., 1988). Neutral polysaccharides that eluted in the void volume of the column were dialysed and lyophilized as described above. Neutral capsular polysaccharide were separated based on their size by Sephadex G50 or Sephacryl S-400HR (Amersham) gel filtration chromatography in the presence of 0.1% ammonium carbonate. The void volume of the column (1.6×90 cm) has been determined with the use of blue dextran or commercial HMW dextrans (Sigma), and presence of carbohydrates in the fractions was determined as described above. Capsular polysaccharide fractions were dialysed and lyophilized and stored at room temperature until further use. EPS of EPS-producing bacteria were isolated by precipitation with three volumes ice cold ethanol, overnight at 4°C. Precipitated EPS was removed by centrifugation at 16 000 *g* for 10 min and the remaining culture medium was 10-fold concentrated in a rotavapor at 40°C (Büchi, Switzerland). Both the precipitated EPS and the concentrated culture medium were dialysed against H_2O and lyophilized. The culture medium of EPS-deficient strains was directly concentrated 10-fold, dialysed and lyophilized. EPS were separated by ion-exchange chromatography on a DEAE Sephadex column followed by Sephadex G50 gel filtration chromatography as described above. GPS were extracted with NaOH from cultures of RBL5523 bacteria grown till an OD_{660} of 1.3 according to Breedveld *et al*. (1990).

Analytical procedures

Glycosyl composition analysis was performed by combined gas chromatography/mass spectrometry (GC/MS) of the per-*O*-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis. Myo-inositol internal standard (20 μg) was added to each dried sample. Methyl glycosides derivatives were prepared from 0.10 mg of dry samples by methanolysis in 1 M HCl in methanol at 80°C (18–22 h), followed by re-*N*-acetylation with pyridine and acetic anhydride in methanol (for detection of amino sugars). The samples were then per-*O*-trimethylsilylated by treatment with Tri-Sil (Pierce) at 80°C (0.5 h). These procedures were carried out as previously described (York *et al*., 1985; Merkle and Poppe, 1994). GC/MS analysis of the TMS methyl glycosides was performed on an HP 5890 GC interfaced to a 5970 MSD, using an Alltech AT-1 fused silica capillary column. Methyl glycoside derivatives of various monosaccharide standards were also prepared and analysed by GC/MS for comparison with the sample peaks.

Glycosyl linkages were determined by methylation analysis. The samples were methylated with methyliodide in dimethylsulphoxide containing dimethylsulphoxide anion according to the Hakomori procedure as previously described by York *et al*. (1985). The methylated samples were then converted to partially methylated alditol acetates as previously described (York *et al*., 1985) and analysed by GC/MS.

Isolation of PSL

Pea lectin was isolated from *P. sativum* cv. Rondo seeds (Cebeco, Rotterdam). Pea seeds were washed and dried and a total of 120 g was ground with a blender and extracted overnight at 4°C in 1200 ml extraction buffer (50 mM Tris, pH 8.2, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$ and 150 mM NaCl). The slurry was filtered through cheese cloth and centrifuged for 60 min at 21 000 *g* at 4°C to remove cellular material. The pH of the supernatant was set at pH 5 to precipitate acid-labile proteins, which are removed by centrifugation for

15 min at 18 000 *g* at 4°C. The pH of the supernatant was raised to a value of 7.3, saturated till 55% $(NH4)_2SO_4$ and incubated overnight at 4°C. Precipitated proteins were collected by centrifugation at 21 000 *g* for 30 min at 4°C, resuspended in 60 ml extraction buffer and dialysed against extraction buffer. The protein extract was applied to a Sephadex G75 (Amersham) column with a bed volume of approximately 150 ml and washed with 20 mM Tris HCl, pH 8.2 containing 0.9% NaCl to remove non-bound proteins. PSL was obtained by elution of the column with 20 mM Tris HCl, pH 8.2 in the presence of 0.9% NaCl and 200 mM D-glucose followed by dialysis and lyophilization. *V. sativa* lectin was purchased from Sigma.

Rhodamine-labelled PSL was produced using a rhodamine protein labelling kit (Pierce). After the labelling reaction, the sample was applied to a desalting column equilibrated with 25 mM Tris HCl, pH 7.5, followed by dialysis against H_2O and lyophilization.

Lectin binding tests

Lectin precipitation assays were performed as described by Van Wauwe *et al*. (1975) with the following modifications. Volumes were scaled down to 300 μl to accommodate microtiter plates. A standard agglutination assay contained 150 μg PSL in 20 mM Tris HCl, pH 7.2, and 15 μg yeast mannan (Sigma) was used as a positive control (Goldstein and Poretz, 1986). Agglutination was measured in the presence of 15 mM galactose or 15 mM 3-*O*-methyl-glucopyranose and was quantified spectrophotometrically in a microplate reader (Bio-Rad) at 405 nm.

Bacterial cultures were grown in B^- minimal medium until an OD_{660} of 0.75. Green fluorescent protein (GFP) was expressed from plasmid pMP4655. Bacteria of approximately 50 μl culture were resuspended in 100 μl 20 mM Tris HCl, pH 7.2, containing 200 μg rh-PSL and when indicated, 20 mM galactose or 3-*O*-methyl-glucopyranose, and incubated for 15 min at room temperature. Bacterial cells were washed three times in 20 mM Tris HCl, pH 7.2, and visualized with a Zeiss Axioplan 2 microscope equipped with a Bio-Rad MRC1024ES scan head. Exitation was performed with a Kr/Ar laser, for GFP at 488 nm (520 nm emission) and for rhodamine at 568 nm (580 nm emission). Germinated *V. sativa* roots were incubated with a bacterial suspension of OD_{660} of 0.1 in 20 mM Tris HCl, pH 7.2, for 30 min at room temperature. Roots were rinsed and incubated in a solution of rh-PSL and visualized as described above.

Nodulation assay

Nodulation assays were performed as described (Laus *et al*., 2004). Jensen medium (Vincent, 1970) was either buffered with 10 mM 2-[N-morpholino]ethanesulphonic acid at pH 5.6 or with 1.3 mM phosphate at pH 7.2. Roots were stained for LacZ activity as described by Boivin *et al*. (1990). Nodules and infection sites were counted from at least 24 roots originating from a minimum of three independent inoculations.

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