Role of Exopolysaccharides of *Rhizobium leguminosarum* bv. *viciae* as Host Plant-Specific Molecules Required for Infection Thread Formation During Nodulation of *Vicia sativa*

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Mutants of *Rhizobium leguminosarum* bv. *viciae* bacteria that are affected in the biosynthesis of exopolysaccharides (EPS) are unable to effectively nodulate their host plants. By studying defined mutants, we show that *R. leguminosarum* bv. *viciae* strains require EPS for formation of infection threads in *Vicia sativa* (vetch) as well as for efficient induction of tight root hair curling. Results of coinoculation experiments with the EPS-deficient *pssDIII* mutant of *R. leguminosarum* bv. *viciae* in combination with heterologous EPS-producing strains indicated that vetch has certain structural requirements for rhizobial EPS to function in symbiosis. We hypothesize that EPS accelerates root hair curling and infection to such an extent that rhizobial root penetration precedes a plant defense response.

Additional keywords: *exo* mutants, Nod factors, root nodules.

Bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, and *Azorhizobium* are able to elicit formation of nodules on the roots of leguminous plants. In these nodules, the rhizobia eventually are present as N₂-fixing organelles maintained by the plant in return for ammonia. One major characteristic of many rhizobia is the production of large amounts of exopolysaccharides (EPS), which show strain-dependent variations in chemical structure. These variations concern sugar composition, condensation linkages, subunit size, and/or noncarbohydrate substitutions. Figure 1 shows the chemical structures of the repeating units of EPS of various rhizobia.

EPS biosynthesis by rhizobia is required for the formation of nitrogen-fixing nodules on indeterminate nodule-type legumes, for example, *Leucaena*, *Medicago*, *Pisum*, *Trifolium*, and *Vicia* spp. (for a recent review see Becker and Pühler 1998). EPS-deficient (*exo*) mutants of *R. leguminosarum* bv. *viciae* normally fail to nodulate pea (Borthakur et al. 1986; Diebold and Noel 1989) and induce small, ineffective nodules

on vetch (van Workum et al. 1995), whereas *exo* mutants of *R. leguminosarum* bv. *trifolii* and *Sinorhizobium meliloti* induce small, white bumps on clover and empty, ineffective nodules on alfalfa, respectively (Leigh et al. 1985; Skorupska et al. 1995; Rolfe et al. 1996). Up to now, the actual function of EPS in symbiosis has not been elucidated.

The establishment of the *Rhizobium*-legume symbiosis requires specific chemical signaling between the symbiotic partners. Restoration of the nodulation efficiency of *exo* mutants of *R. leguminosarum* bv. *trifolii* and *S. meliloti* by addition of low molecular weight fractions of EPS indicated that EPS act as signaling molecules (Djordjevic et al. 1987; Battisti et al. 1992; Urzainqui and Walker 1992; Gonzalez et al. 1996). EPS have long been assumed to represent host-specific signaling molecules because they are located at the rhizobial cell surface and because of the diversity of EPS structures among rhizobia (Ljunggren and Fåhræus 1959; Dudman 1984). Indications that host plants indeed have structural requirements for rhizobial EPS to be biologically active have been obtained for *Leucaena*, clover, and alfalfa species (for example, Djordjevic et al. 1987). *exo* mutants of *Rhizobium* sp. strain NGR234 were able to induce functioning nodules on *Leucaena* plants in the presence of a Sym plasmid-cured but EPS-producing derivative of NGR234 or after the addition of purified NGR234 EPS, but not when coinoculated with a Sym plasmid-cured, EPS-producing *R. leguminosarum* bv. *trifolii* strain (Djordjevic et al. 1987). Further indications that *Leucaena* requires a certain type of EPS came from experiments with hybrid strains of *Rhizobium* sp. strain NGR234 containing *exo* genes from *S. meliloti* that showed that production of succinoglycan did not enable *Rhizobium* sp. strain NGR234 to effectively nodulate *Leucaena* (Gray et al. 1991). For clover, it was found that purified EPS fractions of *R. leguminosarum* bv. *trifolii* could restore nodulation of *exo* mutants of this strain, whereas purified EPS fractions of *S. meliloti* could not (Djordjevic et al. 1987). In the case of alfalfa, the symbiotic deficiencies of *exo* mutants of *S. meliloti* could only be repaired by the addition of the most charged tetramer of the repeating unit of EPS I (Battisti et al. 1992). Furthermore, nonsuccinylated low molecular weight EPS I and high molecular weight EPS I did not repair the nodulation deficiency of an *exo* mutant, nor did low molecular weight

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EPS from *R. leguminosarum* bv. *trifolii* and *Rhizobium* sp. strain NGR234 (Battisti et al. 1992).

Up to now, the inability of *exo* mutants of *R. leguminosarum* bv. *viciae* to properly nodulate the host plant *Vicia sativa* subsp. *nigra* (vetch) has not been studied in detail. Here, we describe the early symbiotic behavior of defined EPS-deficient mutants of *R. leguminosarum* bv. *viciae*. We

show that EPS is required for proper infection thread formation and tight root hair curling, but not for attachment of the rhizobia to vetch root hairs. Furthermore, it appeared to be possible to repair this symbiotic deficiency by coinoculation with certain heterologous EPS-producing rhizobia, but not with others, pointing at structural requirements for rhizobial EPS to function in a successful symbiosis with vetch roots.

Fig. 1. Chemical structure of repeating units of exopolysaccharides (EPS) of *Rhizobium leguminosarum* (Robertsen et al. 1981; McNeil et al. 1986; Dudman et al. 1983), *R. tropici* CIAT899 (Gil-Serrano et al. 1990), *Rhizobium* sp. strain NGR234 (Djordjevic et al. 1986), *Bradyrhizobium japonicum* (Mort and Bauer 1982; Puvanesarajah et al. 1987), and EPS I and EPS II of *Sinorhizobium meliloti* Rm1021 (Åman et al. 1981; Jansson et al. 1977; Her et al. 1990). Non-stoichiometric acetate and 3-hydroxybutanoate substitutions have not been indicated. Abbreviations: Glc, glucose; GlcA, glucuronic acid; Gal, galactose; GalA, galacturonic acid; Man, mannose; Suc, succinate; Me, methyl; Ac, acetyl.

RESULTS

Infection thread formation and root hair curling.

Previously, we showed that *exo* mutants of *R. leguminosarum* bv. *viciae* are at most capable of inducing only partially infected nodules on their host plant vetch (van Workum et al. 1995). To visualize in what stage of the nodulation process these mutants are impaired, we introduced plasmid pXLGD4 containing a fusion of the constitutive *hemA* promoter to the *lacZ* reporter gene (Leong et al. 1985) into parental strain *R. leguminosarum* bv. *viciae* RBL5523 and three defined *exo* mutants: *pssA4*, *pssD111*, and *exoB52*. Mutant *pssA4* lacks a glucosyl-isoprenylphosphate transferase, involved in the first step of the synthesis of EPS repeating units, and produces a small amount of EPS (van Workum et al. 1997; Pollock et al. 1998). Mutant *pssD111* is defective in glucuronosyl-(β 1-4)-glucosyl transferase activity, involved in the second step of the synthesis of EPS repeating units, with total absence of EPS production as a result (van Workum et al. 1997; Pollock et al. 1998). The *exoB* mutant is deficient in the synthesis of a UDP (uridine 5'-diphosphate)-glucose 4'-epimerase (Canter Cremers et al. 1990), and is affected not only in EPS biosynthesis but also in production of other galactose-containing polymers such as lipopolysaccharides (LPS) and capsular polysaccharides (CPS) (Breedveld et al. 1993).

We performed a microscopic study of the nodulation process. The modified strains can convert the colorless 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) substrate into an insoluble blue precipitate (Boivin et al. 1990). After inoculation, vetch plants were grown with the roots in the light, which stimulates ethylene-mediated root hair formation. This facilitates the study of infection thread formation in vetch roots. Roots were removed from vetch plants 5 days after inoculation, fixed, and stained for examination by light microscopy. At least 6 roots per inoculated strain were examined, and the following early infection phenomena were quantified: (i) tightly curled root hairs containing trapped rhizobia, also called infection foci; (ii) immature or aborted infection threads; and (iii) infection threads that successfully entered the root cortex of vetch. Representative photographs are shown in Figure 2. As can be seen in Table 1, parental strain RBL5523 and the three *exo* mutants exhibited notable differences in infection thread formation. Five days after inoculation, parental strain RBL5523 induced on average 43 successful infections per plant, whereas only in the case of the *pssA4* mutant could a few occasional infection threads be found. The *pssD111* mutant was more severely disturbed in its ability to induce infection threads in vetch and, apart from some short or aborted infection threads, only infection foci could be found. Furthermore, the number of infection foci was smaller than in plants inoculated with the *pssA4* mutant. Closer examination of the infection foci induced by the *pssD111* and *pssA4* mutants revealed a thickening of that part of the plant cell wall in immediate contact with the bacteria.

In contrast to the situation with nonpleiotropic *exo* mutants of *R. leguminosarum* bv. *viciae* such as *pssA* and *pssD*, nodulation of vetch by *exoB52* has not been observed (van Workum et al. 1995). In this study, we found that *exoB52* is neither able to induce infection thread formation nor able to form infection foci. Obviously, cell surface polysaccharides of *R. legumi-*

nosarum bv. *viciae* other than EPS play a role in *Rhizobium*-root hair surface interactions.

To test whether EPS is important for the ability of *R. leguminosarum* to attach to root hairs, attachment of the EPS-deficient *pssD111* mutant to vetch root hairs was studied with the attachment assay described by Smit et al. (1986). Briefly, roots were immersed in a suspension of TY- or YMB-grown bacteria and washed 10 times, and attachment was quantified by randomly screening at least 100 developing root hairs with a phase-contrast microscope. *R. leguminosarum* bv. *viciae* RBL5523 as well as the *pssD111* mutant appeared to attach efficiently to vetch root hair tips. We therefore conclude that production of EPS is not necessary for attachment of *R. leguminosarum* to vetch root hair tips.

The finding that rhizobial EPS is required for infection thread formation by *R. leguminosarum* bv. *viciae* is consistent with our earlier observation that carbon-limited (and thus EPS-poor) *R. leguminosarum* bv. *viciae* cells did not form infection threads in pea roots (Kijne et al. 1988). Likewise, carbon-limited *R. leguminosarum* bv. *viciae* cells induce formation of relatively small numbers of infection threads in vetch (data not shown).

Coinoculation with heterologous rhizobia.

Coinoculation experiments with non-nodulating but EPS-producing rhizobia have shown to be reliable assays for testing the biological activity of rhizobial EPS (for example, Chen and Rolfe 1987). We tested which coinoculated partners would allow the *pssD111* mutant to nodulate its host plant vetch. Coinoculation experiments were performed in a plant growth system in which the vetch roots are exposed to the light. AVG was added to minimize ethylene production by the plant, which facilitates nodulation (Zaat et al. 1989). Under these plant growth conditions, EPS-deficient mutants of *R. leguminosarum* are almost unable to form root nodules on vetch. If nodules were present, they were much smaller than nodules induced by wild-type *R. leguminosarum*, almost devoid of bacteroids, and non-nitrogen-fixing (van Workum et al. 1995).

First, coinoculation experiments were performed with RBL5522 as an isogenic EPS⁺ Nod⁻ strain, in order to determine the optimal ratio of EPS⁻ Nod⁺ and EPS⁺ Nod⁻ bacteria. The first set of results (Table 2) shows the nodulation behavior of the *pssD111* mutant in the presence of different numbers of RBL5522 bacteria. It was found that nodulation by the *pssD111* mutant is most efficiently restored when the coinoculated partner is present in a multifold excess. Compared with the results with the parental strain *R. leguminosarum* bv. *viciae* RBL5523, nodulation by these coinoculated EPS⁻ Nod⁺ and EPS⁺ Nod⁻ rhizobia is delayed while the average number of nodules on nodulated plants is smaller.

Coinoculation with the clover symbiont *R. leguminosarum* bv. *trifolii* ANU843, which synthesizes EPS with the same octasaccharide repeating unit, yielded a different result. Although this strain is able to restore nodulation of the *pssD111* mutant, the percentage of nodulated plants and the average number of nodules were smaller than those observed after coinoculation with strain RBL5522, especially when measured 7 days after inoculation (Table 2). Coinoculations were more successful when equal numbers of the EPS⁻ Nod⁺ and EPS⁺ Nod⁻ partners were used. Better results were obtained

when we used *R. leguminosarum* bv. *trifolii* ANU453 as a coinoculation partner. ANU453 is similar to ANU843 but harbors a mutation in the *nodC* gene, and as a result this strain is unable to produce Nod factors. As shown in Table 2, ANU453 is able to restore the nodulation of the *pssD111* mutant as efficiently as the isogenic EPS⁺ Nod⁺ strain RBL5522 if applied in a 1,000-fold excess. Reisolation of bacteria from single nodules of each separate coinoculation experiment (with either strain ANU843 or ANU453) showed that each nodule was occupied by both coinoculated partners. Coinoculation results similar to those obtained with ANU843 and ANU453, respectively, were obtained with Nod⁺ and Nod[−] strains of *R. etli* CE3 (a bean symbiont), which produces EPS of the same chemical structure (data not shown). Apparently, Nod factors produced by these heterologous strains can be perceived by vetch, and in some way hinder the restoration of nodulation by an EPS-deficient *R. leguminosarum* bv. *viciae* strain. This is consistent with the fact that vetch shows the “thick short roots” (TSR) phenotype (van Brussel et al. 1990) after inoculation with either *R. leguminosarum* bv. *trifolii* ANU843 or *R. etli* CE3.

In order to determine at which stage of symbiosis the *pssD111* mutant is restored by either *R. leguminosarum* bv. *trifolii* ANU843 or ANU453, we quantified the early infection events 5 days after (co)inoculation. For these infection studies, we used the *pssD111* mutant carrying plasmid pXLGD4, as was described above. Coinoculation of the *pssD111* mutant with a 1,000-fold excess of ANU843 or ANU453 resulted in an increase in the number of infection foci as well as an increase in the number of (immature/aborted) infection threads (Table 3). Therefore, it appears that EPS produced by the coinoculation partners enhances tight root hair curling and the elicitation of infection thread formation. Interestingly, the relatively small number of observed infection threads induced in the presence of strain ANU453 appears to be sufficient for almost normal nodulation (Table 2).

To test whether vetch has specific structural requirements for EPS to function in symbiosis, we performed coinoculation

experiments with the *pssD111* mutant in combination with heterologous rhizobia that synthesize EPS of a different chemical structure (Table 4). Coinoculation experiments were performed with different numbers of EPS⁺ rhizobia, to iden-

Table 1. Infection thread formation by *Rhizobium leguminosarum* bv. *viciae* RBL5523 and defined Exo[−] mutants

Strain	Foci (no.) ^a	I/AITs (no.) ^a	ITs (no.) ^a
RBL5523	1.4 ± 0.5	0.5 ± 0.5	43 ± 16.4
<i>pssA4</i>	19.5 ± 11.5	2.5 ± 2.3	0.7 ± 1.2
<i>pssD111</i>	10.5 ± 3.7	0.7 ± 0.8	0
<i>exoB52</i>	0	0	0

^a Each value represents the average number of infection events per root. Infection thread formation was studied 5 days after inoculation with rhizobia. Foci = curled root hair containing entrapped rhizobia, without an infection thread; I/AIT = immature or aborted infection thread; IT = infection thread.

Table 2. Coinoculation of the *pssD111* mutant of *Rhizobium leguminosarum* bv. *viciae* with rhizobia producing chemically identical exopolysaccharides (EPS)

Inoculated strains ^a	Inoc. ratio ^b	Nodulation			
		7 days a.i.		21 days a.i.	
		freq. ^c	nod. ^c	freq. ^c	nod. ^c
RBL5523	–	33/36	6.3 ± 2.6	33/36	7.6 ± 2.4
RBL5522: <i>pssD111</i>	1	3/18	1.5 ± 0.7	16/18	6.2 ± 3.7
RBL5522: <i>pssD111</i>	1,000	16/18	4.3 ± 2.1	17/18	5.3 ± 1.3
ANU843: <i>pssD111</i>	1	0/18	0	16/18	3.0 ± 1.4
ANU843: <i>pssD111</i>	1,000	0/18	0	1/18	2.0
ANU453: <i>pssD111</i>	1	0/18	0	15/18	2.2 ± 0.8
ANU453: <i>pssD111</i>	1,000	16/18	3.5 ± 2.6	16/18	5.4 ± 2.2

^a RBL5522: *R. leguminosarum* bv. *viciae* RBL5523 having a deletion in its Sym plasmid (*nod* mutant); ANU843: *R. leguminosarum* bv. *trifolii* ANU843; ANU453: *R. leguminosarum* bv. *trifolii* ANU843 *nodC* mutant.

^b Ratio of the inoculated bacteria. The number of *pssD111* bacteria was constant, i.e., 25 µl of a suspension of 1 to 5 × 10⁵ CFU ml^{−1}.

^c freq. = number of nodulated plants per number of inoculated plants; nod. = average number of nodules per nodulated plant.

Fig. 2. Tight root hair curling and infection thread formation of vetch (*Vicia sativa* subsp. *nigra*) plants inoculated with *Rhizobium leguminosarum* strains containing a fusion of the constitutive *hemA* gene promoter to the *lacZ* reporter gene. Vetch plants were inoculated with (A) the parental strain *R. leguminosarum* bv. *viciae* RBL5523 or (B) *R. leguminosarum* bv. *viciae* *pssD111* mutant, and grown for 5 days, after which the roots were assayed for β-galactosidase activity. A, Insert shows how the blue-stained bacteria are arranged inside the infection thread. Bar = 25 µm.

tify possible interference with heterologous Nod factors. *Rhizobium* sp. strain NGR234, *Bradyrhizobium japonicum* USDA110, and *S. meliloti* Rm1021 (EPS I⁺ EPS II⁻) (see Figure 1) were not able to restore nodulation of the *pssD111* mutant under all conditions tested. Furthermore, coinoculation experiments with strain Rm9000 (EPS I⁻ EPS II⁺) also suggested that EPS II of *S. meliloti* cannot function in the symbiosis of *Rhizobium* and vetch. However, coinoculation experiments with *R. leguminosarum* bv. *trifolii* 4S showed that the presence of EPS that differ in sugar composition and length of the EPS side chains allows the *pssD111* mutant to induce formation of nitrogen-fixing nodules on vetch. Interestingly, it was observed that *R. tropici* CIAT899 restored the nodulation of the *pssD111* mutant even more effectively. The chemical structure of EPS from *R. tropici* CIAT899 has been shown to be different from that of wild-type strains of *R. leguminosarum* bv. *viciae*, with the exception of the presence of

β-1,3-linked pyruvylated glucose and pyruvylated galactose residues in the EPS side chains (Fig. 1). Coinoculation of the *pssD111* mutant with either *R. leguminosarum* bv. *trifolii* 4S or *R. tropici* CIAT899 on vetch was most successful when the coinoculation partners were present in equal numbers, which is indicative of Nod factors, synthesized by the EPS-producing strains, to hinder the restoration of nodulation of the *pssD111* mutant.

To test whether the inability of *Rhizobium* sp. strain NGR234, *B. japonicum* USDA110, and *S. meliloti* Rm1021 and Rm9000 to restore nodulation of the *pssD111* mutant was correlated with plant responses, we studied possible morphological changes in vetch roots due to inoculation with one of these strains. None of these strains induced any root hair curling or root hair deformations, while induction of TSR was also not observed (data not shown). Therefore, we tentatively conclude that the inability of these strains to restore nodulation by the *pssD111* mutant is due to the large difference in structure of their EPS compared with EPS produced by *R. leguminosarum* bv. *viciae* strains (Fig. 1).

DISCUSSION

Previously, we have shown that three defined EPS⁻ mutants of *R. leguminosarum* bv. *viciae*, namely, *pssA4*, *pssD111*, and *exoB52*, are differently affected in their capacity to nodulate vetch. The *pssA4* mutant induces delayed formation of partially infected nitrogen-fixing nodules, the *pssD111* mutant induces delayed formation of almost empty non-nitrogen-fixing nodules, and the *exoB52* mutant is unable to induce nodule formation at all (van Workum et al. 1995). Here we show that these differences in nodulation behavior are correlated with differences in the ability of these mutants to elicit tight root hair curling and root hair infection. Biosynthesis of EPS appeared to be essential for the ability of *R. leguminosarum* bv. *viciae* to effectively induce infection thread formation. This corroborates findings for the *S. meliloti*/alfalfa and *R. leguminosarum* bv. *trifolii*/clover symbioses (Leigh et al. 1985; Rolfe et al. 1996). The *pssA4* mutant, producing residual amounts of EPS, was slightly less affected in its ability to induce the formation of infection threads than was the *pssD111* mutant, unable to produce EPS at all. The pleiotropic *exoB52* mutant was found to be totally ineffective in induction of root hair curling and infection thread formation. It has been shown that this mutant is not affected in Nod factor production nor in its ability to induce the TSR response on vetch (van Workum et al. 1995). Nevertheless, the *exoB52* mutant is not able to induce any root hair curling, and nodules do not develop. Apparently *R. leguminosarum* bv. *viciae* needs certain surface polysaccharides, in addition to Nod factors, to properly interact with its target cells.

In contrast to mutant *exoB52*, mutants *pssA4* and *pssD111* are able to induce tight root hair curling (= infection foci) on vetch. This suggests that EPS are not essential for this process. According to the model of van Batenburg et al. (1986), tight root hair curling is the result of redirection of tip growth of plant root hairs by attached rhizobia. Since EPS-deficient mutants of *R. leguminosarum* bv. *viciae* are able to induce infection foci on vetch, EPS is not expected to play a major role in attachment of *R. leguminosarum* to root hairs. This was confirmed by the observation that the *pssD111* mutant is able

Table 3. Infection thread formation by the *pssD111* mutant of *Rhizobium leguminosarum* bv. *viciae* in the presence of exopolysaccharide (EPS)-producing rhizobia

Inoculated bacteria ^a	Inoc. ratio ^b	Foci (no.) ^c	I/AITs (no.) ^c	ITs (no.) ^c
<i>pssD111</i>	–	11.1 ± 4.9	0.4 ± 0.5	0
ANU843: <i>pssD111</i>	1,000	31.3 ± 10.5	1.5 ± 1.4	1.0 ± 0.6
ANU453: <i>pssD111</i>	1	16.2 ± 6.4	0.2 ± 0.4	0
ANU453: <i>pssD111</i>	1,000	31.2 ± 13.5	3.7 ± 2.0	3.2 ± 2.8

^a ANU843: *R. leguminosarum* bv. *trifolii* ANU843; ANU453: *R. leguminosarum* bv. *trifolii* ANU843 *nodC* mutant.

^b Ratio of the inoculated bacteria. The number of *pssD111* mutant bacteria was constant, i.e., 25 µl of a suspension of 1 to 5 × 10⁵ CFU ml⁻¹.

^c Each value represents the average number of infection events per root. Infection thread formation was studied 5 days after inoculation with rhizobia. Foci = curled root hair containing entrapped rhizobia, without an infection thread; I/AIT = immature or aborted infection thread; IT = infection thread.

Table 4. Coinoculation of the *pssD111* mutant of *Rhizobium leguminosarum* bv. *viciae* with heterologous exopolysaccharide (EPS)-producing rhizobia

Coinoculation partner ^a	Inoc. ratio ^b	Nodulation			
		7 days a.i.		21 days a.i.	
		freq. ^c	nod. ^c	freq. ^c	nod. ^c
4S	1	1/18	1	5/18	1.6 ± 0.9
	1,000	0/18	0	1/18	1.0
CIAT899	1	0/18	0	18/18	4.7 ± 1.1
	1,000	0/18	0	10/18	1.6 ± 0.9
Rm1021	1	0/18	0	0/18	0
	1,000	0/18	0	0/18	0
Rm9000	1	0/18	0	0/18	0
	1,000	0/18	0	0/18	0
NGR234	1	0/18	0	0/18	0
	1,000	0/18	0	0/18	0
USDA110	1	0/18	0	0/18	0
	1,000	0/18	0	0/18	0

^a 4S: *R. leguminosarum* bv. *trifolii* 4S; CIAT899: *R. tropici* CIAT899; Rm1021: *Sinorhizobium meliloti* Rm1021 (EPS I⁺ EPS II⁻); Rm9000: *S. meliloti* Rm9000 (EPS I⁻ EPS II⁺); NGR234: *Rhizobium* sp. strain NGR234; USDA110: *Bradyrhizobium japonicum* USDA110.

^b Ratio of the coinoculation partners (Exo⁺ Nod⁻ : *pssD111*). The number of *pssD111* bacteria was constant, i.e., 25 µl of a suspension of 1 to 5 × 10⁵ CFU ml⁻¹.

^c freq. = number of nodulated plants per number of inoculated plants; nod. = average number of nodules per nodulated plant.

to attach to vetch root hairs as efficiently as parental strain RBL5523. Interestingly, it was observed that the *pssA4* mutant, which produces residual amounts of EPS, induces more infection foci on vetch than does the EPS-deficient mutant *pssDIII*. This implies that EPS plays a role not only in root hair infection but also in the root hair curling process (see also Rolfe et al. 1996). Further support for a role of EPS in root hair curling was obtained by coinoculation experiments with the *pssDIII* mutant and various non-nodulating EPS⁺ strains. The *pssDIII* mutant induced about threefold more infection foci on vetch when coinoculated with *R. leguminosarum* bv. *trifolii* ANU843 or ANU453, producing a homologous type of EPS.

Coinoculation experiments with *pssDIII* and non-isogenic EPS⁺ Nod⁻ strains revealed that strains producing EPS with an octasaccharide repeating unit similar to that produced by *R. leguminosarum* bv. *viciae* RBL5523 can restore nodulation by *pssDIII*. Previously it has been shown that the distribution of *O*-acetyl and hydroxybutanoyl substitutions is different in *R. leguminosarum* bv. *viciae* RBL5523, *R. leguminosarum* bv. *trifolii* ANU843, and *R. etli* CE3 (Canter Cremers et al. 1991a; Orgambide et al. 1992). Apparently, these substitutions do not largely influence the ability of the different strains to “help” *pssDIII* to nodulate vetch. *S. meliloti* Rm1021 (EPS I⁺ EPS II⁻), *S. meliloti* Rm9000 (EPS I⁻ EPS II⁺), *Rhizobium* sp. strain NGR234, and *B. japonicum* USDA110 produce EPS that are totally different in composition from EPS of *R. leguminosarum* bv. *viciae* RBL5523. Coinoculation of each of these strains with *pssDIII* appeared not to restore nodulation. This clearly points at structural requirements for rhizobial EPS to be biologically active on vetch, similar to the situation for *Leucaena* and clover (Djordjevic et al. 1987; Gray et al. 1991). However, these requirements are not very strict. Coinoculation of *pssDIII* with either *R. leguminosarum* bv. *trifolii* 4S or *R. tropici* CIAT899 showed that EPS that are structurally different from EPS of *R. leguminosarum* bv. *viciae* RBL5523 also can function in the symbiosis with vetch. *R. leguminosarum* bv. *trifolii* 4S secretes EPS the repeating unit of which carries a shorter side chain lacking the terminal pyruvylated galactose residue and lacking 3-hydroxybutyryl groups (Philip-Hollingsworth et al. 1989). Apparently, the chemical structure of the side chain can vary while the EPS retains its biological function. Accordingly, the homologous *R. leguminosarum* bv. *viciae* strain 248 secretes EPS, the repeating unit side chain of which also differs in length and sugar composition (Canter Cremers et al. 1991b). EPS produced by *R. tropici* CIAT899 are only structurally related to EPS of *R. leguminosarum* bv. *viciae* RBL5523 by virtue of possessing the same pyruvylated sugar residues at the nonreducing terminus of the side chains. Interestingly, the structure of EPS produced by *R. tropici* CIAT899 resembles that of EPS I from *S. meliloti* Rm1021 (Fig. 1), with a major difference being that in succinoglycan, the nonreducing terminal-sugar residue is glucose instead of galactose, and only one pyruvyl substituent is present on the side chain (Gil-Serrano et al. 1990). At present, the results do not allow for identification of a specific motif required for biological activity of EPS produced by *R. leguminosarum* bv. *viciae*. Future bioassays with purified EPS fractions may enable such an identification.

When performing coinoculation experiments on vetch with *R. leguminosarum* bv. *viciae* strains and heterologous EPS-producing rhizobia, we observed a negative influence on the interaction between *R. leguminosarum* bv. *viciae* and vetch by Nod factors produced by some heterologous strains. This effect was especially obvious when EPS-deficient mutants of *R. leguminosarum* bv. *viciae* were used in the nodulation experiments. Microscopic study showed that heterologous Nod factors produced by *R. leguminosarum* bv. *trifolii* ANU843 influenced infection thread formation rather than root hair curling in vetch. Salzwedel and Dazzo (1993) proposed that elicitation of a rapid increase in peroxidase activity in white clover root hairs by heterologous *R. leguminosarum* bv. *viciae* bacteria might result in increased cross-linking of wall polymers, making the root hair wall more resistant to bacterial penetration. A similar effect may contribute to inhibition of infection thread formation by heterologous Nod factors in vetch, especially when root hair infection is delayed.

Previously, it was hypothesized that EPS may act as a suppressor of the plant defense system during nodulation of alfalfa by *S. meliloti* (Niehaus et al. 1993). The same hypothesis may explain our observation that EPS-deficient mutants of *R. leguminosarum* bv. *viciae* are particularly affected in their ability to induce infection threads in vetch. When the rhizobia are trapped in the root hair curl, EPS might prevent host defense (for example, by suppressing peroxidase activity; Salzwedel and Dazzo 1993), and by this allow formation of infection threads. However, our results indicate that EPS is also involved in redirection of tip growth of root hairs (see also Rolfe et al. 1996). This result is significant because up to now Nod factors produced by rhizobia have generally been considered to be the sole “curling factors.” As an alternative hypothesis, EPS might accelerate the curling and infection process to such an extent that rhizobial root penetration precedes the plant defense response. The molecular mechanism by which this may occur remains to be studied. EPS might be a cooperative signal involved in redirection of root hair growth, or it might enhance the biological activity of the Nod factor. When the rhizobia are quickly trapped, infection thread formation can occur before defense reactions become effective. Preliminary results in our laboratory showed that EPS⁻ mutants of *R. leguminosarum* bv. *viciae* are not affected in their ability to turn on the autoregulation process, which is consistent with observations made for alfalfa (Caetano-Anollés et al. 1990). Therefore, autoregulation and/or plant defense reactions might interfere with slow root hair infection by EPS-mutants of *R. leguminosarum* bv. *viciae*. As a result, the nodulation process is blocked. Timing of defense reactions in relation to nodulation in host plants producing determinate-type nodules, such as soybean and bean, may be different in that even EPS-deficient mutant rhizobia can normally colonize the root tissue (Borthakur et al. 1986; Diebold and Noel 1989).

MATERIALS AND METHODS

Strains, plasmids, and media.

The organisms and the characteristics of the plasmids used in this study are listed in Table 5. *R. leguminosarum* RBL5515 is a Sym plasmid-cured derivative of *R. leguminosarum* bv.

Table 5. Strains and their relevant characteristics

Designation	Relevant characteristics ^a	Source or reference
<i>Rhizobium</i>		
RBL5515	<i>R. leguminosarum</i> bv. <i>trifolii</i> LPR5 cured of its Sym plasmid, <i>str rif</i>	Priem and Wijffelman 1984
RBL5522	<i>R. leguminosarum</i> strain RBL5515 pRL1JI(del) <i>Spc2::Tn1831</i> , <i>str rif</i> (<i>nod</i> mutant)	Pees et al. 1986
RBL5523	<i>R. leguminosarum</i> strain RBL5515 pRL1JI <i>Spc3::Tn1831</i> , <i>str rif</i>	Pees et al. 1986
RBL5807	<i>R. leguminosarum</i> RBL5523 <i>pssA4::Tn5</i> , (<i>exo4</i>), <i>str rif</i>	van Workum et al. 1997
RBL5811	<i>R. leguminosarum</i> RBL5523 <i>exoB52::Tn5</i> , <i>str rif</i>	Canter Cremers et al. 1990
RBL5833	<i>R. leguminosarum</i> RBL5523 <i>pssD111::Tn5</i> , (<i>exo111</i>), <i>str rif</i>	van Workum et al. 1997
Rm1021	<i>Sinorhizobium meliloti</i> SU47, <i>str</i>	Meade et al. 1982
Rm9000	Rm1021, <i>expR101</i> , <i>exoY210::Tn5</i> , <i>str</i>	G. C. Walker
CIAT899	Wild-type <i>R. tropici</i>	Matinez-Romero et al. 1991
ANU843	Wild-type <i>R. leguminosarum</i> bv. <i>trifolii</i>	Rolfe et al. 1980
ANU453	<i>R. leguminosarum</i> bv. <i>trifolii</i> ANU843, <i>nodC</i> mutant, <i>str</i>	Djordjevic et al. 1983
4S	Wild-type <i>R. leguminosarum</i> bv. <i>trifolii</i>	Amemura et al. 1983
NGR234	Wild-type, broad-host-range cowpea <i>Rhizobium</i> sp.	Chen et al. 1988
USDA110	Wild-type <i>Bradyrhizobium japonicum</i>	Tully and Terry 1985
<i>Escherichia coli</i>		
DH5 α	F' <i>endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1</i> $\Delta(lacIZYA-argF)U169 deoR$	Woodcock et al. 1989
Plasmids		
PRK2013	Helper plasmid for mobilization of plasmids, Km ^r	Ditta et al. 1980
PXLGD4	pGD499 prime (<i>IncP</i>) carrying a <i>hemA::lacZ</i> fusion	Leong et al. 1985

^a *str*, streptomycin resistance; *rif*, rifampicin resistance. *Tn1831* codes for spectinomycin- and streptomycin resistance.

trifolii LPR5 (Priem and Wijffelman 1984). Plasmid pRL1JI is the Sym plasmid of *R. leguminosarum* bv. *viciae* 248 (Josey et al. 1979), which was marked with *Tn1831* for selection purposes (Pees et al. 1986). *Rhizobium* spp. were grown at 28°C in the following culture media: TY (Beringer 1974), YMB (Hooykaas et al. 1977), or B⁻ (van Brussel et al. 1977). *Escherichia coli* strains were grown in Luria complete (LC) medium (Maniatis et al. 1982) at 37°C. Tetracycline (Tc) was added to concentrations of 20 and 2 μ g/ml for *E. coli* and *R. leguminosarum*, respectively. Rifampicin (Rif) and spectinomycin (Spc) were added to final concentrations of 20 and 400 μ g/ml, respectively.

Bacterial matings.

Plasmids were transferred from *E. coli* to *Rhizobium* strains by triparental conjugal mating as described previously (Ditta et al. 1980), with pRK2013 as a helper plasmid.

Plant assays and microscopic methods.

Vicia sativa L. subsp. *nigra* (L.) seeds (vetch seeds) were surface disinfected and allowed to germinate as described previously (van Workum et al. 1995). Inoculated plants were cultured in liquid Jensen medium (Vincent 1970) to which, if appropriate, aminoethoxyvinylglycine (AVG) was added to a final concentration of 0.1 mg/liter. Infection of vetch was examined with bacterial strains carrying a fusion of the reporter gene *lacZ* of *E. coli* to the *hemA* gene promoter of *S. meliloti* (courtesy of Georges Truchet). The *hemA* gene is constitutively expressed in free-living *S. meliloti* cells (Leong et al. 1985) as well as under symbiotic conditions (David et al. 1988). β -Galactosidase activity was assayed as described before (Boivin et al. 1990) with X-Gal as a substrate. Rhizobia were reisolated from root nodules according to Vincent (1970). Nodules were immersed in ethanol 96% (30 s), followed by incubation for 2 min in HCl-acidified HgCl₂ (1 g/liter). The nodules were washed six 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.

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