Chemical Characterization of pH-Dependent Structural Epitopes of Lipopolysaccharides from *Rhizobium leguminosarum* Biovar Phaseoli

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Lipopolysaccharide (LPS) was isolated from free-living *Rhizobium leguminosarum* bv. phaseoli CE3 cells grown at pH 4.8 (antigenically similar to bacteroid LPS) and compared with that from cells grown at pH 7.2 (free-living bacteria). Composition analysis revealed that pH 7.2 LPS differs from pH 4.8 LPS in that 2,3,4-tri-*O*-methylfucose is replaced by 2,3-di-*O*-methylfucose. The amount of 2-*O*-methylrhhamnose is greater in the pH 4.8 LPS than in the pH 7.2 LPS. Analysis of the structural components of LPS (O-chain polysaccharide, core oligosaccharides, and the lipid A) revealed that all the composition differences in the various LPSs occur in the O-chain polysaccharide. These structural variations between pH 4.8 and pH 7.2 LPSs provide a chemical basis for the observed lack of cross-reactivity with pH 4.8 LPS of two monoclonal antibodies, JIM28 and JIM29, raised against free-living bacteria grown at pH 7.2. An LPS preparation isolated from bacteroids contained both 2,3,4-tri-*O*- and 2,3-di-*O*-methylfucose residues. This result is consistent with the finding that the two monoclonal antibodies react weakly with bacteroid LPS. It is concluded that methylation changes occur on the LPS O-chain of *R. leguminosarum* bv. phaseoli when the bacteria are grown at low pH and during nodule development.

Rhizobia are gram-negative soil bacteria that form nitrogen-fixing symbiotic associations with leguminous plants. In terms of host selection, the bacteria exhibit a great deal of specificity. The surface carbohydrates of rhizobia, which include the lipopolysaccharide (LPS), extracellular polysaccharide, and capsular polysaccharide, have all been hypothesized to play a role in symbiosis (9, 11, 18, 20, 22, 23). The LPSs of *Rhizobium* species resemble their enterobacterial counterparts in having structurally distinct regions; the O-chain polysaccharide, the core oligosaccharide, and the hydrophobic lipid A (10, 13–17); however, there are many differences in the structural details of *Rhizobium* LPSs compared with enterobacterial LPSs (1–3, 12, 25–27).

Earlier reports on the role of LPSs in symbiosis were directed toward the possibility that LPSs were involved in the specific attachment of the symbiont to the host plant (8, 9, 30). Recent studies indicate involvement of LPSs at a later stage in the nodule development. Mutants which do not have the O-chain region either fail to form normal infection threads or are not released properly from the infection thread into the host root cells (7, 13, 19, 33, 34, 37).

Using monoclonal antibodies to *Rhizobium leguminosarum* bv. viciae bacteroids, it has been shown that there are changes in LPS epitopes which occur during differentiation of bacteria into bacteroids (6, 35, 39, 42). Some of these changes can be produced *ex planta*, by growing the bacteria at low pH or low O₂ tension, conditions which mimic, in part, the microenvironment of the root nodule (28). Similar results have been obtained for another strain of *R. leguminosarum* bv. viciae and for *R. leguminosarum* bv. phaseoli (21, 38). These epitope changes occur only in the complete LPS molecules with intact O-chain regions. Recently, mutants have been isolated which are unable to undergo these

epitope changes (5). Some of these mutants are defective in that the resulting nodules are ineffective (5).

With *R. leguminosarum* bv. phaseoli, the immunochemical studies were done by using monoclonal antibodies raised against the intact bacterial cells. The LPS epitopes for two of these monoclonal antibodies, JIM28 and JIM29, are greatly decreased during bacteroid development and disappear in the LPS from bacteria grown at low pH (4.8) (37a). There are no reports available on the structural characterization of such epitope changes that occur during bacteroid differentiation. The present investigation involves the chemical characterization of LPSs from *R. leguminosarum* bv. phaseoli CE3 cells grown at low pH and from bacteroids-bacteria extracted from the bean nodules.

MATERIALS AND METHODS

Bacterial strain and growth conditions. The strain used in the present study was *R. leguminosarum* bv. phaseoli CE3. Bacteria were grown at 30°C in the minimal glucose-glutamate medium in the presence of MES [2-(*N*-morpholino)ethanesulfonic acid] (37a). The bacteria were grown for 2 days in large flasks vigorously shaken on a gyratory shaker. Bacteria harvested from these cultures were washed in 1% (wt/vol) sodium chloride and then in water (14) before being lyophilized.

Isolation of LPS and its degraded products. LPSs were extracted from the freeze-dried bacterial cells by the hot-phenol-water method (40). The LPS was further purified by treatment with α -amylase, RNase, and proteinase K and then by gel filtration with Sepharose 4B with a triethylamine-EDTA buffer at pH 7.0 (13, 14).

Nodule bacteria were isolated from 1.8 g of frozen nodules as described by Tao et al. (37a). The washed bacteroid pellet was resuspended in water and subjected to hot-phenol-water extraction as described above. The aqueous phase was

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dialyzed, lyophilized, resuspended in Tris buffer for RNase A treatment (14), and extracted again with phenol. The LPS was found in both the water and phenol phases, with the majority in the phenol phase. Since the material from the phenol phase was much less contaminated with other polysaccharides, its analysis is reported here.

The LPS was degraded with 1% acetic acid at 100°C for 2 h. The precipitated lipid A was isolated as a residue after brief centrifugation and repeated washing with warm water and finally with acetone. The supernatant was lyophilized and fractionated on a Bio-Gel P-2 column equilibrated and eluted with 1% acetic acid in deionized water (12). This procedure separates the O-chain polysaccharide from the core oligosaccharides (12).

Polyacrylamide gel electrophoresis (PAGE). Discontinuous slab gel electrophoresis was performed with deoxycholate as the detergent (29). The separating gel and the stacking gel contained 13 and 5% acrylamide, respectively. The gels were visualized by the silver staining procedure as described by Hitchcock and Brown (24).

NMR analysis. ^1H nuclear magnetic resonance (NMR) spectra of the poly- and oligosaccharides were recorded in D_2O with a Bruker AM500 spectrometer. The samples were exchanged several times with D_2O , and the spectrum was recorded at 300 K with trimethylsilylpropionate as the internal standard.

Composition analysis. Total fatty acids were identified and quantified by transesterification, using acid-catalyzed methanolysis (41). The hydroxy fatty acid methyl esters were further derivatized either by trifluoroacetic anhydride (36) or by converting to their trimethylsilyl (TMS) ethers (31). Gas-liquid chromatographic analysis of fatty acid methyl esters was done on a Hewlett-Packard 5890-A gas chromatograph (GC) equipped with a 30-m DB-1 fused silica column (J & W Scientific) and a flame ionization detector. A temperature program of 80°C for 2 min and then 8°C/min to 290°C and holding for 15 min was used.

Neutral sugar compositions of the samples were determined by GC of the alditol acetate derivatives following 2 M trifluoroacetic acid (TFA) hydrolysis at 121°C for 1 h (43). Galacturonic acid in lipid A samples was identified by mild methanolysis in methanolic 1 M HCl (70°C for 3 h) and then reduction with sodium borodeuteride, acid hydrolysis (2 M TFA, 121°C, 2 h), alditol acetate derivatization, and GC-mass spectrometry (MS) analysis. Uronic acid and amino sugars were also characterized by the preparation and GC-MS analysis of TMS methylglycosides as previously described (43). Amino sugars were N-acetylated after methanolysis and prior to trimethylsilylation (43). GC-MS was done by using either a DB-1 (for TMS methylglycosides) or an SP-2330 (for alditol acetates) column on a Hewlett-Packard 5985 GC-MS system with an ionizing voltage of 70 eV. Helium was used as the carrier gas. Colorimetric quantization of uronic acid was done by the method of Blumenkrantz and Asboe-Hansen (4).

Glycosyl linkage analysis. The glycosyl linkages of the O-chain and the core oligosaccharides were determined by the Hakomori methylation procedure according to York et al. (43). The methylated products were purified on Sep-Pak C_{18} cartridges and hydrolyzed with 2 M TFA, 121°C for 2 h, before converting to alditol acetate derivatives. The partially methylated alditol acetate derivatives were separated and identified by GC-MS with a 30-m SP 2330 column (Supelco).

Fast atom bombardment-MS. Fast atom bombardment-MS was performed on a VG ZAB-SE instrument (VG Analytical, Manchester, United Kingdom) operated at 10 kV in the

FIG. 1. Deoxycholate-PAGE of the LPS from *R. leguminosarum* biovar phaseoli CE3 grown at pH 4.8 (lane 1) and pH 7.2 (lane 2). Approximately 15 to 20 μg of LPS was added to each lane.

negative mode. Thioglycerol was used as the fast atom bombardment matrix along with trace amounts of 0.1 M HCl.

RESULTS

Deoxycholate-PAGE analysis of the LPS. Figure 1 shows the PAGE banding pattern of LPSs from cells grown at pH 4.8 and 7.2. There are two banding regions; the region with slower mobility (includes the three major upper bands) is from the complete LPS molecule with O-chains and is termed LPS I, while the second region with faster mobility (includes the two major lower bands) is from the incomplete LPS without O-chains and is termed LPS II. The only difference between the pH 4.8 and pH 7.2 banding patterns is in the migration behavior of LPS I. The pH 7.2 LPS I has a slightly faster mobility compared with that of the pH 4.8 LPS I. No mobility differences are observed for LPS II bands.

The LPS isolated from the root nodules shows a banding pattern similar to the pH 7.2 LPS (data not shown). This LPS, when isolated from the aqueous phase by phenol-water extraction, largely partitions into the phenol phase when reextracted with phenol. Similar results are also obtained with LPS isolated from free-living bacteria. The LPS found in the water layer, after reextraction with phenol, has the same monoclonal antibody-binding properties as that found in the phenol layer (data not shown). Further purification of the root nodule LPS was not possible because of the small amounts isolated.

Chemical analysis of the LPSs. Sugar composition profiles of LPSs purified from bacteria grown at pH 7.2 and pH 4.8 and from nodules are given in Table 1. The LPSs are largely composed of 3-O-methylrhamnose, fucose, and uronic acids. In addition, the LPSs also contain mannose, galactose, 2-amino-2,6-dideoxyhexose, and 3-deoxy-D-manno-2-octulosonic acid. The LPS compositions are similar to that previously reported for CE3 LPS (13), except for the presence of 2,3,4-tri-O-methylfucose and 2-O-methylrhamnose in the pH 7.2 LPS. These methylated fucosyl and rhamnosyl residues were identified by their mass spectra and by comparing their retention times with those of authentic standards.

The major difference in the sugar compositions between

TABLE 1. Monosaccharide composition in mole ratios of LPSs from *R. leguminosarum* bv. phaseoli CE3 isolated from nodules and from free-living cells grown at pH 4.8 and 7.2^a

Monosaccharide	pH 7.2	pH 4.8	Nodule bacteria-bacteroids
2,3,4-Tri- <i>O</i> -methylfucose	4.1		2.4
2,3-Di- <i>O</i> -methylfucose		3.3	2.7
2- <i>O</i> -Methylrhamnose	4.4	6.9	6.6
3- <i>O</i> -Methylrhamnose	28	25	21
Fucose	16	10	9.5
Mannose	7.2	9.4	8.5
Galactose	3.7	7.1	5.5
Uronic acid ^b	25	25	28
2-Amino-2,6-dideoxyhexose	3.3	2.8	4.5
3-Deoxy-D-manno-2-octulosonic acid	8.5	11	9.9

^a The values account for 52 to 60% of mass in LPS preparations.^b Represents both glucuronic acid and galacturonic acid.

pH 4.8 and pH 7.2 LPSs is the absence of 2,3,4-tri-*O*-methylfucose in pH 4.8 LPS and the concomitant presence of 2,3-di-*O*-methylfucose. The LPS from bean nodules contains both 2,3,4-tri-*O*- and 2,3-di-*O*-methylfucose residues in an approximately 1:1 molar ratio. The nodule LPS and pH 4.8 LPS both contain larger amounts of 2-*O*-methylrhamnose and smaller amounts of fucose compared with the pH 7.2 LPS. There are also some small variations in the amounts of mannose, galactose, and uronic acids among the different LPSs.

Lipid A was liberated from pH 4.8 and 7.2 LPSs by mild acetic acid hydrolysis. We did not have large amounts of the nodule LPS; hence, such a study could not be done on that preparation. The degraded polysaccharide was loaded onto a Bio-Gel P-2 column, equilibrated, and eluted with 1% acetic acid in distilled water. For both pH 4.8 and pH 7.2 polysaccharides, a high-molecular-weight fraction and two low-molecular-weight fractions were obtained. The high-molecular-weight fraction is the O-chain and the two low-molecular-weight fractions are the core oligosaccharides. These results are similar to those previously reported (12).

The results of composition analysis of the O-chains from pH 4.8 and pH 7.2 LPSs are given in Table 2. The major difference between the O-chains from pH 4.8 and pH 7.2 LPSs is the presence of 2,3-di-*O*-methylfucose in pH 4.8 O-chain, which is replaced by 2,3,4-tri-*O*-methylfucose in

TABLE 2. Monosaccharide composition in mole ratios of O-chain fractions isolated from *R. leguminosarum* bv. phaseoli CE3 LPSs at pH 4.8 and pH 7.2^a

Monosaccharides	pH 7.2	pH 4.8
2,3,4-Tri- <i>O</i> -methylfucose	4.8	
2,3-Di- <i>O</i> -methylfucose		4.6
2- <i>O</i> -Methylrhamnose	4.5	9.8
3- <i>O</i> -Methylrhamnose	36	40
Fucose	18	14
Mannose	5.0	2.9
Glucuronic acid	25	23
2-Amino-2,6-dideoxy hexose	4.5	2.7
3-Deoxy-D-manno-2-octulosonic acid	2.9	2.5

^a The values account for approximately 95% of mass in O-chain fractions.

the pH 7.2 O-chain. In addition to this major difference, the pH 4.8 O-chain contains significantly larger amounts of 2-*O*-methylrhamnose. These results show that the methylated glycosyl residues affected by growth at low pH or in the nodule are part of the O-chain polysaccharide of the LPS.

Glycosyl linkage analysis of both of these O-chains shows that the methylated rhamnosyl and fucosyl residues are all terminally linked. This was determined by methylation analysis with trideuteromethyl iodide. For example, GC-MS analysis of the partially trideuteromethylated alditol acetate of 3-*O*-methylrhamnose (Fig. 2) shows that it contains trideuteromethyl groups at C-2 and C-4, indicating that this glycosyl residue is terminally linked in the polysaccharide. The terminal linkages of the other methylated fucosyl and rhamnosyl residues were also determined by trideuteromethylation and GC-MS analysis (data not shown).

There are no observable differences in the ¹H NMR spectra between the pH 4.8 and 7.2 O-chain polysaccharides. The spectrum for the pH 7.2 O-chain is shown in Fig. 3. The signals at about δ 1.25 are due to the methyl protons of the various rhamnosyl, fucosyl and 6-deoxyamino sugar residues. The signals at δ 2.1 and 2.0 are due to the methyl protons of *O*- and *N*-acetyl groups, respectively, and show that the O-chain is extensively acetylated. The intense signal at about δ 3.4 is due to the methoxyl protons of the methylated glycosyl residues. The anomeric region of this spectrum is complex because of the size heterogeneity and O-acetylation of this polysaccharide. The structure of the CE3 O-chain is under further investigation.

FIG. 2. MS of the partially trideuteromethylated alditol acetate of the terminally linked 3-*O*-methylrhamnosyl residue found in the O-chain polysaccharide of *R. leguminosarum* biovar phaseoli CE3 LPS. The $m/z = 118$ ion is due to loss of acetic acid (-60) from the $m/z = 178$ ion. Ac, acetyl.

FIG. 3. Proton NMR spectrum of the O-chain polysaccharide from *R. leguminosarum* biovar phaseoli CE3 LPS.

The two low-molecular-weight core oligosaccharides were characterized by composition, methylation, fast atom bombardment-MS, and NMR analyses. The data obtained (not shown) show that the pH 4.8 LPS oligosaccharides are identical in structure to those from pH 7.2 LPS and to those already published for the core oligosaccharides from strain CE3 (1, 12).

The lipid A fractions, obtained as precipitates during mild acid hydrolysis, were also analyzed. Lipid A from nodule bacteria LPS could not be isolated because of the small amount of material available. Lipid A preparations were methanolized and derivatized by trifluoroacetylation or trimethylsilylation. Galacturonic acid and glucosamine are the only detectable sugar components of both lipid A preparations. These two sugars are present in equimolar proportions. The identity in sugar composition of the pH 4.8 and pH 7.2 lipid A preparations is reflected in the GC profiles of their TMS methyl glycosides shown in Fig. 4. The presence of galacturonic acid in lipid A from *R. leguminosarum* biovars has been reported recently (3). The fatty acid compositions are given in Table 3. Very little difference in fatty acid profiles was observed in lipid A's from pH 4.8 and 7.2 LPSs. Only hydroxy fatty acids are found in both lipid A's. 3-Hydroxytetradecanoic acid and 27-hydroxyoctacosanoic acid are the major fatty acids present, along with smaller amounts of 3-hydroxyhexadecanoic and 3-hydroxyoctadecanoic acids. In addition, the lipid A's also contain trace amounts of 3-hydroxy C-15:0. The structure of CE3 lipid A is under further investigation.

DISCUSSION

In this report, it has been shown that LPSs from bacteria grown at pH 4.8 and from bacteria-bacteroids isolated from the bean nodule are altered in structure compared with the LPS of bacteria grown at pH 7.2. The major change in the LPSs from pH 4.8-grown bacteria and from nodule bacteria-bacteroids occurs in the O-chain polysaccharide. This change consists of the disappearance of 2,3,4-tri-*O*-methylfucose and the appearance of terminal 2,3-di-*O*-methylfucose in the pH 4.8 LPS and the presence of both fucosyl residues in the LPS isolated from nodule bacteria-bacteroids. The tri- and di-*O*-methylated fucosyl residues were not reported in our previous communication (13) since the

FIG. 4. GC profiles of the TMS methylglycosides derived from the lipid A of *R. leguminosarum* biovar phaseoli CE3 grown at pH 4.8 (top) and pH 7.2 (bottom). Multiple peaks are often present because of the formation of α - and β -anomers and, in some cases, both pyranose and furanose conformations. GalA, galacturonic acid; GlcN, glucosamine.

temperature program used for GC analysis (190 to 240°C at 10°C/min on a 15-m SP 2330 capillary column from Supelco) resulted in the elution of the alditol acetates of these residues close to the solvent front. The present temperature program (150 to 240°C at 4°C/min) allows the separation and identification of all methylated rhamnosyl and fucosyl derivatives. The presence of both tri- and di-*O*-methylfucosyl residues from the nodule LPS preparation is consistent with the fact that the nodule most likely contains a mixture of bacteria and bacteroids and with the fact that the binding of JIM28 and JIM29 to the nodule preparation is greatly decreased but not absent (37a).

The difference in mobility between pH 4.8 and pH 7.2 LPSs when examined by PAGE is probably not related to the differences in the methylated fucosyl and rhamnosyl

TABLE 3. Fatty acid composition of lipid A from LPSs of *R. leguminosarum* bv. phaseoli grown at pH 4.8 and pH 7.2^a

Component	pH 4.8	pH 7.2
3-OH-14:0	36	37
3-OH-15:0	2.7	2.1
3-OH-16:0	15	14
3-OH-18:0	12	16
27-OH-28:0	36	32

^a Fatty acid components are expressed as relative percentages calculated from the areas of the GC peaks. If all the fatty acid components have the same response factors (hydroxyl groups being derivatized by trifluoroacetylation), then the components listed above account for approximately 76% of lipid A masses.

residues or to differences in monoclonal antibody-binding properties since the LPS from heat-shocked bacteria also loses its ability to bind to JIM28 and, partially, JIM29 monoclonal antibodies but is unaltered in its mobility as determined by PAGE (37a). It was also shown (37a) that LPS from bacteria grown in 25 μ M phosphate loses its ability to bind both JIM28 and JIM29 but is not altered in its PAGE banding pattern.

At present, we cannot state whether the tri-*O*-methylfucosyl residue is required for the binding of both or just one of the JIM28 and JIM29 monoclonal antibodies. Since there are other changes, e.g., the increase in the 2-*O*-methylrhamnose/3-*O*-methylrhamnose ratio, it is possible that a second structural alteration occurs at pH 4.8 growth and during bacteroid formation. Thus, the tri-*O*-methylfucose could be part of the epitope for one of the two monoclonal antibodies, and a second epitope, e.g., involving 2- and/or 3-*O*-methylrhamnose, could be required to bind the other monoclonal antibody. It has recently been suggested that the tri-*O*-methylfucose residue is a binding site for biovar phaseoli CE3 strain-specific antibodies since biovar trifolii and biovar viciae transconjugates that have LPSs with CE3 O-chain sugars but lack tri-*O*-methylfucose only very weakly bind antibodies against strain CE3 (7). In any case, the data show that there are methylation changes in the O-chain during growth at low pH or within the nodule and that these changes correlate with the loss of cross-reactivity with JIM28 and JIM29 monoclonal antibodies.

The role(s) that these LPS epitope changes have in the functioning of the bacteroid is not known. It is possible that such subtle structural changes are required for proper transport of metabolites into and out of the bacteroid. It is also possible that these structural changes are important in providing increased resistance of the bacteroid to a plant defense molecule(s). Recently, it has been shown that growing *Bradyrhizobium japonicum* under conditions which induce *nod* genes confers increased resistance to the soybean phytoalexin, glyceollin (32).

Presently, Noel and coworkers (31a) are developing mutants which are unable to undergo these LPS changes to determine whether these changes are important for nitrogen fixation. The exact chemical nature of these changes and the identification of the genes and gene products responsible for these changes depend on the structural elucidation of the O-chain polysaccharide. This work is in progress.

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