# Production of Cell-Associated Polysaccharides of *Rhizobium fredii* USDA205 Is Modulated by Apigenin and Host Root Extract

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*Rhizobium fredii* USDA205 cells were cultured in the presence of 4',5,7-trihydroxyflavone (apigenin), a compound that has been shown to induce the *nod* genes and other symbiosis-related genes in *R. fredii*. The cell-associated polysaccharides were then extracted with hot phenol/water, separated by repetitive gel filtration chromatography, and analyzed by polyacrylamide gel electrophoresis, nuclear magnetic resonance spectrometry, high-performance anion-exchange chromatography, and gas chromatography. These analyses showed that apigenin effects a modulation in the production of some cell-associated bacterial polysaccharides: 1) The production of a glucan is severely attenuated; 2) the lipopolysaccharide O antigen is modified in composition and $M_r$ distribution; and 3) the ratio of two extracted polysaccharides, which are structurally analogous to group II K antigens (capsular polysaccharides), is altered. Similar effects resulted from the inclusion of host plant root extract in the growth medium.

*Rhizobium* and *Bradyrhizobium* spp. are gram-negative bacteria that are able to induce the formation of morphologically distinct structures (nodules) on the roots of leguminous plants. The symbiotic interaction results in the inclusion of highly differentiated bacterial cells (bacteroids) in host plant nodule cells, where they carry out the reduction of atmospheric dinitrogen to ammonia, which is then utilized by the host plant.

An early phase of the plant-microbe interaction, preceding symbiotic infection, is mediated by an exchange of signal molecules between the host plants and their respective symbionts: Specific flavonoid signals that are secreted by the host plant, in concert with the *trans*-acting gene products of the *nodD* regulatory genes, activate an array of bacterial nodulation (*nod*) genes (Redmond *et al.* 1986; Kondorosi 1992; Denarie *et al.* 1992). The *nod* genes then direct the synthesis of structurally specific lipooligosaccharide signals (Nod factors) that induce root hair deformation and cortical cell division in the region of nodule development on the host plant (Lerouge *et al.* 1990; Denarie and Roche 1992; Denarie *et al.*

1992). In addition to the activation of the *nod* genes, plant-derived flavonoids and plant root exudates have also been shown to affect other bacterial functions, such as chemotaxis (Caetano-Anollés *et al.* 1988a), which may be related to nodulation efficiency, and competitiveness (Caetano-Anollés *et al.* 1988b).

Nod factor induction of root hair deformation and incipient nodule development is necessary but not sufficient for successful symbiosis; a subsequent bacterial infection process is prerequisite for the development of complete nitrogen-fixing nodules. Many reports have demonstrated a correlation between the production of specific bacterial cell-surface lipopolysaccharides (LPSs) and extracellular polysaccharides (EPSs) and the effectiveness of the infection process; these studies are the subject of several recent reviews (Noel 1992; Gray *et al.* 1992; Leigh and Coplin 1992). LPS- and EPS-deficient mutants have been shown to be defective in various symbiotic steps, including infection thread development, bacterial release into the nodule cells, and differentiation of the bacteria into nitrogen-fixing bacteroids.

The LPS of some rhizobial strains has been shown to differ in antibody specificity after the differentiation of free-living bacteria to the bacteroid state (Tao *et al.* 1992; Brewin *et al.* 1986; Wood *et al.* 1989; Sindhu *et al.* 1990), raising the possibility that inducible bacterial functions other than Nod factor production may be related to nodulation. Two recent reports have described specific molecular responses of *R. fredii* to *nod* gene inducers: the stimulation of protein export by *R. fredii* USDA257 (Krishnan and Pueppke 1993) and the modification of EPS production by *R. fredii* USDA193 (Dunn *et al.* 1992). These effects appear to be strain specific and may be related to the host plant selectivity displayed by various *R. fredii* strains (Balatti and Pueppke 1992a,b).

LPSs are not the only cell-associated polysaccharides produced by rhizobia; a class of cell-associated, acidic polysaccharides, which are structurally analogous to the K antigens (capsular polysaccharides) of *Escherichia coli* (Jann and Jann 1990), has recently been identified in cell extracts of two species of rhizobia, *R. meliloti* AK631 and *R. fredii* USDA205 (Reuhs *et al.* 1993). Two polysaccharides of this type have been isolated from *R. fredii* USDA205; each comprises disaccharide subunits of hexose and 3-deoxy-D-*manno*-2-octulosonic acid (Kdo). The complete structure of the polysaccharide from *R. meliloti* AK631 has not yet been determined; however, it also contains Kdo as a major component, and a correlation has been established between the

production of this polysaccharide and the effective nodulation of alfalfa by strain AK631 (Reuhs et al. 1993; Petrovics et al. 1993; Williams et al. 1990; B. L. Reuhs and M. N. V. Williams, unpublished). Evidence is presented in this report that, in addition to LPS and the Kdo-rich polysaccharides, R. fredii USDA205 also produces other cell-associated, high molecular weight (HMW) polysaccharides, composed of glucose and mannose, as well as low molecular weight (LMW) 1,2-linked β-D-glucans, which are common to rhizobia (Dell et al. 1983; Miller et al. 1988).

The effect of gene induction agents on the production of the cell-associated polysaccharides produced by R. fredii USDA205, the microsymbiont of Peking soybeans (among other hosts), was investigated. This report describes a comparison of the purified and partially characterized polysaccharides from R. fredii USDA205 cells cultured either in the presence or in the absence of 4′,5,7-trihydroxyflavone (apigenin), an inducer of the R. fredii nod genes (Peters and Verma 1990) and other symbiosis-associated genes (Sadowsky et al. 1988). Biochemical analyses of cell extracts demonstrated that apigenin effected a modulation in the production of the cell-associated polysaccharides: 1) The production of a glucan was severely attenuated; 2) the LPS was modified in $M_r$ distribution and O antigen composition; and 3) the production of the Kdo-containing polysaccharides was altered. Peking soybean root extract, also an inducer of nod genes and other symbiosis-related genes (Olson et al. 1985), elicited similar effects.

## RESULTS

### Induction conditions and bacterial growth.

The culture conditions for nod gene induction were established with R. fredii USDA201::Mu-d (KAN, lac) 11G2, a Nod⁻ mutant that showed β-galactosidase induction in the presence of nod gene inducers. β-Galactosidase activity in cultures that contained root extract or apigenin reached similar levels, approximately eight- to 10-fold higher than that of controls. Maximum β-galactosidase induction occurred with Peking soybean root extract at a concentration of 500 µg/ml. Root extract concentrations as low as 100 µg/ml showed induction to 75% of the maximum, and induction to 25% of the maximum was possible with concentrations as low as 20 µg/ml. Maximum β-galactosidase induction with apigenin occurred at a concentration of 3 µg/ml (11.1 µM).

### PAGE analysis of crude cell extracts.

Aliquots of the crude cell extracts from cultures induced with 11.1 µM apigenin and from noninduced cultures were analyzed by polyacrylamide gel electrophoresis (PAGE), and the gels were silver-stained to various degrees (Fig. 1A and B). LPS I comprises lipid A and core oligosaccharide. LPS IIA and IIB comprise lipid A, core, and various forms of O antigen. This banding pattern is similar to those of other Rhizobium LPSs (Carlson 1984; Carrion et al. 1990; Carlson et al. 1992). The bands in region III are due to a distinct glucan (glucan III), rather than a different form of LPS (see below, under Composition analysis and HPAEC analyses). There are three distinct differences between the PAGE banding patterns of the extracts from the apigenin-induced culture and those of the control (lanes 1 and 2, respectively, in Fig. 1A and B):

1) The region III bands are virtually absent in lane 1; 2) the LPS IIA band is predominant in lane 2, whereas the LPS IIB band is more intense in lane 1; and 3) an additional LPS form that migrated between LPS I and LPS II is apparent only in lane 1, which implies a reduction in the LPS O antigen chain length.

Figure 1C shows the LPS region from a PAGE analysis of the crude extracts from cells cultured in the presence or absence of Peking soybean root extract. The effects of the root extract were similar those of apigenin: Induction resulted in a modulation of O antigen production, from the LPS IIA form to the LPS IIB form. In addition, the LPS from moderately induced cells displayed a PAGE pattern that is intermediate between those of the others; thus, the degree of modification appears to be correlated with the concentration of root extract. The intermediate LPS bands, which are apparent in Figure 1B, do not appear in Figure 1C, because that gel was lightly stained to enhance the resolution; this also resulted in the lightly stained LPS II region in lane 1. Glucan III was absent from lane 3 (data not shown).

### Sepharose 4B and Sephadex G-150 chromatography.

The crude cell extracts from cultures induced with 11.1 µM apigenin and from noninduced cultures were chromatographed over Sepharose 4B (Fig. 2A and B). LPS is the only component of the cell-associated polysaccharides that was found to contain glycosyluronic acids (Table 1), and the presence of uronic acids in the Sepharose 4B fractions parallels the presence of LPS, as determined by PAGE (LPS-containing fractions are represented by the solid bars in Fig. 2A and B). The LPS from the noninduced culture eluted into fractions 36–72 (Fig. 2A), which appeared to be a result of aggregation: PAGE analyses of Sepharose 4B chromatography fractions have shown that this purification step has little sizing effect on the extracted LPS (Reuhs et al. 1993). To verify this, fractions 62–72 (peak 4B-2 in Fig. 2A) were pooled and rechromatographed under identical conditions.



**Fig. 1.** Silver-stained polyacrylamide gels of crude cell extracts from cultures of *Rhizobium fredii* USDA205 grown either in the presence of apigenin or soybean root extract (induced cultures) or in their absence (nonnduced cultures). **A** and **B**, Two similar gels stained to different degrees. The lanes contain the crude cell extract from cells induced with 11.1 µM apigenin (lane 1) and from noninduced cells (lane 2). The designations for the banding region are explained in the text. **C**, The LPS banding region of crude cell extract from *R. fredii* USDA205 cultured with Peking soybean root extract: lane 1, control; lane 2, moderate induction with root extract (70 µg/ml); lane 3, full induction with root extract (500 µg/ml).

The LPS again eluted into fractions 36–72 (Fig. 2C), demonstrating that the LPS elution pattern was, in fact, due to aggregation. This second Sepharose 4B chromatography step also served to further separate the LPS from the peak 4B-3 polysaccharides (discussed below).

Figure 2B shows that the LPS from the induced cultures did not aggregate to the same extent as that from the noninduced cultures (note the uronic acid elution profile, peak 4B-2). Sufficient LPS was present in earlier fractions to be weakly detected by PAGE (solid bar), but the majority of the LPS was found in fractions 60–74. The 4B-2 pool from this preparation was also rechromatographed to further separate the LPS from the peak 4B-3 polysaccharides. The elution profile of this LPS pool (not shown) was identical to that in Figure 2B; i.e., the LPS did not appear to aggregate.

There was also a difference in the elution of the Kdo-rich polysaccharides from the induced and the noninduced cultures (peak 4B-3 in Fig. 2A and B): The median elution point of those from the former is seven fractions prior to those from the latter. These polysaccharides are discussed in detail in the introduction and below, under NMR analysis of the Kdo-rich polysaccharides.

The crude cell extract from *R. fredii* USDA205 also contained several HMW neutral polysaccharides and LMW 1,2-linked β-D-glucans (see below, under Composition analysis, and Table 1). The elution profiles of the HMW polysaccharides from the induced and the noninduced culture extracts were slightly different (peak 4B-1 in Fig. 2A and B), although, based on this data alone, any significance is unclear. The elution of the LMW 1,2-linked β-D-glucans (peak 4B-4 in Fig. 2A and B) from the induced culture extracts was not conspicuously different from that of the noninduced culture extracts. The extraction and chromatography protocol was repeated on three separate cultures of induced cells and four separate cultures of noninduced cells, with no significant differences in the Sepharose 4B profiles from preparation to
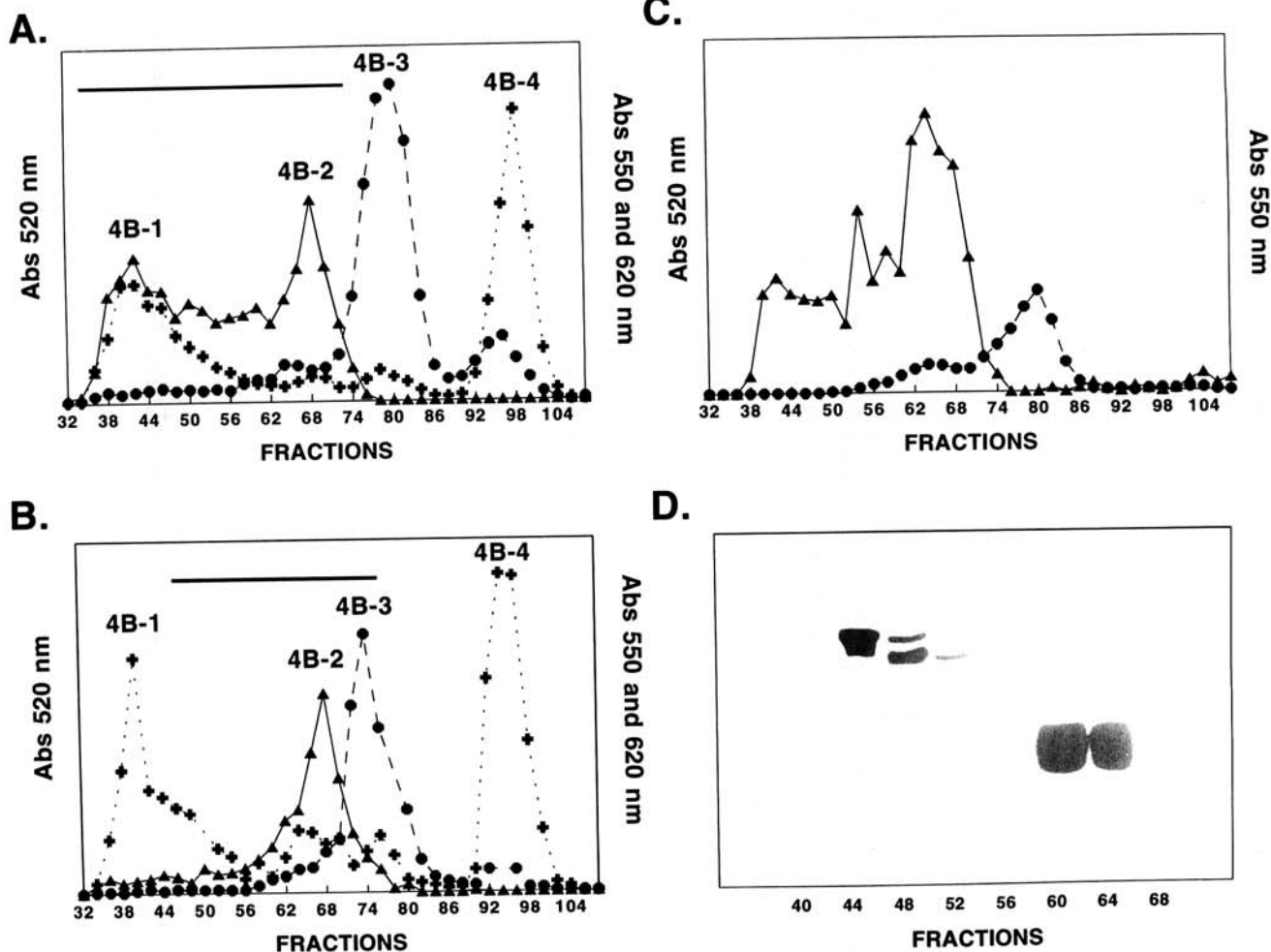


Fig. 2. A and B, Elution profiles of crude cell extracts from *Rhizobium fredii* USDA205, obtained by Sepharose 4B gel filtration in a solution of 30 mM triethylamine and 10 mM EDTA: A, control (extracts from noninduced cultures); B, extracts from cultures induced with 11.1 μM apigenin. The fractions were assayed for the presence of 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) (circles, $A_{550}$), uronic acids (triangles, $A_{520}$), and hexoses (plus signs, $A_{620}$). The inset bar represents fractions that contained lipopolysaccharides (LPSs), as determined by polyacrylamide gel electrophoresis (PAGE). C, Sepharose 4B profile of rechromatographed 4B-2 pool from the noninduced bacterial extract (fractions 62–72 in A). The fractions were assayed for the presence of Kdo (circles, $A_{550}$) and uronic acids (triangles, $A_{520}$). D, PAGE gel showing fractions 40–68 (every fourth fraction) from the Sephadex G-150 separation of LPS I and LPS II (from the 4B-2 pool of LPS from the induced cells). This step also resulted in a partial separation of LPS IIA and LPS IIB, as seen in lanes 2 and 3 (fractions 44 and 48).

preparation.

The LPS pools from Sepharose 4B chromatography were subsequently chromatographed on Sephadex G-150 with 0.25% deoxycholic acid, which prevents LPS aggregation, included in the eluent. This resulted in the separation of the LPS from glucan III, as well as the different forms of LPS (i.e., LPS I and II). Figure 2D shows a polyacrylamide gel of column fractions from the Sephadex G-150 chromatography of LPS; the separation of LPS I and LPS II is evident in this figure. LPS IIA and LPS IIB were also partially separated by repeated Sephadex G-150 chromatography.

**Composition analysis.**

The results of composition analyses are presented in Table 1. LPS I from both induced and noninduced cultures contained the β-hydroxy fatty acids commonly associated with rhizobial LPS (Carlson et al. 1992), and LPS I fractions from both cultures were similar in glycosyl composition. In contrast, the major O antigen–containing LPS fractions showed a significant difference in glycosyl composition: The noninduced cells produced an O antigen moiety (LPS IIA) that was composed primarily of glucose, whereas the predominant O antigen moiety produced by the induced cells (LPS IIB) contained much higher levels of xylose and mannose. Although the β-hydroxy fatty acid content of the LPSs could not be quantified, because of a lack of authentic standards, a comparison of the integration values for the β-hydroxy fatty acids from LPS I and those from LPS II showed that the lipid A–core moiety (i.e., LPS I) accounted for ~20–30% of LPS II. If the relative percent compositions of LPS IIA and LPS IIB are adjusted to account for the glycosyl components that are specific to the core oligosaccharides (i.e., if the core oligosaccharide contribution to the LPS II composition is subtracted from the total), the difference in the O antigens is more evident: The O antigen of LPS IIA consisted of glucose and mannose in a molar ratio of ~8:1, whereas the O antigen of LPS IIB consisted of glucose, mannose, and xylose in a molar ratio of ~1:1:1.

In the analysis of the region III glucan, which was absent in the induced cell preparations (and therefore is not presented

in Table 1), only glucose was detected; neither the uronic acids nor the fatty acids that were components of the LPS core oligosaccharides and the lipid A region, respectively, were detected in that fraction. Although the PAGE banding pattern appears to be that of a higher molecular weight form of the LPS, the absence of even trace amounts of the LPS-specific components would suggest that this is not so.

The composition analysis of the 4B-1 pools supported the Sepharose 4B chromatography data; i.e., LPS-specific sugars and fatty acids were detected in the 4B-1 pool of the noninduced cell extract, but not in the 4B-1 pool of the induced cell extract. The compositions of the 4B-1 pools, however, were similar in other respects: both contained high levels of glucose and mannose, which were found to be the components of two different polysaccharides (data not shown).

Glucose was the only glycosyl component detected in the composition analysis of the peak 4B-4 pools, and there were no apparent differences between the induced and the noninduced preparations. The glucosyl linkage was determined by per-O-methylation, hydrolysis, reduction, and acetylation, followed by gas chromatography–mass spectrometry analysis. The electron-impact mass spectrum of the only glucitol peak was that of a 2-linked hexopyranosyl, i.e., 3,4,6-tri-O-methyl-hexitol acetate, identified by primary fragments at $m/z$ 161 and $m/z$ 190. Terminal glucose was not detected. This analysis identified this component as the cyclic 1,2-linked β-D-glucans that are commonly associated with rhizobia (Dell et al. 1983). Peak 4B-4 also contained high levels of phosphate, as determined by colorimetric analysis (data not shown), suggesting that these polysaccharides were substituted with glycerol phosphate, which is not unusual (Miller et al. 1988).

**HPAEC analyses.**

The LPS forms purified with Sephadex G-150 (i.e., LPS I, LPS IIA, and LPS IIB) were treated with dilute acid, which releases the LPS core from lipid A and O antigen, and the core oligosaccharides were analyzed by high-performance anion-exchange chromatography with a pulsed amperometric detector (HPAEC-PAD). Figure 3A shows the HPAEC chromatogram of the core oligosaccharides released from the LPS

**Table 1.** Percent sugar composition and relative fatty acid content of purified polysaccharides from noninduced and induced cell extracts of Rhizobium fredii USDA205

| Glycosyl component[a] | Noninduced cell extract | | | | Induced cell extract | | | |
| | LPS[b] | | 4B pool[c] | | LPS | | 4B pool | |
| | I | IIA | 4B-1 | 4B-4 | I | IIB | 4B-1 | 4B-4 |
|---|---|---|---|---|---|---|---|---|
| Kdo[d] | 21 | 9 | 2 | ... | 20 | 5 | ... | ... |
| Glucose | 39 | 68 | 74 | 100 | 41 | 39 | 79 | 100 |
| Mannose | 4 | 9 | 16 | ... | 2 | 21 | 21 | ... |
| Xylose | ... | 4 | ... | ... | ... | 20 | ... | ... |
| Galactose | 12 | 5 | 3 | ... | 10 | 10 | ... | ... |
| Galacturonic acid | 10 | 2 | 3 | ... | 12 | 2 | ... | ... |
| Glucuronic acid | 7 | 2 | 1 | ... | 7 | 2 | ... | ... |
| Glucosamine | 7 | 2 | 1 | ... | 8 | 1 | ... | ... |
| β-OH fatty acids[e] | +++ | + | + | ... | +++ | + | ... | ... |

[a] The compositions were determined by gas chromatography–mass spectrometry of the alditol acetate and trimethylsilyl methyl glycoside derivatives; retention times and response factors were established with identically treated authentic standards.
[b] Purified lipopolysaccharides LPS I and LPS II from the peak 4B-2 pools (Fig. 2A and B) rechromatographed on Sephadex G-150 (see Fig. 2D and text).
[c] Sepharose 4B pools from Figure 2A and B.
[d] Kdo = 3-deoxy-D-manno-octulosonic acid.
[e] The presence and relative abundance of the β-OH $C_{14:0}$, β-OH $C_{16:0}$, and β-OH $C_{18:0}$ fatty acids were determined by gas chromatography–mass spectrometry of the β-O-trimethylsilyl methyl ester derivatives.

I of the noninduced cell extract; HPAEC of the LPS I core oligosaccharides from the induced cell extracts resulted in an identical chromatogram (data not shown). The complexity of the chromatogram is a result of heterogeneity in carbohydrate composition and noncarbohydrate (e.g., phosphate) substitution, which has been verified by fast atom bombardment mass spectrometry of the hydrolysates (B. L. Reuhs, unpublished), and the acid-catalyzed conversion of some reducing-end Kdo residues of each oligosaccharide to various anhydrous forms (Carlson and Bhagyalakshmi 1992). The HPAEC chromatograms of the core oligosaccharides released from LPS IIA and LPS IIB are shown in Figure 3B and C. The similarity of these chromatograms to one another and to Figure 3A shows
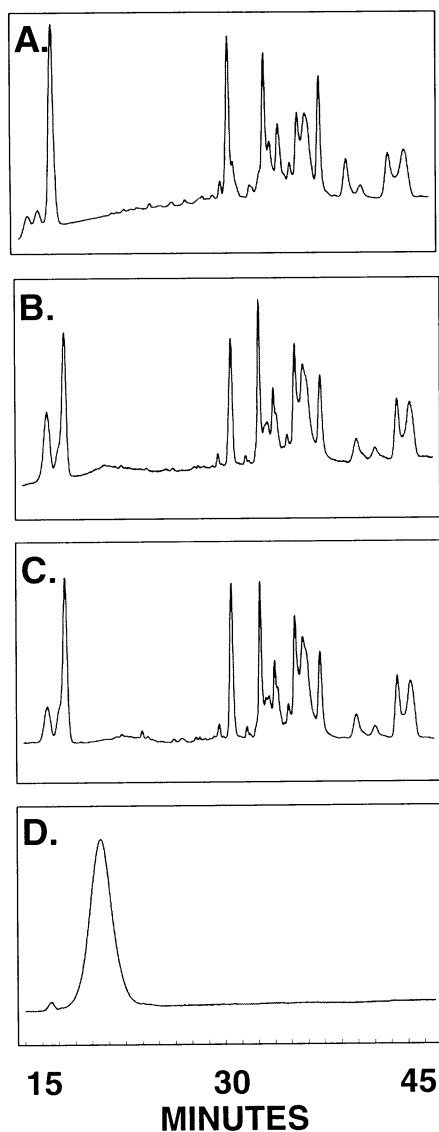


**Fig. 3. A,** High-performance anion-exchange chromatography (HPAEC) of the lipopolysaccharide (LPS) core oligosaccharides released from LPS I from noninduced *Rhizobium fredii* USDA205 purified by Sephadex G-150 chromatography. The core oligosaccharides were released from the lipid A moiety by pretreatment with dilute acid (see text). **B** and **C,** HPAEC of the oligosaccharides from LPS II purified by Sephadex G-150 chromatography: LPS IIA from noninduced cell extracts (**B**) and LPS IIB from induced cell extracts (**C**). **D,** HPAEC of identically treated glucan III. See text for details.

that LPS I and LPS II are, in fact, related molecules, and that the core regions of the LPS II components from the noninduced cell extracts did not differ significantly from those of the induced cell extracts. The O antigens, which consisted primarily of neutral glycosyl residues (discussed above), were not retained under these conditions and were eluted in the void volume. The components that were responsible for the PAGE bands between LPS I and LPS II (Fig. 1B) were also confirmed to be LPSs by this method (data not shown).

The glucan III component was treated and chromatographed under identical conditions. The HPAEC chromatogram of that fraction displayed none of the LPS core oligosaccharide peaks (Fig. 3D), which is further evidence that it is not a form of LPS.

The peak 4B-4 pools (Fig. 2A and B), which contained phosphate-substituted, cyclic 1,2-linked β-D-glucans, were analyzed by HPAEC, without the dilute acid pretreatment. The HPAEC analyses showed no differences between induced and noninduced cell extracts (data not shown), which supported the Sepharose 4B chromatography results.

**NMR analysis of the Kdo-rich polysaccharides.**

The Kdo-rich material from both noninduced and induced cell extracts consisted of two polysaccharides of similar nature; i.e., both polysaccharides found in the 4B-3 pools were K antigen analogues that comprised Kdo and hexose in a molar ratio of 1:1. One polysaccharide consisted of galactose-Kdo repeating units and the other of 2-O-methylmannose-Kdo repeating units (Reuhs et al. 1993). The peak 4B-3 polysaccharides from both induced and noninduced cell extracts accounted for ~25–30% (w/w) of the recovered material purified with Sepharose 4B; hence, the overall production of these polysaccharides appeared to remain constant. The peak 4B-3 pools were examined by $^1$H nuclear magnetic resonance (NMR) to determine what differences might account for the altered chromatographic elution of the polysaccharides from the induced cells (see Fig. 2). Figure 4 shows the $^1$H-NMR spectrum of the peak 4B-3 pool from the noninduced cell extract: The major resonance at chemical shift (δ) 5.05 (coupling constant $J = 3.93$ Hz) is from the anomeric proton of the galactosyl residues of the galactose-Kdo polysaccharide. The resonances of the Kdo methylene (H3$_a$ and H3$_e$) protons are found at δ 1.8 (H3$_a$) and δ 2.4 (H3$_e$). The resonance at δ 4.7 was identified as the anomeric resonance of the 2-O-methylmannosyl residues; the very small coupling constant ($J < 2.0$ Hz) associated with this resonance is indicative of mannose. The integration values for the anomeric protons showed that the ratio of galactose to 2-O-methylmannose was ~10:1. The inset in Figure 4 shows the anomeric region of the peak 4B-3 pool from the induced cell extract: The ratio of galactose to 2-O-methylmannose in this preparation was found to be ~3:1 (the Kdo methylene resonances from this spectrum were identical to those discussed above). This confirmed that a modulation in the production of these polysaccharides resulted from the inclusion of apigenin in the growth media.

## DISCUSSION

This report has presented evidence that the inclusion of apigenin, an inducer of *R. fredii nod* genes (Peters and Verma 1990) and other symbiosis-associated genes (Sadowsky et al.

1988), in the growth medium of cultured cells of *R. fredii* USDA205 results in a modulation of polysaccharide production. The cell extracts from induced cells displayed carbohydrate elution profiles clearly different from those of noninduced cells when chromatographed on Sepharose 4B (Fig. 2). PAGE analyses (Fig. 1) showed that the $M_r$ distribution of the LPS from induced cells was decreased, that the predominant form of O antigen–containing LPS II was different, and that LPS I was unaffected. These results indicate that apigenin modulates the production of the LPS O antigen. Similar results were obtained by the use of host plant (soybean) root extract, which has also been shown to induce *nod* genes and other symbiosis-related genes in *R. fredii* (Olson *et al.* 1985). Composition analysis showed that the LPS I components of induced and noninduced cells were virtually identical, whereas the LPS II fraction from the induced cells was enriched in xylose and mannose. HPAEC analysis confirmed that the observed differences were not in the core–lipid A portion of the LPS. It may be concluded that apigenin effects a modulation of LPS O antigen production, from a glucan to a xylomannan.

These results raised another question: Is this phenomenon unique? The possibility that *nod* gene inducers may have ancillary effects on the LPS production of other rhizobial species has been investigated: There were no apparent differences observed in PAGE studies of the LPS from cell cultures of *R. meliloti* and *Bradyrhizobium japonicum* that were grown in the presence of specific *nod* gene–inducing flavonoids (R. W. Carlson and G. Stacey, unpublished; B. L. Reuhs and G. Petrovics, unpublished). It is possible that LPS

production by some strains of these species, other than those tested, might be affected by *nod* gene inducers, or that there were differences too subtle to be detected by PAGE, or that different flavonoids might be required. It is also possible that *R. fredii* is unusual in this regard.

The LPS was not the only cell-associated polysaccharide affected by induction: PAGE showed that a series of low-mobility bands (glucan III) was absent in the induced cell extracts, and chromatography showed a difference in the elution the Kdo-rich polysaccharides, which are similar to *E. coli* K antigens (Jann and Jann 1990; Reuhs *et al.* 1993). Subsequent NMR studies showed that there was a much higher proportion of a 2-*O*-methylmannose-Kdo polysaccharide in the induced cell extract.

The production of 1,2-linked β-D-glucans and HMW neutral polysaccharides, composed of glucose and mannose, was not affected by apigenin. This suggests that the differences in LPS and glucan III were not simply pleiotropic effects, reflecting a modification of glucose metabolism.

The mechanism and biological significance of the observed modifications are unknown; however, two of the three components have been shown to be of symbiotic importance in some systems, i.e., the LPS (see the introduction) and the K antigen–like polysaccharides (Reuhs *et al.* 1993; Petrovics *et al.* 1993; Williams *et al.* 1990; B. L. Reuhs and M. N. V. Williams, unpublished). It is possible that the induced modifications may be a significant factor in the host selectivity shown by *R. fredii* strains (Balatti and Pueppke 1992a,b).

In light of a recent report by Vázquez *et al.* (1993), which describes a similarity between the group II K antigen export
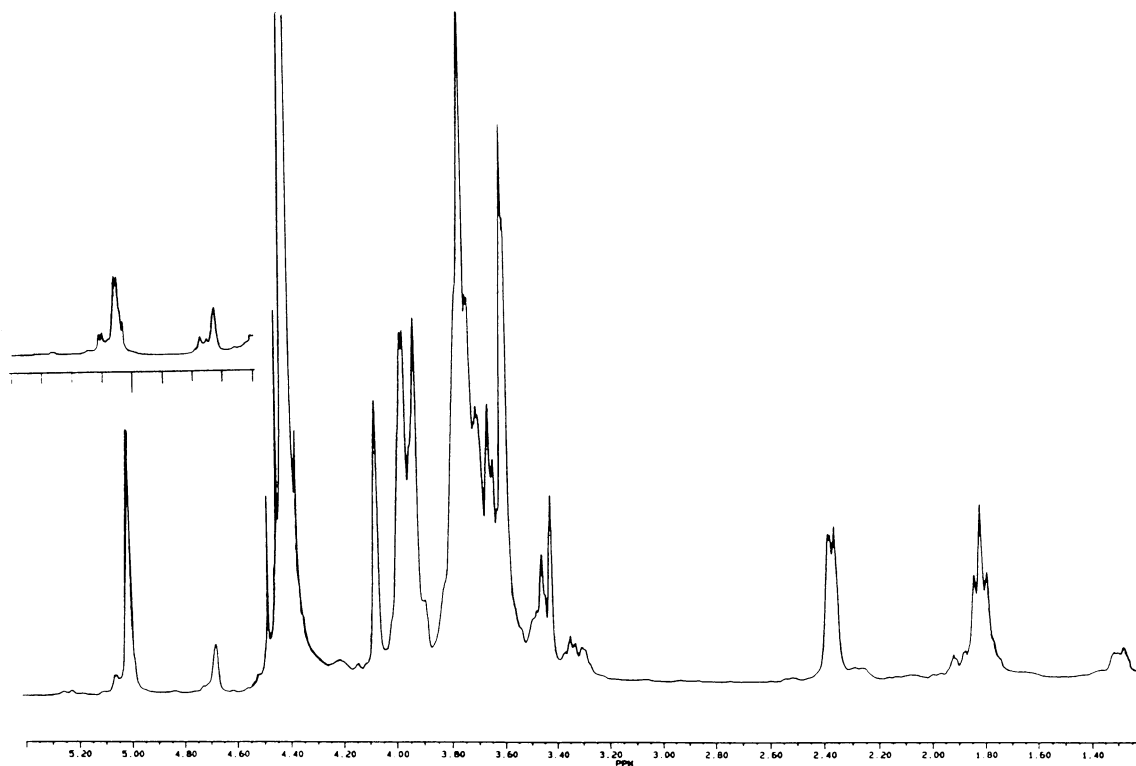


**Fig. 4.** ¹H-nuclear magnetic resonance (NMR) spectrum of the peak 4B-3 pool from noninduced cell extract of *Rhizobium fredii* USDA205 (Fig. 2A). The resonances at δ 5.05 and δ 4.7 are due to the anomeric protons of the galactosyl and 2-*O*-methylmannosyl residues, respectively. The ratio of galactose to 2-*O*-methylmannose, as determined by integration of the anomeric resonances, was ~10:1. The inset shows the anomeric region of the ¹H-NMR spectrum of the peak 4B-3 pool from the induced cell extract (Fig. 2B); the ratio of galactose to 2-*O*-methylmannose in this preparation was calculated to be ~3:1.

proteins (KpsT and KpsM) from *E. coli* and the inducible NodI and NodJ proteins from *Rhizobium*, it would be interesting to determine if the observed modulation in *R. fredii* polysaccharide production by apigenin was *nodD*-dependent. At this time, however, *R. fredii nodD1 nodD2* double mutants are not available.

## MATERIALS AND METHODS

### Root extract preparation.

Root extract was prepared by freezing and grinding the roots of Peking soybean plants grown (from seeds donated by Jack Paxton, University of Illinois, Urbana) in presterilized pearl white and nourished with a nitrogen-deficient nutrient solution. The ground root material was dialyzed against $H_2O$ in 6,000–8,000 molecular weight cut-off tubing, and the dialysate was collected, reduced in volume, and lyophilized.

### Organisms and growth conditions.

*R. fredii* USDA205 was obtained from Barry Rolfe (Australian National University, Canberra). *R. fredii* USDA201::Mu-d (KAN, *lac*) 11G2 (Nod⁻) was provided by Desh Pal S. Verma (Ohio State University, Columbus).

*R. fredii* USDA201::Mu-d (KAN, *lac*) 11G2 was tested for *nod* induction by growth in YEM broth ($K_2HPO_4$, 0.5 mg/ml; $MgSO_4\cdot7H_2O$, 0.2 mg/ml; NaCl, 0.1 mg/ml; mannitol, 5.0 mg/ml; gluconate, 5.0 mg/ml; yeast extract, 0.5 mg/ml) containing kanamycin (50 μg/L) and varied concentrations of 4′,5,7-trihydroxyflavone (apigenin; Sigma Chemical Co., St. Louis, MO) or root extract. Aliquots (0.5 ml) were removed at timed intervals and assayed for β-galactosidase activity (Miller 1972).

Based on the results of the induction assays, the induced *R. fredii* USDA205 cells were grown in YEM containing 3 μg of apigenin or 0.5 or 0.07 mg of root extract per milliliter. The optimal bacterial harvest point was established in studies of the induction kinetics; peak β-galactosidase activity occurred at $OD_{600}$ 0.85–0.95. Culture pH was assayed at inoculation and harvest. The pH of the induced cultures did not differ from that of the noninduced cultures at the time of inoculation or harvest. The cells were harvested by low-speed centrifugation. The bacterial pellets were washed with phosphate-buffered saline and immediately frozen.

### Polysaccharide extraction and purification.

The bacterial polysaccharides were extracted by the hot phenol-water extraction method (Westphal and Jann 1965), as modified by Carlson *et al.* (1978).

Initial chromatographic separations were performed on a Sepharose 4B (Sigma) gel filtration column (1 m × 3 cm) in a solution of 30 mM triethylamine and 10 mM EDTA, pH 7. The fractions were chemically assayed for the presence of Kdo, uronic acids, and hexoses (reviewed by York *et al.* [1985]) and assayed for the presence of LPS by PAGE (described below). A second chromatographic step was performed on a Sephadex G-150 superfine column (Pharmacia, Uppsala, Sweden), 1 m × 1.5 cm, in a deoxycholic acid buffer (0.2 M NaCl, 1 mM EDTA, 10 mM Tris base, and 0.25% deoxycholic acid, pH 9.25). The pooled fractions were dialyzed against 4 mM Tris base, 0.25% NaCl, and 10% ethanol (pH 9.1) for three changes; this was followed by exhaustive dialysis against $H_2O$. This step was essential for removal of the deoxycholic acid.

### HPAEC analysis of degraded polysaccharides.

HPAEC of oligosaccharides was performed on a Dionex Metal-free BioLC (Dionex Corp., Sunnyvale, CA) using a CarboPac PA1 anion-exchange column (4 × 250 mm) and a pulsed amperometric detector with a gold working electrode. The detector was operated at a sensitivity of 1,000 nA, with the following pulse potentials and durations for detection of carbohydrates: E1 = 0.05 V ($T_1$ = 500 ms), E2 = 0.60 V ($T_2$ = 80 ms, and E3 = −0.60 V ($T_3$ = 50 ms). The response time of the detector was 1 sec.

HPAEC eluents were prepared with ultra-pure water, carbonate-free liquid 50% NaOH, and $CH_3COONa\cdot3H_2O$ (Baker Analyzed HPLC Reagent), filtered (through 0.2-μm Nylon 66 membranes), and degassed with helium. Eluent A consisted of 100 mM NaOH, and eluent B consisted of 1M sodium acetate in 100 mM NaOH. The gradient used was produced by varying the concentration of eluent B as follows: 0 min, 10% B; 10 min, 10% B; 30 min, 50% B; 35 min, 50% B; 40 min, 60% B; 45 min, 100% B, 50 min, 100% B; 55 min, 10% B; 60 min, 10% B. The flow rate was 1 ml/min at ambient temperature.

LPS samples were prepared for HPAEC-PAD by mild acid hydrolysis (1% acetic acid at 100° C for 3–5 hr) followed by dilution in ultra-pure water and filtration through 0.2-μm Nylon-66 Microfilterfuge Tubes (Rainin Instrument Co., Woburn, MA).

### Polysaccharide analysis.

Sample composition was analyzed by gas chromatography (Hewlett-Packard 5890A GC) of the alditol acetate derivatives (York *et al.* 1985) using a 15-m Supelco SP2330 fused silica column, and of the trimethylsilyl methyl glycosides (York *et al.* 1985) using a 30-m J&W Scientific DB1 fused silica column.

¹H-NMR analyses were conducted on Bruker 250- or 500-MHz NMR spectrometers in ²H₂O at 314 K.

### PAGE.

PAGE was performed using 15% acrylamide and deoxycholic acid as a detergent (Krauss *et al.* 1988). Gels were developed by the silver-staining method of Tsai and Frisch (1982).

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