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Ethylene Prevents Nodulation of *Vicia sativa* ssp. *nigra* by Exopolysaccharide-Deficient Mutants of *Rhizobium leguminosarum* bv. *viciae*

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Exopolysaccharide-deficient mutants (Exo mutants) of *Rhizobium leguminosarum* bv. *viciae* are usually impaired in root nodule formation on their host plants. However, we found that *Vicia sativa* ssp. *nigra* (vetch) could be nodulated by such mutants if ethylene production by the host plant root, resulting from rhizobial inoculation, was minimized. Under these circumstances, Exo mutants induced delayed formation of partially infected nodules. Exo mutants did not induce abnormally large amounts of ethylene in host roots nor showed abnormal production of lipo-oligosaccharide Nod signals; thus, impaired nodulation could not be ascribed to these features. The nodulation ability of *R. leguminosarum* bv. *viciae* Exo mutants only affected in EPS synthesis could be restored completely by coinoculation with a Nod$^-$ Exo$^+$ strain, indicating that impaired nodulation is indeed caused by the absence of EPS. Our results are consistent with the following hypothesis: In addition to other nodulation-related phenomena, rhizobial Nod signals also induce ethylene formation in host plant roots. By influencing root cell growth, ethylene inhibits proper root infection by rhizobia. In case of delayed nodulation, for instance, due to EPS deficiency, ethylene formation precedes root infection and as a result nodulation is impaired.

Additional keywords: defense, lipopolysaccharide, symbiosis.

One of the best-studied examples of a relationship between microorganisms and plants is the symbiosis between *Rhizobium* bacteria and legumes. Rhizobia induce formation of nitrogen-fixing nodules on legume roots in a host-specific way, and factors determining this specificity are of particular interest. Host plant-specificity of nodulation is determined by the structure of Nod factors, acylated *N*-acetylglucosamine oligosaccharides carrying specific decorations (Lerouge et al. 1990; Spaink et al. 1991). These Nod factors are produced by rhizobia and elicit formation of nodule primordia in the host plant root. In addition, surface polysaccharides produced by *Rhizobium*, such as exopolysaccharides (EPS), capsular polysaccharides (CPS), and lipopolysaccharides (LPS), play a role in the nodulation process. The acidic EPS of *R. leguminosarum* is a polymer containing glucose, glucuronic acid and galactose in the ratio 5:2:1 (McNeil et al. 1986), and usually consists of high molecular weight and low molecular weight (a few repeating units) fractions. CPS of *R. leguminosarum* is a large neutral gel-forming polysaccharide consisting of glucose, galactose, and mannose in the ratio 1:4:1 (Zevenhuizen and Van Neerven 1983), which was found in many different *R. leguminosarum* strains (Zevenhuizen 1984). LPS of *R. leguminosarum* usually appears in two different forms: LPS-I is the long-chain LPS consisting of *O*-antigen attached via the core to lipid A, and LPS-II only consists of core-lipid A molecules (Carlson et al. 1987).

The symbiotic importance of EPS and LPS has been the subject of many studies, and has been reviewed recently (Leigh and Walker 1994). Indications that EPS might have a host-specific signaling function have been obtained by Djordjevic et al. (1987), who found that nodulation induced by Exo mutants of *Rhizobium* sp. strain NGR234 and Exo mutants of *R. l.* bv. *trifolii* in their respective hosts *Leucaena* and clover could be restored by addition of homologous EPS or EPS-derived oligosaccharides but not by addition of heterologous EPS. Likewise, the symbiotic deficiencies of Exo mutants of *R. meliloti* could be overcome by the addition of low molecular weight (LMW) EPS at the time of inoculation (Battisti et al. 1992; Urzainqui and Walker 1992). High molecular weight (HMW) acidic EPS did not promote invasion of Exo mutants of *R. meliloti*, and of the LMW EPS fractions only the tetrameric form of the repeating EPS unit was active (Battisti et al. 1992). It was also found that nonsuccinylated LMW EPS could not promote root infection of alfalfa by Exo mutants of *R. meliloti*. Therefore, it was hypothesized that (LMW) EPS is actively involved in root infection by *Rhizobium*, for instance, by specifically activating enzymes involved in root hair curling or infection-thread initiation and growth (Battisti et al. 1992). EPS may also act as a suppressor of the plant defense system during nodule development. Evidence for this was presented by Niehaus et al. (1993), who found significant plant defense responses in root nodules of alfalfa induced by an Exo mutant of *R. meliloti*.

Ethylene is known to play an important role in the regulation of plant defense responses of plants infected by incompatible pathogens (for a review, see Ohashi and Ohshima 1992). Furthermore, ethylene was found to inhibit nodulation of both *Pisum sativum* and *Vicia sativa* ssp. *nigra* (e.g., Lee and LaRue 1992a; Lee and LaRue 1992b; Zaat et al. 1989). In

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the present project, we tested the working hypothesis that the inability of EPS-deficient *R. l.* bv. *viciae* mutants to nodulate *V. sativa* ssp. *nigra* (vetch) and *P. sativum* is correlated with an inhibitory action of ethylene. We describe the nodulation behaviour of several Exo mutants of *R. leguminosarum* on two different hosts, common vetch and *T. repens* (white clover). It will be shown that Exo mutants of *R. leguminosarum* are able to nodulate vetch only if ethylene formation is repressed.

RESULTS

Synthesis of EPS, CPS, and LPS.

Mutants of *R. leguminosarum* strain RBL5515 affected in exopolysaccharide synthesis (Exo mutants) were obtained by transposon mutagenesis (Canter Cremers et al. 1988). Cell surface polysaccharide synthesis by *R. leguminosarum* RBL5515 and three of these Exo mutants, exo4, exoB, and exo344, has been recently described (Breedveld et al. 1993). By using the same methods, we studied cell surface polysaccharide synthesis by three other mutants, exo2, exo5, and exo370. The combined data of these studies are listed in Table 1 (for extracellular polysaccharide [EPS] and capsular polysaccharide [CPS]) and shown in Figure 1 (for lipopolysaccharide [LPS]).

Exo mutants of *R. leguminosarum* strain RBL5515 could be distinguished with respect to several characteristics. Mutants exo5 and exo370 were not able to produce a detectable amount of EPS, whereas the other Exo mutants produced 5 to 20% of the amount of EPS produced by the parental Exo$^+$ strain RBL5515. Mutants exo2 and exo4 produced EPS consisting of the same repeating units as does EPS from RBL5515, but exo2 is producing slightly more EPS than exo4 (Canter Cremers 1990). EPS synthesized by mutants exo344 and exoB lacked galactose (Breedveld et al. 1993; Canter Cremers et al. 1990). The gelling neutral CPS (Zevenhuizen and Van Neerven 1983) appeared to be produced in normal amounts by each mutant strain with the exception of exoB (Canter Cremers et al. 1990) and exo370. After electrophoretical analysis, mutants exo2, exo4, exo5, and exo344 showed an LPS profile identical to that of parental *R. l.* bv. *viciae* strain RBL5515 (Fig. 1). Introduction of Sym-plasmid pRL1JI::Tn1831 (lane 8) or pSym5::Tn1831 (not shown) did not alter the LPS profile. The LPS profile of the parental strain consists of two components, LPS-I and LPS-II. LPS-II presumably consists of lipid A and core LPS, whereas LPS-I contains O-antigen in addition (Carlson et al. 1987). In contrast to the other mutants, exoB (Canter Cremers et al. 1990) and exo370 lacked the O-antigen containing LPS-I.

Table 1. Cell surface polysaccharide production by *Rhizobium leguminosarum* RBL5515 and six Exo mutants

Strain	EPS production[a]	EPS structure[b]	CPS production[c]
RBL5515[d]	++	WT	+
exo2[e]	+	WT	+
exo4[d,e]	+	WT	+
exo5	–		+
exo344[d]	+	gal$^-$	+
exo370	–		–
exoB[d]	+	gal$^-$	–

[a] ++, Wild-type EPS production; +, 5 to 20% of wild-type EPS production; –, no EPS detectable.
[b] WT, wild-type EPS structure of *R. leguminosarum* RBL5515 (Breedveld et al. 1993; Canter Cremers et al. 1990); gal$^-$, EPS lacking galactose (Canter Cremers *et al.* 1990).
[c] +, Wild-type CPS production; –, no CPS detectable.
[d] Data from Breedveld et al. (1993).
[e] Data from Canter Cremers (1990).

Nodulation tests on vetch and white clover.

Previously, it was reported that mutant exo4 containing the biovar *viciae* Sym plasmid pRL1JI::Tn1831 failed to induce formation of root nodules on vetch, whereas the same mutant containing the biovar *trifolii* Sym plasmid pSym5::Tn1831 instead of pRL1JI was able to induce formation of nitrogen-fixing nodules on white clover plants (Canter Cremers 1990). To test if other Exo mutants were specifically disturbed in their ability to nodulate plants of the pea cross-inoculation group, nodulation tests were performed on vetch and white clover using mutants harboring Sym plasmid pRL1JI::Tn1831 and pSym5::Tn1831, respectively. When vetch plants were grown in the standard way with the roots in the light, only

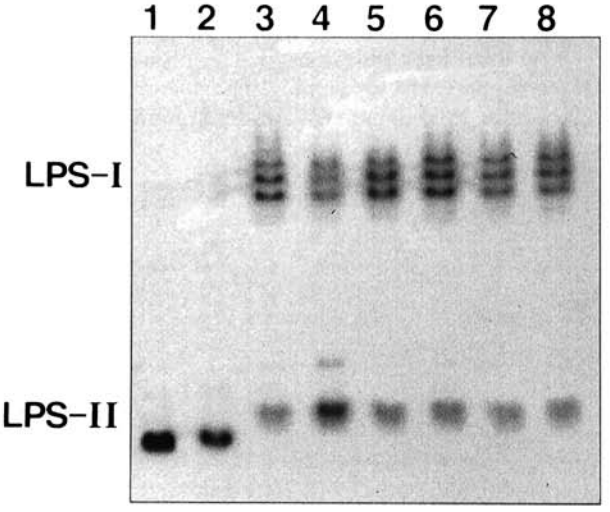

Fig. 1. LPS profiles of Exo mutants of *Rhizobium leguminosarum* bv. *viciae*. 1) exoB, 2) exo370, 3) exo344, 4) exo5, 5) exo4, 6) exo2, 7) parental Exo$^+$ RBL5515, 8) parental Exo$^+$ RBL5515 pRL1JI::Tn1831.

Table 2. Nodulation by Exo mutants of *Rhizobium leguminosarum* harboring Sym plasmid pRL1JI::Tn1831 of vetch roots under different plant culture conditions, as compared to white clover nodulation by these mutants when harboring pSym5::Tn1831

	Nodulation of vetch[a]				
Strain	Roots in the light	Roots in the light + AVG	Roots in the dark	Roots in the dark + AVG	Nodulation of white clover, roots in the light
RBL5515	++	++	++	++	++
exo2	++	++	++	++	++
exo4	–	+	+	+	+
exo5	–	+/–	+/–	+/–	+/–
exo344	–	+	+	+	+
exo370	–	–	–	–	+/–
exoB	–	–	–	–	+/–

[a] ++ = Normal nodulation, large nitrogen fixing nodules, + = delayed nodulation, large nitrogen fixing nodules, +/– = delayed nodulation, small non-nitrogen-fixing nodules, – = no nodulation.

grown in the standard way with the roots in the light, only mutant exo2 was able to induce formation of nitrogen-fixing nodules. In contrast, mutants exo2, exo4, and exo344 were able to induce formation of nitrogen-fixing nodules on white clover grown with the roots in the light, whereas the other Exo mutants were able to induce formation of small non-nitrogen-fixing bumps on these roots (Table 2). In comparison with nodulation by exo2, nodulation of the other Exo mutants on white clover appeared to be slightly delayed (3 to 4 days). These results suggest that white clover is more permissive with regard to nodulation by Exo mutants of *R. leguminosarum* than vetch.

Zaat et al. (1989) have shown that nodulation of vetch is impaired due to induction of ethylene formation, yielding the "thick and short roots" (Tsr) phenotype. To test the possible influence of ethylene on nodulation ability of Exo mutants of *R. leguminosarum*, nodulation tests were performed on vetch using plant culture conditions with a suppressive effect on ethylene production. This was done by adding the ethylene inhibitor aminoethoxyvinylglycine (AVG) to the plant growth medium or by growing the vetch plants with the roots in the dark in a specially devised system (dark glass containers). Previously it has been shown that the Tsr phenotype of vetch could be suppressed by shielding the roots from the light, which resulted in an improved nodulation by *R. l.* bv. *viciae* (Van Brussel, unpublished results). We found that mutants exo4 and exo344 were able to induce formation of nitrogen-fixing nodules in vetch roots under these conditions, whereas mutant exo5 was now able to induce very small non-nitrogen-fixing nodules. Nodulation by exo4, exo344, and exo5 was delayed about 7 days when compared to nodulation by RBL5515 pRL1JI::Tn1831 or exo2, while a smaller number of nodules was observed. When AVG was added to vetch roots grown in darkness, mutant exo5 induced slightly larger non-nitrogen-fixing nodules. However, mutants exoB and exo370 were not able to induce nodules on vetch, not even when the plants were grown with the roots in the dark in the presence of AVG. These data have been summarized in Table 2.

The results of these experiments show that the nodulation ability of Exo mutants of *R. leguminosarum* on vetch is strongly dependent on the plant culture conditions. Suppression of the ethylene-dependent Tsr phenotype of vetch (Van Brussel et al. 1986) allowed infection and nodulation by several Exo mutants of *R. leguminosarum*, most notably those producing residual amounts of EPS.

Nodules formed on vetch by parental RBL5515 pRL1JI::Tn1831 and mutants exo2, exo4, exo344, and exo5 were fixed, sectioned, and examined by light microscopy. Photographs of representative sections are presented in Figure

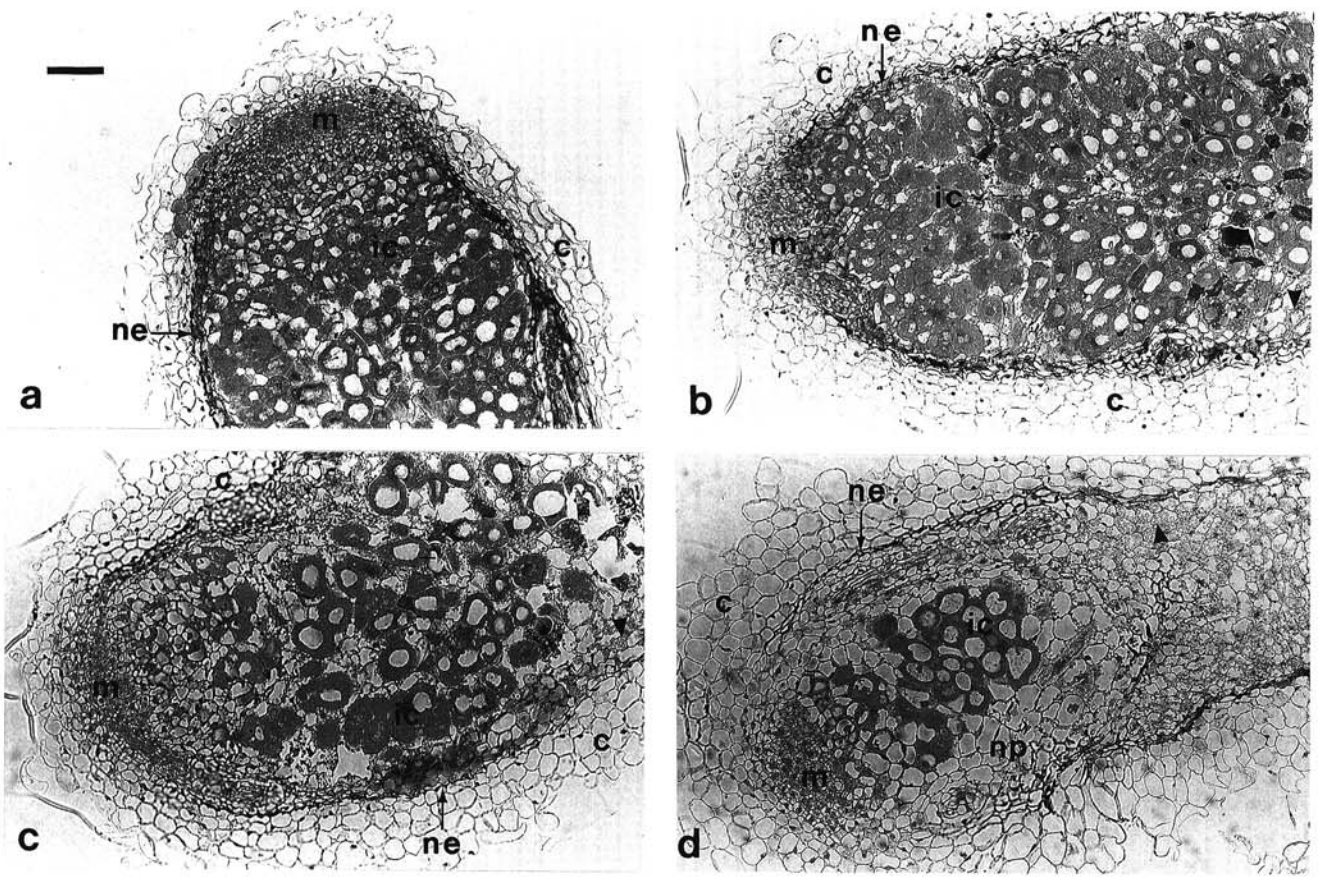

Fig. 2. Light microscopy of 7-µm sections of vetch nodules. **(A)** Eighteen-day-old vetch nodule induced by parental Exo$^+$ *Rhizobium leguminosarum* RBL5515 pRL1JI::Tn1831. **(B)** Eighteen-day-old vetch nodule induced by *R. leguminosarum* exo2 pRL1JI::Tn1831. **(C)** Twenty-four-day-old vetch nodule induced by *R. leguminosarum* exo4 pRL1JI::Tn1831. **(D)** Twenty-four-day-old vetch nodule induced by *R. leguminosarum* exo5 pRL1JI::Tn1831. Bar indicates 0.1 mm, ne = nodule endodermis, ic = infected cells, m = nodule meristem, c = nodule cortex, np = nodule parenchyma. Starch granules in noninfected cells are indicated by arrowheads.

2. A nodule induced by exo2 is similar to a nodule induced by parental RBL5515 pRL1JI::Tn1831. An active dividing meristem can be observed and most of the cells in the nitrogen-fixing zone of the nodule are infected by *Rhizobium*. A part of the noninfected cells contain starch granules. In comparison, nodules induced by both mutants exo4 and exo344 show a well-developed meristem, but less infected cells in the nitrogen-fixing zone. Furthermore, many noninfected cells contain starch granules. Nodules induced by mutant exo5 are usually much smaller than nodules induced by RBL5515 pRL1JI::Tn1831, exo2, exo4, or exo344. They contain fewer dividing cells in the meristematic zone, only a few infected cells in the nitrogen-fixing zone, and many noninfected cells containing starch granules. The infected cells of nodules induced by exo5 contained bacteroids, but apparently these do not fix nitrogen.

Ethylene production.

It was unclear whether the differences in nodulation ability of the Exo mutants on vetch plants are caused by differences in induction of ethylene production in the roots. Therefore, we examined ethylene production by roots of vetch when inoculated by pRL1JI::Tn1831-containing Exo strains and compared it with ethylene production in the presence of parental *R. leguminosarum* strain RBL5515 harboring pRL1JI::Tn1831 and noninoculated vetch roots. Inoculated vetch plants were cultured with the roots in the light, the condition under which maximal differences in nodulation behavior could be observed. Roots were harvested 2, 3, 4, and 5 days after inoculation, respectively, and ethylene production was measured after incubation of the roots in small vials under reduced pressure for 2 h at room temperature. The results have been listed in Table 3.

Roots inoculated with *R. leguminosarum* produced significantly more ethylene than did noninoculated roots, with a maximal production at 48 h after inoculation. At this time point, vetch roots inoculated with either parental RBL5515 pRL1J::Tn1831, exo2, exo4, exo5, exo344, exo370, or exoB produced about 2.0 times more ethylene than did noninoculated roots. These results are consistent with the observation that mutants exo2, exo4, exo5, exo344, exo370, and exoB induced a Tsr phenotype identical to that induced by parental *R. leguminosarum* RBL5515 pRL1JI::Tn1831 in vetch roots. Significantly, roots inoculated with RBL5522 (a strain with an extensive deletion in Sym plasmid pRL1JI::Tn1831 which removes many essential nodulation and nitrogen fixation genes [Pees et al. 1986]) produced about the same amount of ethylene than did noninoculated roots, and did not induce the Tsr phenotype. Roots inoculated with parental RBL5515 pRL1JI::Tn1831 in the presence of AVG produced significantly less ethylene than did noninoculated roots, demonstrating that AVG indeed is inhibiting ethylene production in this system. Inoculated roots which were shielded from light produced about the same amount of ethylene than did noninoculated roots, indicating that this shielding indeed results in reduced ethylene formation. Spaink et al. (1991) have shown that the Tsr phenotype is induced by rhizobial Nod factors. Likewise, these factors induce the INI phenotype, that is production of additional flavonoid inducer molecules by the host plant root (Van Brussel et al. 1990; Spaink et al. 1991). By using the same methods, we were able to show that all six Exo mutants produced Nod factors in about the same amounts as did the parental strain, and that these factors induced the INI phenotype at the same level (data not shown). Taken together, the results are inconsistent with the hypothesis that impaired nodulation by Exo mutants results from induction of an abnormally high amount of ethylene.

Coinoculation experiments on vetch.

Previously, it has been reported that a mixed inoculum consisting of an Exo^- Nod^+ strain and a Exo^+ Nod^- strain could normally induce formation of nitrogen-fixing nodules on clover (Rolfe et al. 1980) and pea (Borthakur et al. 1988). To test if impaired nodulation by the Exo mutants used in this study was caused by the lack of EPS, coinoculation experiments were performed on vetch using strain RBL5522 as an isogenic EPS^+ Nod^- strain. Coinoculation experiments were performed on vetch plants with the roots in the light, representing the most disadvantageous condition for nodulation. It was found that coinoculation of either exo4, exo344, or exo5 (all harboring pRL1JI::Tn1831) with RBL5522 resulted in the timely formation of normal nitrogen-fixing nodules on vetch. Reisolation of bacteria from 10 single nodules of each separate coinoculation experiment showed that each nodule was occupied by both coinoculation partners. In contrast, the Nod^- phenotype of mutants exo370 and exoB could not be rescued by coinoculation with strain RBL5522. Apparently, coinoculation is not sufficient to complement for the lack of CPS and the O-antigen containing LPS-I. However, coinoculation of these strains with RBL5522 resulted in the formation of nonnitrogen-fixing nodules, if the plants were grown with the roots in the dark. These small, underdeveloped nodules were only formed at the site of emergence of lateral roots, and reisolation of bacteria from these nodules showed that they were solely occupied by RBL5522. These results show that restoration of nodulation by Exo mutants by addition of an isogenic EPS-producing strain also applies to the vetch symbiotic system.

DISCUSSION

Several studies have demonstrated that acidic exopolysaccharide (EPS) is required for an indeterminate type of nodulation of legumes by *Rhizobium* bacteria (Chakravorty et al.

Table 3. Ethylene production by inoculated vetch roots relative to ethylene production by noninoculated roots (All plants were grown with the roots in the light, unless indicated otherwise)[a]

Strain	2 days a.i.[b]	3 days a.i.	4 days a.i.	5 days a.i.
Noninoculated	1.0	1.0	1.0	1.0
RBL5515 pRL1JI::Tn1831	2.3	1.5	1.3	1.4
RBL5515 pRL1JI::Tn1831 + AVG	0.2	0.2	0.1	0.2
RBL5515 pRL1JI::Tn1831 (dark)	0.9	1.0	0.9	1.0
RBL5522 (Nod^-)	1.0	0.9	1.1	1.0
exo4 pRL1JI::Tn1831	2.1	1.4	1.3	1.4
exo5 pRL1JI::Tn1831	2.1	1.3	1.6	1.8
exoB pRL1JI::Tn1831	2.0	1.8	1.2	1.4
exo370 pRL1JI::Tn1831	2.2	1.4	1.1	1.3

[a] Ethylene concentrations are given relative to the amount produced by noninoculated roots, that is 2.5 $pMol.plant^{-1}\ h^{-1}$.

[b] a.i., After inoculation.

1982; Leigh et al. 1985; Borthakur et al. 1986). However, in some cases it was observed that the same *exo* mutation influenced nodulation of different indeterminate host plants in a different way. Diebold and Noel (1989) found that several *exo* mutations in *R. l.* bv. *viciae* caused a Nod$^-$ phenotype on pea, whereas *R. leguminosarum* bv. *trifolii* carrying the same *exo* mutations was able to induce small white bumps on clover roots. Likewise, it was observed that a Tn*5*-insertion in the *pss* gene of *R. l.* bv. *viciae*, such as in mutant exo4, affected nodulation of pea but not of white clover, whereas a mutation in the promoter of the same gene (such as in mutant exo2) affected EPS synthesis, but did not affect nodulation of both pea and white clover (Canter Cremers 1990). The results from the present study suggest that differences between nodulation behavior of *Rhizobium* strains on different host plants might result from a different response of the host plant to Nod factors, dependent on the culture conditions of the roots. Roots of vetch plants, when grown in the light, respond to the presence of rhizobial Nod factors by production of ethylene to an extent, that root growth as well as nodulation are affected (the Tsr phenotype; Van Brussel et al. 1986; Zaat et al. 1989).

Exo mutants studied could be classified into three groups, (i) rhizobia producing residual amounts of EPS, (ii) rhizobia producing no detectable amounts of EPS, and (iii) pleiotropic mutants, also affected in production of CPS and LPS. Mutant exo2 belongs to the first group and is showing in all cases the same nodulation behavior as parental RBL5515. Apparently exo2 is producing enough EPS for efficient nodulation of vetch. Other mutants which are able to produce some residual amount of EPS, like exo4 and exo344, induce formation of nitrogen-fixing nodules in vetch roots only under the proper conditions, resulting in nodules containing less infected cells. It should be noted that exo4 is a *pss* mutant which was previously reported not to be able to induce nodule formation on plants of the pea cross-inoculation group (Borthakur et al. 1986; Canter Cremers 1990). In contrast, mutant exo5, a *R. leguminosarum* strain which is not able to produce any EPS at all, induced at most delayed formation of small non-nitrogen-fixing nodules, in which only few cells were infected. The same observations were made when other EPS-negative mutants were used (data not shown). The two pleiotropic mutants studied, exo370 and exoB, were not able to nodulate vetch, not even under ethylene-suppressive conditions. From these results, it can be concluded that differences in nodulation behavior of Exo mutants of *R. leguminosarum* preferably should be tested under ethylene-suppressive conditions (with roots growing in the dark, in the presence of an ethylene inhibitor) in order to optimize performance of the mutants and to smooth out differences in host plant response to Nod factors.

Mutants exo4, exo344 (EPS-deficient), and exo5 (EPS-negative) were normally able to nodulate if coinoculated with Exo$^+$ Nod- strain RBL5522. This strongly suggests that the nodulation phenotype of these strains is caused by a defect in EPS synthesis, and that production of EPS by another strain can compensate for this defect (see also Rolfe et al. 1980; Borthakur et al. 1988). After coinoculation, nitrogen-fixing nodules were occupied by both strains. This result does not corroborate the findings of Borthakur and coworkers (1988), who reported that coinoculation of a Nod$^-$ Exo$^+$ strain and a *pss* mutant strain (such as exo4) resulted in nodules solely occupied by the Nod$^-$ Exo$^+$ strain. Coinoculation of the pleiotropic mutants exoB or exo370 with RBL5522 only resulted in nodule formation under Tsr-suppressing conditions. It should be noted, however, that nodules resulting from coinoculation of the latter strains with RBL5522 appeared very late and were poorly developed. Moreover, in these nodules only the Exo$^+$ Nod$^-$ strain was found. These results suggest that presence of another strain cannot compensate for deficiencies in somatic polysaccharides rather than for diffusible exopolysaccharides, and that CPS and/or LPS is necessary for proper infection and nodule formation on vetch. As several studies have indicated that LPS-I is not required for infection or nodule formation (e.g., De Maagd et al. 1989), it can be concluded that the neutral gelling CPS or a related component might be important for (one of) these processes. Furthermore, it is obvious from these coinoculation experiments that it is possible to complement for the inability of RBL5522 to produce Nod factor by coinoculating with a noninfective, Nod factor producing strain if ethylene production by the host plant root was minimized. This would imply that it might be possible to complement a *R. leguminosarum* Nod- strain under certain conditions by adding purified Nod factor in an inoculation experiment. This is supported by the recent observation that it is possible to complement Nod mutants of the broad-host-range *Rhizobium* sp. NGR234 and *Bradyrhizobium japonicum* strain USDA110 by adding purified Nod factors in nodulation experiments (Relic et al. 1994).

Table 4. *Rhizobium leguminosarum* strains and Sym-plasmids used in this study

Strain or plasmid	Relevant characteristics
RBL5515[a]	*R. leguminosarum* bv. *trifolii* LPR5 cured of its Sym plasmid, *str rif*[b]
RBL5823[c]	*R. leguminosarum* RBL5515 *exoB52*::Tn5, *str rif*[b]
RBL5857[c]	*R. leguminosarum* RBL5515 *exo2*::Tn5, *str rif*[b]
RBL5851[c]	*R. leguminosarum* RBL5515 *exo4*::Tn5, *str rif*[b]
RBL5858[c]	*R. leguminosarum* RBL5515 *exo5*::Tn5, *str rif*[b]
RBL5825[c]	*R. leguminosarum* RBL5515 *exo370*::Tn5, *str rif*[b]
RBL5852[c]	*R. leguminosarum* RBL5515 *exo344*::Tn5, *str rif*[b]
RBL5522	*R. leguminosarum* RBL5515 pRL1JI(del)[d] Spc2::Tn1831, *str rif*[b]
pRL1JI::Tn1831	pRL1JI Spc3::Tn1831 Sym-plasmid of *R. leguminosarum* bv. *viciae*[d]
pSym5::Tn1831	pSym5 Spc51::Tn1831 Sym-plasmid of *R. leguminosarum* bv. *trifolii*[d]

[a] Priem and Wijffelman 1984.
[b] *str*, streptomycin resistance gene; *rif*, rifampicin resistance gene.
[c] Canter Cremers et al. 1988.
[d] Pees et al. 1986.
[e] In the text we refer to RBL5823 as exoB, RBL5857 as exo2, RBL5851 as exo4, RBL5858 as exo5, RBL5825 as exo370, and RBL5852 as exo344.

From our results, it can be concluded that rhizobial EPS is not a prerequisite for nodule initiation on vetch, but plays an important role in the nodule development as has been described before for clover (Chakravorty et al. 1982). The differences found before between nodulation behavior of Exo mutants on plants of the pea cross-inoculation group and white clover can largely be contributed to the action of ethylene in plants of the pea cross-inoculation group. This is reflected by the fact that the nodulation ability of Exo mutants of *R. l.* bv. *trifolii* is much less dependent on plant culturing conditions (roots in the dark/addition of AVG). Such plant-dependent responses might also explain the observation that an Exo mutant of the broad host range *Rhizobium* sp. GRH2 is not able to nodulate vetch or clover roots, whereas the same mutant can nodulate the indeterminate host *Acacia cyanophylla* (López-Lara et al. 1993). It should be noted that differences in nodulation behavior between Exo mutants of the three different groups could not be related to the amount of ethylene production by the host root.

We hypothesize that the Nod factors of *Rhizobium* act as elicitors, causing morphological changes (Van Brussel et al. 1992) and hormonal changes in the plant root, including production of ethylene. The ethylene produced by vetch after inoculation with *Rhizobium* causes hypertrophy in correlation with a rearrangement of microtubules in root cortical cells (Van Spronsen et al., manuscript in preparation; Van Brussel et al. 1992). By this mechanism, ethylene might inhibit nodulation of parental *R. l.* bv. *viciae* RBL5515 pRL1JI::Tn1831 and totally prevent nodulation of Exo mutants of RBL5515 pRL1JI::Tn1831. Obviously the threshold of vetch against rhizobial infection is lowered if ethylene production by the root is prevented. Another explanation could be that ethylene is positively regulating the host plant defense response and therefore inhibits nodulation. However, host defense responses like those found in root nodules of alfalfa induced by a EPS^- mutant of *R. meliloti* (Niehaus et al. 1993) might also be a side effect of ethylene production by the host plant root, therefore not responsible for the inability of Exo mutants to form effective nodules.

Our results are consistent with the following hypothesis. Next to nodule primordia, preinfection thread structures and other nodulation-related phenomena, rhizobial Nod signals induce formation of ethylene in host plant roots. By influencing root cell growth, ethylene inhibits proper root infection by rhizobia. In case of delayed nodulation, for instance due to EPS deficiency, ethylene formation precedes root infection and nodulation is impaired. If root infection is in time, the amount of ethylene is sufficiently small to allow for proper infection thread formation.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

A list of the organisms and the characteristics of the plasmids used in this study is shown in Table 4. *Rhizobium* species were maintained on solid A^+ medium (Van Brussel et al. 1977), and grown in TY (Beringer 1974) or YMB medium (Hooykaas et al. 1979).

Isolation and characterization of polysaccharides.

High molecular weight EPS was precipitated from the supernatant by adding 3 volumes of ethanol, as described previously (Borthakur et al. 1988). CPS was isolated by alkaline extraction followed by ethanol precipitation, and quantified by the antrone-sulfuric acid method (Breedveld et al. 1990). LPS in crude cell extracts was analyzed by PAGE using 18% acrylamide gels with deoxycholic acid as the detergent (Krauss et al. 1988), and was silver-stained (Tsai and Frisch 1982).

Plasmid transfer.

Plasmids were transferred by the method of Beringer et al. (1978).

Plant cultures.

Trifolium repens L. seeds (white clover seeds) were surface disinfected by immersion in concentrated sulphuric acid (5 min) followed by rinsing with demineralized water and immersion in undiluted commercial bleach for 10 min. To remove the bleach, at least 6 washes with sterile demineralized water during 6 h were performed. The seeds were imbibed by overnight incubation in a 1% dilution of commercial bleach in water, and germinated on 1% Jensen agar plates at 28°C after 3 washes with sterile demineralized water. *Vicia sativa* L. ssp. *nigra* (L.) seeds (vetch seeds) were treated like white clover seeds, with the exception that the incubation time in concentrated sulfuric acid was 45 min. After disinfection, vetch seeds were incubated for at least 7 days at 4°C in the dark to improve germination. Germinated seeds with roots approximately 2 cm long were used to cultivate plants under the conditions described before (Van Brussel et al. 1982) in liquid Jensen medium, a mineral medium without fixed nitrogen (Vincent 1970). The germinated seeds were inoculated by adding 25 μl of a thousandfold diluted bacterial suspension of an A_{660} of 0.1, which corresponds to 1 to 5×10^5 CFU ml^{-1}. The bacteria used for the inoculations were grown on YMB plates. The plants were cultured in the usual way with the roots in the light or in special glass containers in which the roots were shielded from light. If appropriate, aminoethoxyvinylglycine (AVG) was added to a final concentration of 0.1 mg/liter.

The plants were grown in growth chambers (20°C; 70% relative humidity) at a light intensity at the table surface of approximately 15,000 to 20,000 lx (Philips TLF 60W/33 fluorescent tubes). The day length was 16 h.

N_2-fixation assays and ethylene production.

Nitrogenase activity was measured with acetylene reduction assays. Reaction tubes containing six plants were capped with Suba seal rubber stoppers and 10% of the air was replaced by acetylene. Ethylene production was measured with use of a gas chromatograph, as previously described (Van Brussel et al. 1982).

Reisolation of rhizobia.

Reisolation of rhizobia from root nodules was done according to Vincent (1970). Nodules were immersed in ethanol 96% (30 s), followed by incubation for 2 min in HCl-acidified $HgCl_2$ (1 g $liter^{-1}$). The nodules were washed 6 times with sterile water, crushed in TY medium, and plated on TY medium. Single colonies (20 to 30) were then tested for their respective markers on YMB medium.

Microscopy.

Nodules were processed for thin sectioning as described previously (Bakhuizen et al. 1985), with the exception that

the first and second fixation periods were reduced to 1 h and 45 min, respectively. Transverse serial sections of 7 μm were made of 18- or 24-day-old nodules and were investigated by light microscopy.

Bioassay for *nod* gene-inducing activity.

The presence of *nod* gene inducers in the root exudate was quantified as described by Van Brussel et al. (1990).

Isolation and detection of Nod metabolites.

The production of Nod metabolites by the *R. leguminosarum* strains was analyzed as described by Spaink et al. (1992).

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LITERATURE CITED

Bakhuizen, R., van Spronsen, P. C., Sluiman-den Hartog, F. A. J., Venverloo, C. J., and Goosen-de Roo, L. 1985. Nuclear envelope radiating microtubules in plant cells during interphase mitosis transition. Protoplasma 128:43-51.

Battisti, L., Lara, J. C., and Leigh, J. A. 1992. Specific oligosaccharide form of the *Rhizobium meliloti* exopolysaccharide promotes nodule invasion in alfalfa. Proc. Natl. Acad. Sci. USA 89:5625-5629.

Beringer, J. E. 1974. R factor transfer in *Rhizobium leguminosarum.* J. Gen. Microbiol. 84:188-198.

Beringer, J. E., Hoggan, S. A., and Johnston A. W. B. 1978. Linkage mapping in *Rhizobium leguminosarum* by means of R plasmid-mediated recombination. J. Gen. Microbiol. 104:201-207.

Borthakur, D., Barber, C. E., Lamb, J. W., Daniels, M. J., Downie, J. A., and Johnston, A. W. B. 1986. A mutation that blocks exopolysaccharide synthesis prevents nodulation of peas by *Rhizobium leguminosarum* but not of beans by *R. phaseoli* and is corrected by cloned DNA from *Rhizobium* or the phytopathogen *Xanthomonas*. Mol. Gen. Genet. 203:320-323.

Borthakur, D., Barker, R. F., Latchford, J. W., Rossen, L., and Johnston, A. W. B. 1988. Analysis of *pss* genes of *Rhizobium leguminosarum* required for exopolysaccharide synthesis and nodulation of peas: Their primary structure and their interaction with *psi* and other nodulation genes. Mol. Gen. Genet. 213:155-162.

Breedveld, M. W., Canter Cremers, H. C. J., Batley, M., Posthumus, M. A., Zevenhuizen, L. P. T. M., Wijffelman, C. A., and Zehnder, A. J. B. 1993. Polysaccharide synthesis in relation to nodulation behavior of *Rhizobium leguminosarum*. J. Bacteriol. 175:750-757.

Breedveld, M. W., Zevenhuizen, L. P. T. M., and Zehnder, A. J. B. 1990. Excessive excretion of cyclic β-(1,2)-glucan by *Rhizobium trifolii* TA-1. Appl. Environ. Microbiol. 56:2080-2086.

Canter Cremers, H. C. J. 1990. Role of exopolysaccharide in nodulation of *Rhizobium leguminosarum* bv. *viciae*. Ph.D. thesis. Leiden University, Leiden, The Netherlands.

Canter Cremers, H. C. J., Batley, M., Redmond, J. W., Eydems, L., Breedveld, M. W., Zevenhuizen, L. P. T. M., Pees, E., Wijffelman, C. A., and Lugtenberg, B. J. J. 1990. *Rhizobium leguminosarum exoB* mutants are deficient in the synthesis of UDP-glucose 4′-epimerase. J. Biol. Chem. 265:21122-21127.

Canter Cremers, H. C. J., Wijffelman, C. A., Pees, E., Engels, M., Hoogerbruggen, F., Stevens, K., van Dijk, M., and Lugtenberg, B. J. J. 1988. Chromosomal nodulation genes of *R. leguminosarum* biovar *viciae*, Page 484 in: H. Bothe, F. J. de Bruijn, and W. J. Newton, eds. Nitrogen Fixation: Hundred Years After. Gustav Fisher, Stuttgart, Germany.

Carlson, R. W., Kalembasa, S., Thurowski, D. T., Pachori, P., and Noel, K. D. 1987. Characterization of the lipopolysaccharide from a *Rhizobium phaseoli* mutant that is defective in infection thread development. J. Bacteriol. 169:4923-4928.

Chakravorty, A. K., Zurkowski, W., Shine, J., and Rolfe, B. G. 1982. Symbiotic nitrogen fixation: Molecular cloning of *Rhizobium* genes involved in exopolysaccharide synthesis and effective nodulation. J. Mol. Appl. Genet. 1:585-596.

De Maagd, R. A., Rao, A. S., Mulders, I. H. M., Goosen-De Roo, L., Loosdrecht, M. C. M. van, Wijffelman, C. A., and Lugtenberg, B. J. J. 1989. Isolation and characterization of mutants of *Rhizobium leguminosarum* bv. *viciae* 248 with altered lipopolysaccharides: Possible role of surface charge or hydrophobicity in bacterial release from the infection thread. J. Bacteriol. 171:1143-1150.

Diebold, R., and Noel, K. D. 1989. *Rhizobium leguminosarum* exopolysaccharide mutants: Biochemical and genetic analyses and symbiotic behavior on three hosts. J. Bacteriol. 171:4821-4830.

Djordjevic, S. P., Chen, H., Batley, M., Redmond, J. W., and Rolfe, B. G. 1987. Nitrogen fixation ability of exopolysaccharide synthesis mutants of *Rhizobium* sp. strain NGR234 and *Rhizobium trifolii* is restored by the addition of homologous exopolysaccharides. J. Bacteriol. 169:53-60.

Hooykaas, P. J. J., Klapwijk, P. M., Nuti, M. P., Schilperoort, R. A., and Rörsch, A. 1979. Transfer of the *Agrobacterium tumefaciens* Ti plasmid to avirulent agrobacteria and to *Rhizobium* ex planta. J. Gen. Microbiol. 98:477-484.

Krauss, J. H., Weckesser, J., and Mayer, H. 1988. Electrophoretic analysis of lipopolysaccharides of purple nonsulfur bacteria. Int. J. Syst. Bacteriol. 38:157-163.

Lee, K. H., and LaRue, T. A. 1992a. Ethylene as a possible mediator of light- and nitrate-induced inhibition of nodulation of *Pisum sativum* L. cv Sparkle. Plant Physiol. 100:1334-1338.

Lee, K. H., and LaRue, T. A. 1992b. Exogenous ethylene inhibits nodulation of *Pisum sativum* L. cv Sparkle. Plant Physiol. 100:1759-1763.

Leigh, J. A., Signer, E. R., and Walker, G. C. 1985. Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. Proc. Natl. Acad. Sci. USA 82:6231-6235.

Leigh, J. A., and Walker, G. C. 1994. Exopolysaccharides of *Rhizobium*: Synthesis, regulation and symbiotic function. Trends Genet. 10:63-67.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J. C., and Dénarié, J. 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature 344:781-784.

López-Lara, I. M., Orgambide, G., Dazzo, F. B., Olivares, J., and Toro, N. 1993. Characterization and symbiotic importance of acidic extracellular polysaccharides of *Rhizobium* sp. strain GRH2 isolated from acacia nodules. J. Bacteriol. 175:2826-2832.

McNeil, M. J., Darvill, J., Darvill, A., Albersheim, P., Van Veen, R., Hooykaas, P., and Schilperoort, R. A. H. 1986. The discernible structural features of the acidic exopolysaccharides secreted by different *Rhizobium* species are the same. Carbohydr. Res. 146:307-326.

Niehaus, K., Kapp, D., and Pühler, A. 1993. Plant defense and delayed infection of alfalfa pseudonodules induced by an exopolysaccharide (EPS I)-deficient *Rhizobium meliloti* mutant. Planta 190:415-425.

Ohashi, Y., and Ohshima, M. 1992. Stress-induced expression of genes for pathogenesis-related proteins in plants. Plant Cell Physiol. 33:819-826.

Pees, E., Wijffelman, C. A., Mulders, I. H. M., van Brussel, A. A. N., and Lugtenberg, B. J. J. 1986. Transposition of Tn*1831* to Sym plasmids of *Rhizobium leguminosarum* and *Rhizobium trifolii*. FEMS Microbiol. Lett. 33:165-171.

Priem, W. J. E., and Wijffelman, C. A. 1984. Selection of strains cured of the *Rhizobium leguminosarum* Sym-plasmid pRL1JI by using small bacteriocin. FEMS Microbiol. Lett. 25:247-251.

Relic, B., Perret, X., Estrada-García, M. T., Kopcinska, J., Golinowski, W., Krishnan, H. B., Pueppke, S. G., and Broughton, W. J. 1994. Nod factors of *Rhizobium* are a key to the legume door. Mol. Microbiol. 13(1):171-178.

Rolfe, B. G., Gresshoff, P. M., Shine, J., and Vincent, J. W. 1980. Interaction between a nonnodulating and an ineffective mutant of *Rhizobium trifolii* resulting in effective (nitrogen-fixing) nodulation. Appl. Environ. Microbiol. 39:449-452.

Spaink, H. P., Aarts, A., Stacey, G., Bloemberg, G. V., Lugtenberg, B. J. J., and Kennedy, E. P. 1992. Detection and separation of *Rhizobium* and *Bradyrhizobium* Nod metabolites using thin-layer chromatography. Mol. Plant-Microbe Interact. 5:72-80.

Spaink, H. P., Sheeley, D. M., van Brussel, A. A. N., Glushka, J., York, W. S., Tak, T., Geiger, O., Kennedy, E. P., Reinhold, V. N., and

Lugtenberg, B. J. J. 1991. A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium*. Nature 354:125-129.

Tsai, C., and Frisch, C. E. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. 119:115-119.

Urzainqui, A., and Walker, G. C. 1992. Exogenous suppression of the symbiotic deficiencies of *Rhizobium meliloti* exo mutants. J. Bacteriol. 174:3403-3406.

Van Brussel, A. A. N., Bakhuizen, R., van Spronsen, P. C., Spaink, H. P., Tak, T., Lugtenberg, B. J. J., and Kijne, J. W. 1992. Induction of pre-infection thread structures in the leguminous host plant by mitogenic lipo-oligosaccharides of *Rhizobium*. Science 257:70-72.

Van Brussel, A. A. N., Planqué, K., and Quispel, A. 1977. The wall of *Rhizobium leguminosarum* in bacteroid and free-living forms. J. Gen. Microbiol. 101:51-56.

Van Brussel, A. A. N., Recourt, K., Pees, E., Spaink, H. P., Tak, T., Wijffelman, C. A., Kijne, J. W., and Lugtenberg, B. J. J. 1990. A biovar-specific signal of *Rhizobium leguminosarum* bv. *viciae* induces increased nodulation gene-inducing activity in root exudate of *Vicia sativa* subsp. *nigra*. J. Bacteriol.172:5394-5401.

Van Brussel, A. A. N., Tak, T., Wetselaar, A., Pees, E., and Wijffelman, C. A. 1982. Small leguminosae as test plants for nodulation of *Rhizobium leguminosarum* and other *Rhizobia* and *Agrobacteria* harboring a leguminosarum plasmid. Plant Sci. Lett. 27:317-325.

Van Brussel, A. A. N., Zaat, S. A. J., Canter Cremers, H. C. J., Wijffelman, C. A., Pees, E., Tak, T., and Lugtenberg B. J. J. 1986. Role of plant root exudate and Sym plasmid-localized nodulation genes in the synthesis by *Rhizobium leguminosarum* of Tsr factor, which causes thick and short roots on common vetch. J. Bacteriol. 165:517-522.

Vincent, J. M. 1970. A Manual for the Practical Study of the Root Nodule Bacteria. IBP Handbook no. 15. Blackwell Scientific Publications, Ltd., Oxford. Pages 75-76.

Zaat, S. A. J., van Brussel, A. A. N., Tak, T., Lugtenberg, B. J. J., and Kijne, J. W. 1989. The ethylene-inhibitor aminoethoxyvinylglycine restores normal nodulation by *Rhizobium leguminosarum* biovar *viciae* on *Vicia sativa* subsp. *nigra* by suppressing the thick and short roots phenotype. Planta 177:141-150.

Zevenhuizen, L. P. T. M. 1984. Gel-forming capsular polysaccharide of fast-growing bacteria: occurrence and rheological properties. Appl. Microbiol. Biotechnol. 20:393-399.

Zevenhuizen, L. P. T. M., and Van Neerven, A. R. W. 1983. Gel-forming capsular polysaccharide of *Rhizobium leguminosarum* and *Rhizobium trifolii*. Carbohydr. Res. 124:166-171.