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The transition of *Rhizobium fredii* lipopolysaccharides induced by soybean root exudation

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Abstract. The chemical compounds contained in leguminous plant root exudation induce structural and compositional transitions of the lipopolysaccharides of *Rhizobium* that occur during rhizobia-legume symbiotic infection processes. The root exudate was extracted from the seed germinated solution and seedling roots of soybean. We added the exudation to bacteria cultured medium. *Rhizobium* and *Bradyrhizobium* strains were purified through gel filtration chromatography followed by SDS-PAGE analysis. Different chromatographic and SDS-PAGE patterns among the different rhizobia strains were obtained. We determined by means of the gel filtration chromatography that soybean root exudations induced a molecular weight range change in the lipopolysaccharides of these strains. The band of this LPS-II region exhibited diffused patterns in SDS-PAGE as a result of soybean root secretion. The LPS-I region of four strains cultured in TY medium with root exudates exhibited clearer and denser bands than those of rhizobia from TY medium without added soybean root exudates. Based on the composition analysis, we found that the KDO ratio increased in *R. fredii* TU6, *R. meliloti* ATCC 9930, *R. leguminosarum* bv. *viciae* ATCC 10004 and *B. japonicum* USDA 110, but decreased in *R. fredii* KR23, *R. loti* Lu7, and *R. loti* ATCC 33669 when soybean root exudation was added to the medium. The hexouronate ratio decreased except in *R. loti* Lu7. This result was consistent with the SDS-PAGE profile. The LPS-I region of *R. loti* LU7 showed little variation. It was therefore ascertained that soybean root secretion induces a variation in the structure of rhizobial lipopolysaccharides.

Keywords: *Bradyrhizobium japonicum*; Lipopolysaccharide; *Rhizobium fredii*; Root exudation.

Abbreviations: LPS, lipopolysaccharide; **Hex,** hexose; **HexA,** hexuronic acid or hexuronate; **KDO,** 2-keto-3-deoxyoctanate; **O-Ag,** O-antigen; **MWCO,** molecular weight cut off; **SDS-PAGE,** sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Introduction

Lipopolysaccharide (LPS) is a component of the outer membrane of Gram negative bacteria and is composed of O-antigen (O-Ag), core oligosaccahride, and lipid A. In the nitrogen fixing bacteria *Rhizobium*, it has been suspected of playing a role in early symbiotic processes. It has been pointed out in some research results that LPS can affect the ability of rhizobia to nodulate leguminous hosts. For instance, an LPS mutant of *Bradyrhizobium japonicum*, defective in O-Ag, cannot infect soybean root hair (Puvanesarajah et al., 1987). LPS mutants of *R. phaseoli* also form a defective infectious thread, so that rhizobial cells cannot be released and form nodules (Cava et al., 1989). Thus, rhizobial LPS is known to be an essential factor in nodule forming processes.

Leguminous root exudations were previously thought to be an important factor in the infection processes and to contain a variety of different molecular weight fractions. Recently, some reports have showed that flavonoid chemicals, secreted in root exudates, can activate nod genes and regulate the nod gene product as part of a signal compound participating rhizobia-host recognition reaction (Peter et al., 1986; Peter and Long, 1988; Lerouge et al., 1990). The signal compound of *R. meliloti* was identified as a tetrasaccharide which could be derived from cell wall (Lerouge et al., 1990). In rhizobial extracellular polysaccharides, some alterations in composition, structure, and molecular weight of exopolysaccharides were induced by 1 μM genistein (Dunn et al., 1992). No one has yet reported on the effect on LPS by low molecular weight root exudates although LPS plays an important role in infectious processes.

In this study, we decided to investigate the effect of a mixture, instead of any single chemical substance, because the low molecular weight fraction was always a mixture in the rhizosphere. According to the results of Gaworzeska and Carlile (1982), the low molecular fractions are secreted during 0–3 days of germinating seedling. We therefore collected the root secretion of 3-day-old soybean seedlings for our research. In this paper, we will use the results of gel filtration chromatography and SDS-PAGE to demonstrate that the transition of LPS can be induced by those small molecules.

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Materials and Methods

Bacterial Strains

Rhizobium fredii KR23 and TU6 were isolated from indigenous soils of Taiwan and stored in yeast-mannitol agar at 4°C in our laboratory. *Rhizobium loti* Lu7, an isolate in Taiwan, was provided by Prof. C.C. Young, Dept. of Soil Science, National Chung Hsing University, Taichung, Taiwan. *Bradyrhizobium japonicum* USDA110 was donated by USDA. *Rhizobium meliloti* ATCC 9930, *R. leguminosarum* bv. *viciae* ATCC 10004 and *R. loti* ATCC 33669 were purchased from the American Type Culture Collection, Rockville, MD, USA. The rhizobial strains and their host are described in Table 1.

Table 1. Strains and their symbiotic hosts.

Bacteria	Host genus
Rhizobium fredii	*Glycine*
KR23	
TU6	
Bradyrhizobium japonicum	*Glycine*
USDA 110	
R. loti	*Leucaena*
Lu7	
ATCC 33669	
R. meliloti	*Medicago*
ATCC 9930	
R. leguminosarum bv. *viciae*	*Pisum*
ATCC 10004	

Soybean and Preparation of Root Exudate

Soybean seeds (*Glycine max*) were obtained from the Tainan Agricultural Experiment Station, Tainan, Taiwan. Surface sterilized seeds were incubated and allowed to germinate on a sterilized petri dish with a stainless grid. N-free Fahraeus' solution was used for plant incubation. Preparation of the root secretions were modified from the method of Wall and Favelukes (1991).

The seedlings were cultured in a plant growth chamber, in the dark, at 28°C for 3 days. The incubation solution was used as one source of root secretion. The roots of seedlings were cut off, and washed in distilled water. Two of these root exudations were combined and dialyzed (MWCO<3000, Spectrum Med. Ind. Inc., CA, USA) against distilled water. The dialyzable fraction was collected, concentrated, and lyophilized for further incubating preparation.

Bacteria were grown in TY medium at 28°C until the late log phase. The medium was composed of 0.5% tryptone, 0.3% yeast extract, and 10 mM $CaCl_2$. Root exudation was added to the TY medium at a 93 μg/ml concentration. As a control, bacteria were incubated in the TY medium only. In order to avoid root-exudations from denaturation during autoclaving, a 0.22 μm membrane (Millipore Co., Bedford, MA, USA) was used for sterilization by filteration through this membrane.

LPS Purification and Analysis

The LPS was extracted from bacteria by phenol-water method as modified by Carlson et al. (1978) and Carrion et al. (1990). Crude LPS was purified through Fractogel TSK HW-65 (F) (Merck, Germany) gel filtration chromatography with 0.1 M EDTA-0.3 M triethylamine buffer, pH 7.0. Fractions were collected and assayed for hexose (Hex) by the phenol-sulfuric acid method, hexuronic acid (HexA) by the *m*-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen, 1973), and 2-keto-3-deoxyoctanate (KDO) by the thiobarbituric acid (Karhanis et al., 1978).

The purified LPS was analyzed by 12.5% SDS-PAGE (16×16 cm), 20 mA/gel in a Hoeffer SE600 electrophoresis apparatus (Hoeffer Scientific Instruments, San Francisco, CA). The gels were stained with silver nitrate solution according to the method of Tsai and Frasch (1982).

Results

The crude rhizobial LPS was purified using a Fractogel TSK HW-65 (F) gel filtration column (Figure 1). The gel filtration chromatography was performed with an EDTA-triethylamine buffer (pH 7.0), which separates the LPSs from residual capsular polysaccharides, extracellular polysaccharides, and smaller molecular glucans (Carlson, 1984). From these results, we determined that soybean root exudates induce alterations in LPS expression. A later eluted peak corresponded to the position of *R. fredii* cultured in TY-soybean root exudation containing medium. As shown in Figure 1, we also found some transitional peaks in the early eluted regions.

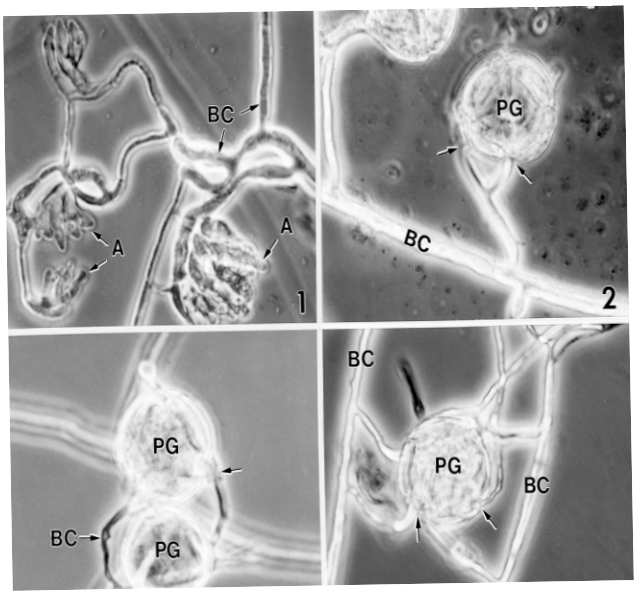

Figure 1. TSK HW-65 gel filtration chromatography of LPS from *Rhizobium fredii* species. Column size 82×1.6 cm, 4.0 ml each fractions, eluted with EDTA-triethylamine. (l) LPS extracted from rhizobia incubated in medium without soybean root exudations, and (¡) extracted from rhizobia incubated in medium with soybean root exudations. (A) *R. fredii* KR23, (B) *R. fredii* TU6.

Our compositional analysis revealed that hexose, hexuronate, and KDO were ubiquitously present in each strain and that the Hex/HexA and Hex/KDO ratio of each strain had changed in the root exudates as shown in Table 2. We found that the Hex/HexA ratio always increased, except in *R. loti* Lu7, when the medium contained soybean root exudation. The Hex/KDO ratios were divided into two groups, one with an increased and the other with a decreased ratio. Four strains belonged to the increased Hex/KDO group—*R. fredii* TU6, *R. meliloti* ATCC 9930, and *R. leguminosarum* bv. *viciae* ATCC 10004, as well as *B. japonicum* USDA 110. By contrast, *R. fredii* KR23, *R. loti* Lu7 and *R. loti* ATCC 33669 exhibited decreased Hex/KDO ratios.

We obtained different LPS patterns by SDS-PAGE analysis (Figure 2). Based on the position of lysozyme used as the molecular weight standard, we identified two regions: LPS-I and LPS-II. The LPS-I region, with slower mobility than that of lysozyme, is derived from the complete LPS molecule with O-antigen while the faster-moving LPS-II region represents the LPS without O-chain (Bhat and Carlson, 1992; Carlson, 1984). Each strain showed quite a different electrophoretic pattern. Except for *R. loti* Lu7, differences were seen between the LPS structures derived from cultures with and without soybean exudates. Thus, a major difference was observed in the LPS-II region of *R. fredii* species. In *R. fredii* KR23, variations in LPS involved a decrease in molecular weight (Figure 2) and a different SDS-PAGE expression while *R. fredii* TU6 exhibited different LPS-II SDS-PAGE patterns. Other rhizobial strains showed different transitions in the LPS-I and LPS-II regions. There were fewer LPS-I bands in *B. japonicum* USDA 110 and *R. meliloti* ATCC 9930 after incubation with soybean root exudation medium. The number of LPS-I bands increased in *R. leguminosarum* bv. *viciae* ATCC10004 and *R. loti* ATCC 33669. As to the difference in band forms, we found a very prominent doublet band in the LPS-I region of *R. loti* ATCC 33669 not present in the TY medium without root secretion. The transition in LPS-I region seems to indicate the heterogeneity in size induced by soybean root exudates.

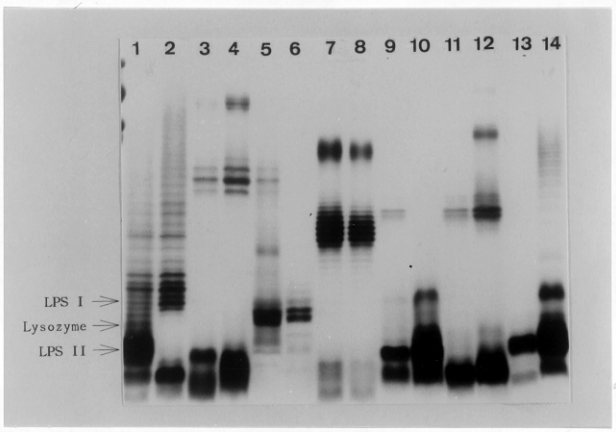

Figure 2. SDS-PAGE analysis of lipopolysaccharides. The LPS samples loaded are Lane 1–2, *Rhizobium fredii* KR23; Lane 3–4, *R. fredii* TU6; Lane 5–6, *Bradyrhizobium japonicum* USDA 110; Lane 7–8, *R. loti* Lu7; Lane 9–10, *R. meliloti* ATCC 9930; Lane 11–12, *R. leguminosarum* bv. *viciae* ATCC 10004; Lane 13–14, *R. loti* ATCC 33669. The odd lanes loaded with LPS were extracted from rhizobia incubated in medium with soybean root exudations. The even lanes loaded with LPS were extracted from rhizobia incubated in medium without soybean root exudations.

Discussion

Rhizobia will move to the surface of root hairs from the rhizosphere because of the chemotaxis caused by leguminous plant root exudation. They then tend to bind to the root hair surfaces (Bergman et al., 1988; Gaworzeska and Carlile, 1982). Root secretions contain many compounds that are capable of inducing *nod* gene expression determining infection specificity. Flavonoids have been identified to trigger *nod* gene expression (Puppke, 1984; Firmin et al., 1986; Peter et al., 1986; Djordjevic et al., 1987). In those studies, different *Rhizobium* species were affected by different types of chemical compounds. Isoflavones induced activation of *B. japonicum nod* genes, but not those of *R. leguminosarum* bv. *trifolii* (Kosslake et al., 1987). In addition to these effects, *nodD* gene encodes a regulatory

Table 2. Soybean root exudate influences the variation of rhizobial lipopolysaccharides on hexose/hexuronate and hexose/KDO.

Bacteria	Without root exudate		With root exudate	
	Hex/HexA[a]	Hex/KDO	Hex/HexA[a]	Hex/KDO
Rhizobium fredii				
KR23	57.7	27.2	111.7	10.8
TU6	15.0	9.2	30.9	21.2
Bradyrhizobium japonicum				
USDA 110	19.9	10.5	34.5	34.5
R. loti				
Lu7	7.3	206.9	8.1	59.3
ATCC 33669	7.4	154.8	24.1	43.3
R. meliloti				
ATCC 9930	5.0	20.0	15.3	50.8
R. leguminosarum bv. *viciae*				
ATCC 10004	10.4	16.1	20.7	31.1

[a]The abbreviations: Hex, hexose; HexA, hexuronate; KDO, 2-keto-deoxy-octanic acid.

protein which not only controls the expression of other *nod* genes but also autoregulates *nodD* itself (Pinero et al., 1988; Rossen et al., 1985; Sherman et al., 1986). These data strongly suggest that infection recognition is a multi-step process.

Once rhizobial *nod* genes are activated, rhizobia produce a signal compound, which correlates with a specific recognition reaction. The *nodH* and *nodPQ* genes of *R. meliloti* direct the synthesis of a signal compound, nodRm-1, which has been identified as *N*-acetyl-D-glucosamine responsible for root hair curling (Lerouge et al., 1990; Truchet et al., 1991). This sugar, a component of lipid-A of LPS, is released from LPS. *Bradyrhizobium japonicum* produces a nonsulfated pentaglucosamine as a signal compound (Verma, 1992). In other studies, the *nodF* gene of *R. leguminosarum* has been shown to be involved in the synthesis of acyl-carrier protein and fatty acids. The *nod* gene is thought to induce the synthesis of proteins, fatty acids and acylation of LPS (Lipman, 1971; Sherman et al., 1986).

Rhizobial extracellular polysaccharides also play an important role in infection processes (Clover et al., 1989; Goosen-de Roo et al., 1991; Kato et al., 1979, 1980; Long, 1989). As to the alterations flavonoids produce in the extracellular polysaccharides, Dunn et al. (1992) found that genistein in the medium alters their composition, structure, and molecular weight.

Priefer (1989) conjugated a *R. leguminosarum* LPS-I defect mutant with *Lps* cosmid, which caused the mutant to synthesize LPS-I and form effective nodules in *Vicia hirsuta*. He proposed that LPS-I can serve as a signal for successful nodulation. Dazzo et al. (1991) suggested that LPS is a positive signal molecule for successful infection processes.

In this study, we found that both the molecular weight and structure of LPS were altered by root secretions. These alterations included molecular weight range, composition, and structure as exhibited by gel filtration chromatography, compositional analyses, and SDS-PAGE results. Comparing the Hex/HexA and Hex/KDO ratios, these results showed that Hex/HexA increased except for *R. loti* Lu7 when the medium contained soybean root exudation. These results were consistent with the LPS-I region of the SDS-PAGE profile. This alteration of the LPS-I pattern reflects the alteration of O-Ag. We also obtained a variation in KDO as derived from the change in the Hex/KDO ratio (Table 2 and Figure 2). The transition pattern of rhizobial strains showed that the composition, structure, and molecular weight of LPS were influenced by root exudates during degradation or post-synthesis modification in LPS synthetic processes.

In general, Gram negative bacteria, irrespective of symbiotic interactions, undergo modification of their LPSs in ways that best cope with environmental changes; however, rhizobia will respond to conditions more specific to the plant environment. According to some reports, researchers are concerned with LPS changes induced by environmental factors (Bhat and Carlson, 1992; Tao et al., 1992). Tao et al. (1992) cultured rhizobia under a variety of different conditions including low pH, high temperature, low phosphate, and low oxygen concentration. Antigenic changes at low pH were dependent on growth of the bacteria, but independent of nitrogen and carbon sources and the rich or minimal quality of the medium. This means that these LPSs will have less or no reaction to antibodies but will have the same mobility in SDS-PAGE, except when rhizobia are incubated in a low pH. Bhat and Carlson (1992) studied the pH-dependent structural epitopes of LPS from *R. leguminosarum* bv. *phaseoli*. The results showed that the methylated glycosyl residues were either affected by growth pH or were part of the O-chain polysaccharide of LPS in the nodule when the bacteria were incubated in a pH 4.8 medium. These research results showed that the LPS changes were affected by conditions of low pH and that these changes only showed up in the LPS-I region. In addition, LPSs are involved at a later stage in the nodule development, such as forming infection threads and releasing bacteroids from infection threads. We found that the changes are expressed not only in the LPS-I region but also in the LPS-II region, and showed transitions in the SDS-PAGE pattern. We can therefore conclude that alterations in LPSs will be affected by plant exudates in the rhizosphere environment and that the changes are related to the recognition or attachment in the infection processes.

In our agronomy experiments, we observed that soybean infected by *R. fredii* KR23 had a 50% higher nodulation rate than *R. fredii* TU6. When comparing the LPS-I regions by SDS-PAGE, the band of *R. fredii* KR23 showed more alterations than that of *R. fredii* TU6, indicating that LPSs play an important role in infection processes, especially after transitions are induced through root secretions. We therefore conclude from both agronomic and biochemical results that LPSs are important determinants for successful infection between soybean and *R. fredii*.

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