

## Isolation and Characterization of the Lipopolysaccharides from *Bradyrhizobium japonicum*

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The lipopolysaccharide (LPS) of *Bradyrhizobium japonicum* 61A123 was isolated and partially characterized. Phenol-water extraction of strain 61A123 yielded LPS exclusively in the phenol phase. The water phase contained low-molecular-weight glucans and extracellular or capsular polysaccharides. The LPSs from *B. japonicum* 61A76, 61A135, and 61A101C were also extracted exclusively into the phenol phase. The LPSs from strain USDA 110 and its Nod<sup>-</sup> mutant HS123 were found in both the phenol and water phases. The LPS from strain 61A123 was further characterized by polyacrylamide gel electrophoresis, composition analysis, and <sup>1</sup>H and <sup>13</sup>C nuclear magnetic resonance spectroscopy. Analysis of the LPS by polyacrylamide gel electrophoresis showed that it was present in both high- and low-molecular-weight forms (LPS I and LPS II, respectively). Composition analysis was also performed on the isolated lipid A and polysaccharide portions of the LPS, which were purified by mild acid hydrolysis and gel filtration chromatography. The major components of the polysaccharide portion were fucose, fucosamine, glucose, and mannose. The intact LPS had small amounts of 2-keto-3-deoxyoctulosonic acid. Other minor components were quinovosamine, glucosamine, 4-O-methylmannose, heptose, and 2,3-diamino-2,3-dideoxyhexose. The lipid A portion of the LPS contained 2,3-diamino-2,3-dideoxyhexose as the only sugar component. The major fatty acids were β-hydroxymyristic, lauric, and oleic acids. A long-chain fatty acid, 27-hydroxyoctacosanoic acid, was also present in this lipid A. Separation and analysis of LPS I and LPS II indicated that glucose, mannose, 4-O-methylmannose, and small amounts of 2,3-diamino-2,3-dideoxyhexose and heptose were components of the core region of the LPS, whereas fucose, fucosamine, mannose, and small amounts of quinovosamine and glucosamine were components of the LPS O-chain region.

Bacteria of the genera *Rhizobium* and *Bradyrhizobium* form important nitrogen-fixing systems through symbiotic infection of various legumes. Previous investigations suggest that bacterial polysaccharides, including extracellular polysaccharides (EPSs), capsular polysaccharides, and lipopolysaccharides (LPSs), play an important role in the symbiotic infection process (2, 7, 16).

*Rhizobium* LPSs have been reported to bind specifically to the host root. Recently, specific binding of *Rhizobium meliloti* to alfalfa roots was reported to occur at low symbiont concentrations (6). This specific binding was inhibited by *R. meliloti* LPS preparations (24). Lectins from the host plant have also been implicated in binding to the LPSs from *Rhizobium* symbionts (see references 2, 7, and 15 for reviews).

In addition to the binding studies, it has been shown that alterations in the LPS structure result in symbiotic defects and also that there are alterations in the LPS from bacteroids as compared with the LPS from bacteria. An intact LPS is required to form an effective nodule. *Rhizobium leguminosarum* biovar *phaseoli*, *trifolii*, and *viciae* mutants, which have defective LPSs that lack the O-chain polysaccharide, are defective in nodulation (5, 10, 14, 30, 32). These mutations result in aborted infection threads (30) or in nodules that prematurely senesce (32). It has also been reported that a *B. japonicum* mutant that lacks the LPS O chain does not form nodules (33). Thus, an intact LPS is important to form an effective nodule. In other recent reports (20, 36, 42), monoclonal antibodies have been generated that are specific to the LPS found in the nodule bacteroids and that do not

interact with another type of LPS found in the bacteria. Thus, there are subtle changes that occur in the LPS when bacteria differentiate to bacteroids.

Structurally, LPSs are complex molecules that can vary significantly, even among strains of a single species (7, 11, 46). *Rhizobium* LPSs are similar to the LPSs of other gram-negative bacteria in that they usually contain a lipid A, a core oligosaccharide(s), and a polysaccharide known as the O chain (7, 8, 13). However, the specific structure of the lipid A and the type of core oligosaccharides present are very different from those of enteric LPSs. Unlike the glucosamine disaccharide backbone of most enteric lipid A's, the *R. leguminosarum* lipid A's consist largely of a galacturonic acid-glucosamine disaccharide and have 27-hydroxyoctacosanoic acid as a major fatty acyl residue in addition to the usual 3-hydroxymyristic acid found in most lipid A's (17; Bhat and Carlson, manuscript in preparation). Recently the structures of two core oligosaccharides released by mild acid hydrolysis of the LPSs from *R. leguminosarum* strains were determined (8a, 9, 18) One is a trisaccharide component consisting of two galacturonosyl residues and a 3-deoxy-D-manno-2-octulosonic acid (KDO) residue (8a). The other is a tetrasaccharide consisting of galactose, galacturonic acid, mannose, and KDO (18). The LPSs from *R. leguminosarum* are very different from *Bradyrhizobium* LPSs, since the latter have been shown to contain 2,3-diamino-2,3-dideoxyglucose (2,3-DAG) in their lipid A's (27).

In this report we describe the isolation and characterization of the LPS from *Bradyrhizobium japonicum* 61A123. Furthermore, we show that, in general, the LPSs from *B. japonicum* strains are extracted into the phenol layer during phenol-water extraction.

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## MATERIALS AND METHODS

**Organisms and growth conditions.** Strains 61A123 and 61A76 were obtained from Wolfgang D. Bauer of the Agronomy Department at Ohio State University. Strains 61A101C and HS123 were obtained from Gary Stacey of the Microbiology Department at the University of Tennessee. Strain USDA 110 was obtained from Harold Keyser of the U.S. Department of Agriculture. The bacteria were maintained on solid yeast extract-mannitol-gluconate medium and stored at 4°C.

Bacteria were grown in a medium developed by R. E. Tulley (35). This medium was used because it inhibits EPS production, which interferes with LPS isolation. Bacteria were grown at 28°C to the midlog phase and harvested by centrifugation. The cell pellets were frozen until ready to use.

**Isolation of LPS and its degraded products.** LPSs were extracted from the bacterial pellets by the phenol-water method (39) as modified by Johnson and Perry (19). Both the phenol and water layers were dialyzed against deionized water, digested with proteinase K (100 µg/ml) at 37°C for 2 h, dialyzed again, and lyophilized. The material from the phenol layer was suspended in water by using a blender and centrifuged at about 12,000 × *g* for 15 min. The supernatant was decanted and saved. This procedure was repeated on the pellet a total of three times. The supernatants were combined and lyophilized. Further purification was accomplished by gel filtration column chromatography with a 112-by 1.0-cm column of Sepharose 4B in phosphate-buffered saline-Tween buffer at pH 7.4. Fractions were collected and assayed for their ability to inhibit an enzyme-linked immunosorbent assay (ELISA) (see below) and for hexose by using the anthrone assay.

The complete and incomplete (i.e., without the O chain) forms of the LPS were separated by gel filtration chromatography (Sephadex G150) with deoxycholic acid as previously described (31).

The LPS was degraded with 1% acetic acid at 100°C for 5 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 later fractionated on a Sephadex G-50 column equilibrated with deionized water.

**Immunochemical techniques.** Polyclonal antisera to strain 61A123 was prepared by injecting saline-washed 61A123 bacteria into the marginal ear vein of female New Zealand White rabbits. ELISAs, ELISA inhibition assays, and immunoblots of polyacrylamide gels were performed as previously described (12). In the ELISA inhibition procedure the fraction to be tested was preincubated with polyclonal 61A123 antiserum to see whether the amount of antiserum that bound to the bacterial antigen was reduced.

**Composition analysis.** The hexose compositions of the samples were determined by gas chromatography (GC) of the alditol acetate derivatives (44) on a Hewlett-Packard 5890A gas chromatograph equipped with a 15-m SP2330 fused silica column (Supelco) and a flame ionization detector. Amino sugars were quantitated by hydrolysis in 4 N HCl at 100°C for 16 h, N acetylation, preparation of the alditol acetates (44), and GC analysis with a 30-m DB1 fused silica column (J&W Scientific). Where possible, identification and quantitation were made by comparison to authentic stan-

dards, with inositol as the internal standard. Alditol acetate derivatives of the glycosyl residues were also identified by GC-mass spectrometry by using either the SP2330 or DB1 column and a Hewlett-Packard GC-mass spectrometry system. Fatty acids were identified by GC-mass spectrometry (with the DB1 column) of their methyl esters prepared by acid-catalyzed methanolysis (38).

After hydrolysis with 1 M HCl at 100°C for 30 min, KDO was determined by the thiobarbituric acid method (37). Uronic acid was quantitated by the method of Blumenkrantz and Asboe-Hansen (3).

**Polyacrylamide gel electrophoresis (PAGE).** Discontinuous slab gel electrophoresis was performed with sodium dodecyl sulfate (23) or deoxycholate (21). The separating gel and stacking gel contained 15 and 5% acrylamide, respectively. The gels were silver stained by the method of Hitchcock and Brown (16).

**NMR analysis.** <sup>1</sup>H and <sup>13</sup>C nuclear magnetic resonance (NMR) spectra of the polysaccharide were recorded in D<sub>2</sub>O with a Bruker AM500 spectrometer. About 12 mg of the sample was exchanged with D<sub>2</sub>O several times and then dissolved in D<sub>2</sub>O, and the spectrum was recorded at 300 K with trimethylsilylpropionate as the internal standard.

## RESULTS

**Purification of the LPS.** The material extracted into the water layer from phenol-water extraction of strain 61A123 did not contain any detectable KDO, nor did it inhibit the interaction between 61A123 antiserum and the bacterial antigen as determined by ELISA inhibition. Glycosyl composition analyses of the water layer material and of crude EPS showed that they both consisted of mannose-galactose-glucose-uronic acid (1:1:2:2). When the bacteria were grown on medium that repressed EPS production, the material in the water layer consisted largely of glucose. However, the material extracted into the phenol phase and further purified by dialysis, treatment with proteinase K, and centrifuged to remove insoluble debris did contain small amounts of KDO and gave 90% ELISA inhibition at a concentration of 250 µg/ml. Sepharose 4B chromatography of this KDO-positive material resulted in two hexose peaks eluting near the excluded volume of the column. Analysis by PAGE of the material in these peaks resulted in identical banding patterns for both peaks (Fig. 1A). Glycosyl composition (discussed further below) analysis gave similar results for both peaks, except that the first peak contained excess amounts of galactose and glucose, indicating that the material in this peak may be a mixture of some EPS and LPS. Thus, all further analyses were done on the second of the two peaks.

**Chemical characterization of the LPS.** The glycosyl and fatty acyl composition of the LPS is given in Table 1. The major glycosyl components were fucose, mannose, glucose, fucosamine, and 2,3-diamino-2,3-dideoxyhexose (2,3-DAH). Minor components were quinovosamine, glucosamine, 4-O-methylmannose, heptose, and KDO. Both fucose and fucosamine have been reported to be components of the LPS from *B. japonicum* USDA 110 (33), and 2,3-DAH is a component of the lipid A region of the LPS from strain USDA 110 (27). 2,3-DAH was identified by its mass spectrum, since an authentic standard was not available. This mass spectrum showed characteristic primary fragments at *m/z* 288, 215, and 144 together with a number of secondary fragments observed in published reports of 2,3-DAG (43). KDO, a characteristic glycosyl component of LPSs, was present in small amounts (less than 1%). A similar result was

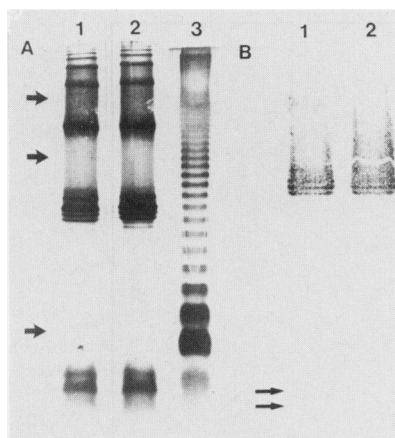


FIG. 1. Lanes 1 and 2 (in both A and B) contain about 20  $\mu\text{g}$  each of the two LPS-containing peaks (the first and second peaks, respectively) from the Sepharose 4B column. Lane 3 contains 15  $\mu\text{g}$  of *Salmonella minnesota* LPS as a control. (B) Immunoblot stained with polyclonal antiserum to strain 61A123. The arrows from top to bottom in panel A show the mobilities of bovine serum albumin, ovalbumin, and lysozyme, respectively. The arrows in panel B show the location of LPS II, which is observed if the gel is silver stained.

also reported for the LPS from strain USDA 110 (33). The fatty acid components were identified as lauric, 3-hydroxy-lauric, myristic, 3-hydroxymyristic, palmitic, and stearic acids. An unsaturated  $\text{C}_{18:1}$  fatty acid was also present. The 3-hydroxy fatty acid methyl esters were identified by characteristic mass fragments of  $m/z$  M-18, M-50, 74, and 103 (M is the molecular ion). The unsaturated fatty acid showed

TABLE 1. Composition of *B. japonicum* 61A123 LPS and its derivatives, LPS I and LPS II<sup>a</sup>

Glycosyl component	% Polysaccharide			
	Intact LPS	From 1% acetic acid hydrolysis of:		
		LPS	LPS I	LPS II
Fucose	44	48	57	12
4-O-Methylmannose	2.8	2.2	2.2	12
Mannose	12	8.5	10	38
Glucose	16	16	4.8	34
Fucosamine	17	16	19	+
2,3-DAH	8.3	6.5	NF	+
Quinovosamine	Tr	Tr	Tr	NF
Glucosamine	Tr	Tr	Tr	NF
Heptose	Tr	Tr	Tr	Tr
KDO	Tr	Tr	ND	ND
Uronic acid	Tr	Tr	ND	ND
Fatty acids				
$\text{C}_{12:0}$	12			
3-OH- $\text{C}_{12:0}$	9.5			
$\text{C}_{14:0}$	10			
3-OH- $\text{C}_{14:0}$	13			
$\text{C}_{16:0}$	18			
$\text{C}_{18:1}$	29			
$\text{C}_{18:0}$	7.9			
27-OH- $\text{C}_{28:0}$	Tr			

<sup>a</sup> The glycosyl components are given as relative percentages, and the fatty acyl components as relative area (from the GC profile) percent. The glycosyl components account for 30% of the LPS mass. The quantities of all the amino sugars were corrected by using the GC response factor for glucosamine. ND, Not determined; NF, not found; Tr, present at less than 1% of the mass; +, present but not quantitated.

fragments at  $m/z$  71 and 74 with that at  $m/z$  71 of greater intensity, and fragments at  $m/z$  M<sup>+</sup>, M-18, and M-50. A minor component also present was 27-hydroxyoctacosanoic acid. Phosphate was not detected in this LPS. The fact that not all of the mass is accounted for can be explained by incomplete hydrolysis of the polysaccharide due to the stability of amino sugar glycoside bonds and to the fact that accurate response factors for fucosamine and 2,3-DAH were not available because of the lack of authentic standards.

Hydrolysis in 1% acetic acid is a common method by which the glycosyl bond between KDO and the lipid A portion of LPSs can be broken. In the case of *B. japonicum* 61A123, the LPS required hydrolysis at 100°C for 5 h, much longer than the usual 1-h treatment for most LPSs but identical with the result obtained for the LPS from strain USDA 110 (33). Figure 2 shows Sephadex G-50 gel filtration profiles of the LPS before and after hydrolysis. After hydrolysis the polysaccharide portion of the LPS was significantly reduced in molecular weight compared with that of the intact LPS. The lipid A, which precipitated during hydrolysis, was removed by centrifugation. The composition of the polysaccharide is shown in Table 1; it contained all the glycosyl components found in the intact LPS. All of the LPS fatty acyl components were found in the lipid A portion of the LPS and not in the polysaccharide. The only glycosyl component in the lipid A was 2,3-DAH. Thus, this glycosyl residue was present in both the polysaccharide and lipid A portions of the LPS. The presence of 2,3-DAH in the polysaccharide was probably not due to contamination by lipid A, since NMR analysis did not reveal any resonances that could be attributable to fatty acyl residues. In addition, no fatty acyl residues could be detected in the polysaccharide.

<sup>1</sup>H and <sup>13</sup>C NMR spectra of the polysaccharide moiety are shown in Fig. 3. The <sup>1</sup>H NMR spectrum contained signals around  $\delta$  1.25 which can be attributed to the methyl protons of fucose and fucosamine. The signals at  $\delta$  2.20 and 2.09 were due to the methyl protons of O- and N-acetyl groups, respectively. The anomeric region of the <sup>1</sup>H spectrum was not clearly resolved. The <sup>13</sup>C spectrum showed signals at  $\delta$  176 and at 23.0 and 25.1, which were due to the carbonyl and methyl carbons, respectively, of the acetyl groups. Other diagnostic signals in the <sup>13</sup>C spectrum included those around  $\delta$  18.0, attributable to C-6 of fucose and fucosamine, three signals at  $\delta$  50 through 58, due to C-2 carbons carrying the acetamido groups, and four to six signals at  $\delta$  90 through 110, which are attributable to the anomeric carbons. The complexity of the anomeric region suggests that there is some heterogeneity in the structure of this polysaccharide fraction.

**PAGE analysis of the LPS.** Figure 1A shows the PAGE banding pattern of the 61A123 LPS. There were two banding regions, one region with a slower mobility than that of lysozyme (LPS I) and a second region with a faster mobility than that of lysozyme (LPS II). The LPS I region contained several clusters of bands that appeared to be separated by regular molecular weight intervals. These different clusters are most probably due to different aggregation states of the LPS, since they collapsed into one cluster when the gel was run in deoxycholate instead of sodium dodecyl sulfate (data not shown). Each banding cluster in this region contained multiple bands, which may reflect the heterogeneity of the polysaccharide portion of this LPS. The LPS II region contained three bands, which also indicates heterogeneity in this lower-molecular-weight form of LPS.

It has been shown for other *Rhizobium* strains that the

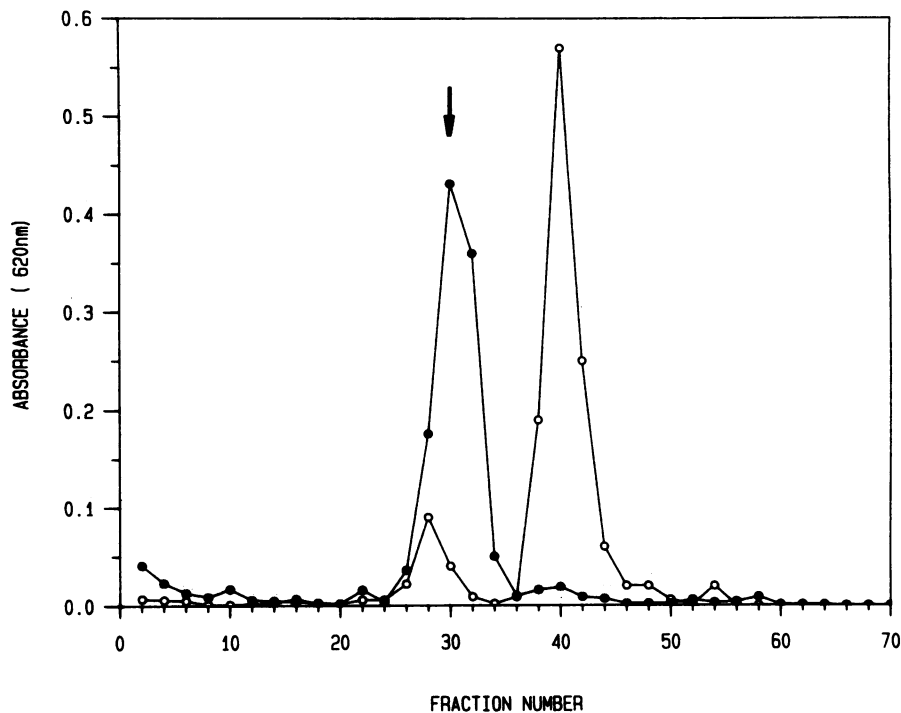


FIG. 2. Sephadex G-50 elution profile of 61A123 LPS before (●) and after (○) hydrolysis in 1% acetic acid at 100°C for 5 h. The arrow marks the location of the void volume. The size of the column is 92 by 2.2 cm, and each fraction is 4.0 ml.

LPS II region is due to incomplete LPS molecules, i.e., those without the O-antigen polysaccharide chain and containing only the lipid A and core oligosaccharide, whereas the LPS I region contains the complete LPS molecules (8, 10, 12). To determine which banding region contains the

O-antigen polysaccharide chain, an immunoblot with 61A123 polyclonal antiserum was performed. Only the LPS I region bound 61A123 antiserum (Fig. 1B), a result analogous to those obtained for other *Rhizobium* LPSs (10, 12).

To determine which sugars are in the O-antigen chain and

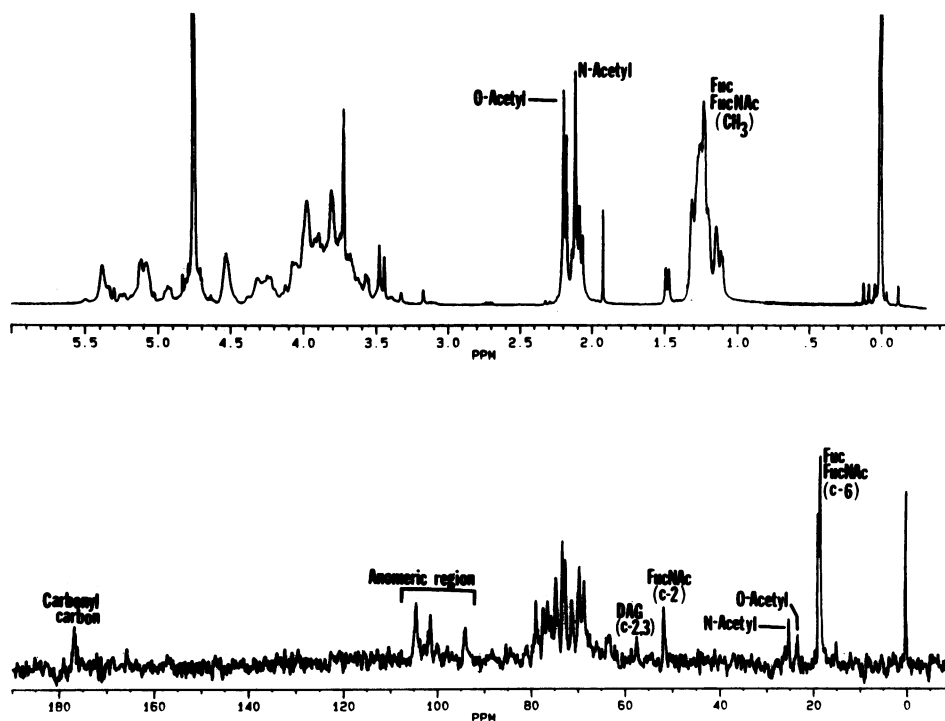


FIG. 3. The proton (top) and carbon (bottom) NMR spectra of the polysaccharide released from 61A123 LPS by mild acid hydrolysis.

which comprise the core region of the LPS, LPS I and LPS II were separated by gel filtration chromatography with a buffer containing deoxycholate (31). Separation of these regions was verified by PAGE analysis of the column fractions (data not shown). Both LPS I and LPS II were hydrolyzed in 1% acetic acid at 100°C for 5 h, and the released polysaccharides were obtained from the supernatant. The glycosyl compositions of the polysaccharides released from LPS I and LPS II are given in Table 1. Since only very small amounts of these LPS I and II polysaccharides were available, the largest portion of each polysaccharide was analyzed for neutral sugar content. This procedure also gave values for fucosamine and quinovosamine but not for glucosamine or 2,3-DAH. Analysis for amino sugars on the small amount of remaining material permitted the detection but not the quantitation of glucosamine and 2,3-DAH. Not enough of these polysaccharide preparations was available to perform the KDO and uronic acid determinations. The LPS II polysaccharide, compared with LPS I, was greatly increased in mannose, glucose, and 4-*O*-methylmannose, indicating that these residues are components of the LPS core region. The presence of 2,3-DAH in the LPS II polysaccharide and the inability to detect its presence in the LPS I polysaccharide indicate that it is also part of the core region. The slight presence of fucose in the LPS II polysaccharide is probably due to some contamination by LPS I polysaccharide. The major components of the O chain appear to be fucose, mannose, and fucosamine, since they were present in much larger amounts in the LPS I than in the LPS II polysaccharide. Glucosamine and quinovosamine were found in the LPS I but not in the LPS II polysaccharide, indicating that they are minor components of the O chain.

**PAGE analysis of the LPSs from other *B. japonicum* strains.** Strains 61A76, 61A101C, USDA 110 and its Nod<sup>-</sup> mutant HS123, and 61A135 were examined to see whether the extraction of *B. japonicum* LPSs into the phenol layer is a general feature of *B. japonicum* strains. Strains 61A76, 61A101C, and USDA 110 are representative of distinct *B. japonicum* serogroups, whereas strains 61A135 and 61A123 are members of the same serogroup. Each strain was grown in approximately 10 ml of medium and centrifuged, and the bacterial pellet was subjected to phenol-water extraction. After dialysis, the materials extracted into the phenol and water layers were treated with proteinase K and analyzed by PAGE. The results (data not shown) showed that LPS banding patterns were obtained in the phenol layer from each strain. In the case of strain USDA 110 and its mutant, HS123, the LPSs were found in both the phenol and water layers. An earlier report describes the characterization of the strain USDA 110 and HS123 LPSs, which were purified from the water layer of phenol-water extraction (33).

## DISCUSSION

The properties of the LPS from *B. japonicum* 61A123 can be summarized as follows: (i) it is extracted into the phenol phase during phenol-water extraction, (ii) it contains a lipid A and a polysaccharide portion that are joined by a mild acid-labile bond (presumably via KDO), (iii) it contains a small amount of KDO, and (iv) it is present in both complete (LPS I) and incomplete (LPS II) forms.

The extraction of *B. japonicum* 61A123 LPS into the phenol layer may be a property that is common to LPSs from all *B. japonicum* strains, since we found that this was true for the LPSs from strains representing several serogroups of *B.*

*japonicum*. The water layer is composed largely of EPS and also contains some neutral glucans. Only in the case of strain USDA 110 was some LPS found in the water layer. Extraction of LPSs into the phenol layer, although unusual, has also been reported for *Rhodopseudomonas palustris* strains (40) and for *Brucella abortus* (22, 25). In the latter species, the LPS I is extracted into the phenol phase and the LPS II is extracted into the aqueous phase (22, 25). The reasons for these observations are not known. Perhaps, in the case of *B. japonicum* LPSs, the predominance of heavily N- and O-acetylated 6-deoxyhexoses in the O chain cause the LPSs to have enough hydrophobicity to be extracted into the phenol layer.

The KDO content of 61A123 LPS is lower (<1%) than that (>3%) reported for other *Rhizobium* LPSs (7, 11, 46). In addition, 61A123 LPS is more resistant to mild acid hydrolysis than are other LPSs, requiring 5 h instead of the normal 1 h in 1% acetic acid at 100°C to separate the lipid A from the polysaccharide. As described above, similar results have been reported for the LPS from *B. japonicum* USDA 110 (33). Low levels of KDO and more stable KDO glycosidic bonds have also been reported for the LPSs from other gram-negative bacteria. The LPSs from *Haemophilus influenzae* and *Vibrio cholerae* have apparently low levels of KDO and increased stability of the lipid-polysaccharide bond due to a phosphorylated KDO (4, 45). However, in our case phosphate was not detected in the *B. japonicum* 61A123 LPS. Thus, the reasons for the low level of KDO and the increased stability of the lipid-polysaccharide bond in *B. japonicum* LPSs are still not clear.

The data in this report and the data reported for USDA 110 LPS (33) indicate that the O chains of 61A123 and USDA 110 LPSs vary in structure but that the core regions may have a common structure. As with 61A123 LPS, and O chain from *B. japonicum* USDA 110 (33) contains acetylated fucose and fucosamine as major glycosyl components. However, it also contains xylose and arabinose, sugars which are not present in 61A123 LPS. In addition, antiserum to 61A123 does not cross-react with USDA 110 LPS. Thus, these two O chains do not appear to have any common immunodominant structures. A previous report showed that mannose and glucose are major components of the LPS from strain HS123, a mutant of USDA 110 that lacks the O chain of the LPS (33). Since mannose and glucose are major core components of strain 61A123, it is possible that these *B. japonicum* strains may have common LPS core structures. The presence of 4-*O*-methylmannose was not reported for strain HS123 LPS; however, the packed column used to analyze that LPS would not have resolved 4-*O*-methylmannose from mannose. Analysis of the LPSs from several strains of *B. japonicum* is required to determine whether LPSs from this species contain a common core structure.

The lipid A portion of *B. japonicum* 61A123 LPS contains 2,3-DAH as the only glycosyl residue. 2,3-DAG is also present in the lipid A of *B. japonicum* USDA 110 and in *Bradyrhizobium lupini* (27). Additionally, it has been shown that DAG-containing lipid A types are also found in two species of *Pseudomonas* (41), three species of *Rhodopseudomonas* (1, 34), two species of *Nitrobacter* (26), *Phenyllobacterium immobile* (38), and *Brucella melitensis* (29). The 2,3-DAG may be in the form of a monosaccharide or a 2,3-DAG disaccharide or may be present along with glucosamine to form a mixed lipid A (28). It has been shown that bacteria with the unusual DAG-containing lipid A are phylogenetically related (27). Thus, the presence of DAG in the LPS from strain 61A123 further supports the relatedness

of *Bradyrhizobium* strains to these other DAG-containing bacteria.

The lipid A of strain 61A123 also contains 27-hydroxyoctacosanoic acid, which has been reported a major fatty acyl component of *R. leguminosarum* biovar *trifolii* LPS (17). In the case of *B. japonicum* 61A123, this fatty acid is present but as a minor component. The distribution of this fatty acyl component in the LPSs from a variety of gram-negative bacteria is under investigation.

We are presently screening Tn5-generated symbiotic mutants of *B. japonicum*, provided by Gary Stacey at the University of Tennessee, to determine whether they are defective in their LPSs. The LPSs from defective mutants will be structurally characterized.

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