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Host-Symbiont Interactions

III. PURIFICATION AND PARTIAL CHARACTERIZATION OF *RHIZOBIUM* LIPOPOLYSACCHARIDES[1]

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ABSTRACT

The lipopolysaccharides of three strains each of *Rhizobium leguminosarum*, *R. phaseoli*, and *R. trifolii* have been purified and partially characterized. The last step in the purification procedure is gel filtration column chromatography using Sepharose 4B with an elution buffer consisting of ethylenediaminetetraacetic acid and triethylamine. Each of the lipopolysaccharides reported in this paper elutes as a symmetrical peak in the partially included volume of this Sepharose 4B column. The ratio of 2-keto-3-deoxyoctonate acid (a sugar which is characteristic of lipopolysaccharides) to hexose is constant throughout the carbohydrate-containing peaks as they elute from the Sepharose 4B. The compositions and immunodominant structures of the purified lipopolysaccharides vary as much among strains of a single *Rhizobium* species as among the different species of *Rhizobium*. There is no obvious correlation between the nodulation group to which a *Rhizobium* belongs and the chemical composition or immunochemistry of the *Rhizobium*'s lipopolysaccharide. There is extensive cross-lysis by phage of strains of *R. trifolii*, *R. phaseoli*, and *R. leguminosarum*. This suggests that the receptors for these cross-lysing phage reside either in nonlipopolysaccharide structures or in common structures within the lipopolysaccharide which are not detected by compositional or immunochemical analysis.

The molecular basis for the interaction between *Rhizobium* and their legume hosts is not understood. This interaction is characterized by a degree of selectivity (9, 10, 25). For example, *Rhizobium trifolii*, the symbiont of clover, does not form a symbiotic relationship with soybeans, and *Rhizobium japonicum*, the symbiont of soybeans, does not form a relationship with clover.

Cell surface molecules are known to function in cell-cell interactions (1, 19). Therefore, the cell surface molecules of *Rhizobium* are likely to participate in the establishment of symbiotic relationships between *Rhizobium* and legumes (10, 31). An early interaction between *Rhizobium* and their legume hosts takes place between the cell surfaces of the legume root hairs and the *Rhizobium* (8). The cell surface of Gram-negative bacteria, like *Rhizobium*, includes extracellular polysaccharides, capsular polysaccharides, LPS,[6] and flagella proteins (17-19, 24). This paper describes the purification and some of the properties of the LPS from several strains of *Rhizobium*.

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[6] Abbreviations: LPS: lipopolysaccharide(s); TEA: triethylamine.

The LPS of Gram-negative bacteria are known to be involved in the interactions of Gram-negative bacteria with other organisms (6, 12, 16, 28, 32, 35). The LPS are primary antigenic determinants of Gram-negative bacteria (17-19). For example, the LPS are species-specific antigenic determinants of *Salmonella* and are strain-specific determinants of *Escherichia coli* (17-19). In addition, the LPS serve as phage receptors (6, 32, 37) and are also known to cause systemic toxic effects in mammalian hosts (16, 17, 28).

The LPS of *Rhizobium* have not been widely studied. The partial compositions of the LPS from several strains of *R. trifolii* and *R. leguminosarum* have been reported (14, 15, 21, 22, 38). Immunochemical studies of the LPS of the two *R. trifolii* strains have suggested that these LPS are strain-specific antigenic determinants of these bacteria (14). The properties of these LPS have indicated that their gross structure is similar to the structures of the other Gram-negative bacteria.

The LPS of *Rhizobium* may participate in the establishment of symbiosis. Van Brussel *et al.* (29) have published data which show that the cell wall of *R. leguminosarum* bacteroids, the nitrogen-fixing form of the bacteria in the nodule, is altered in that the cell wall is more permeable and contains less polysaccharide than the cell wall of the cultured *Rhizobium*. Van Brussel (29) suggested that his results may reflect a decrease in the polysaccharide portion of the LPS of bacteroids. These results, together with the diversity in composition, structure, and antigenic specificity of the LPS from a variety of Gram-negative bacteria, and earlier work in this laboratory (36), convinced us of the need to characterize structurally the LPS of *Rhizobium*.

MATERIALS AND METHODS

Rhizobium Strains. *R. japonicum* R54a, *R. leguminosarum* 128C63, and *R. phaseoli* 127K24 were obtained from H. Evans and S. Russell of Oregon State University; *R. trifolii* strain 2S from K. C. Marshall, University of Tasmania, Australia; *R. trifolii* 0403 from G. Fahraeus, Agricultural College, Uppsala, Sweden; *R. trifolii* 162S7 from J. Burton of the Nitragin Company, Milwaukee; and *R. leguminosarum* 3HOQ1 from D. Weber from USDA, Beltsville, Md. *R. trifolii* strains 162S7, 0403, and 2S were grown on a defined media as previously described (3). The remaining *Rhizobium* strains were grown on defined media as described by Vincent (30) with 100 μg of thiamine HCl, 100 μg of calcium pantothenate, 200 μg of biotin, and 10 g of mannitol/l of solution. The bacteria were harvested in late exponential phase by centrifugation at 13,000*g* for 15 min, yielding 2 to 11 g wet weight/l of culture. The pelleted cells were stored at −20 C.

LPS Isolation. The LPS were extracted from the *Rhizobium* by the phenol-water method (34) with the following modifications. Thirty to 80 g wet weight of bacteria were washed in 1 liter of 0.5 M NaCl, and centrifuged at 13,000*g* for 10 min. The washing procedure was repeated three times in order to remove the extracellular polysaccharides.

The washed bacteria were suspended in 200 ml of 65 C H_2O to which 200 ml of 65 C phenol were added. This slurry was stirred for 15 min at 65 C, cooled on ice for 15 min, and centrifuged at 13,000*g* for 20 min at 5 C. The water phase was set aside and the phenol phase was heated to 65 C. Two hundred ml of 65 C H_2O were added to the hot phenol phase and the above procedure was repeated. The H_2O layers were combined and dialyzed at 5 C against two 25-liter changes of distilled H_2O.

The dialyzed LPS solution was passed through a 10-ml bed volume of Dowex AG 1 X 1 (Bio-Rad) in the acetate form. The material which passed directly through the Dowex column was concentrated to about 100 ml by evaporation under reduced pressure at 40 C. The concentrated solution was made 10 mM in $MgSO_4$ and 50 mM in Tris·HCl, and adjusted to pH 7.0. One mg of DNase I (1,000 Kunitz units/mg) and 1 mg of RNase A (100 Kunitz units/mg), both purchased from Sigma, were added. This solution was stirred overnight at 5 C and then lyophilized.

The lyophilized LPS-containing preparation was dissolved in 5 ml of a buffer containing 20 mM imidazole HCl (pH 7.0), and 100 mM NaCl and chromatographed on a column of Bio-Gel A-1.5m (4 × 60 cm) which had been equilibrated with the imidazole buffer. The LPS voids this column while a contaminating polysaccharide fraction elutes in the included volume. However, because the LPS preparations from *R. phaseoli* 127K24, *R. phaseoli* 127K14, *R. leguminosarum* 128C53, *R. leguminosarum* 3HOQ1, *R. trifolii* 0403 and *R. trifolii* 162S7 were exceptionally viscous, it was not feasible to pass these solutions directly through the A-1.5m column. The viscous LPS preparations were first pelleted by ultracentrifugation at 100,000*g* for 3 hr in the imidazole-NaCl buffer. The supernatant solutions from the ultracentrifugations remained viscous and contained polysaccharides which were not LPS. The pelleted LPS-containing preparations were dissolved in the imidazole buffer and applied to the A-1.5m column. The LPS-containing material, which elutes in the void volume of the A-1.5m column, was dialyzed and lyophilized.

Preparation of Antisera. Rabbit antisera were raised against the LPS from *R. phaseoli* 127K17 and from *R. leguminosarum* 128C53 as described by Humphrey and Vincent (13, 14).

Immunodiffusion Procedure. Micro double diffusion tests were performed as described by Crowle (7). The diffusion plates (microscope slides) were prepared with 0.75% Noble agar (Difco) in 10 mM sodium phosphate (pH 7.0) containing 137 mM NaCl. The diffusion gels were allowed to develop for 48 hr at 4 C before being submerged overnight at room temperature in a solution of 10 mM sodium phosphate (pH 7.0) containing 500 mM NaCl. The diffusion gels were then placed in agitated distilled H_2O for 30 min and stained for 15 to 30 min in a solution composed of water-isopropyl alcohol-acetic acid (13:5:2) containing 0.05% Coomassie blue (Sigma). The gels were destained for about 60 min in a solution composed of water-isopropyl alcohol-acetic acid (8:1:1).

Analysis of Sugar Compositions. Aldoses and amino sugar compositions of the LPS were quantitatively analyzed by gas chromatography of the alditol acetate derivatives. The LPS samples were hydrolyzed for 60 min at 121 C in 2 N trifluoroacetic acid. The resulting monosaccharides were converted into their corresponding alditol acetates and the alditol acetates were identified by combined gas chromatography-mass spectrometry (4) and, when possible, by comparison of their gas chromatographic retention times to the retention times of standards (2, 27). The amount of each alditol acetate was determined by integrating the curve generated by flame ionization of the gas chromatographic effluent. The integration was accomplished with a computer data system using spline function base line correction. This integration method was designed by H. Albert in this laboratory (unpublished procedure). The amount of each alditol acetate has been corrected for differences in the flame ionization detector responses using a correction factor determined for the appropriate standard (26). This correction factor was calculated from the ratio of the area obtained for a known amount of the standard sugar to a known amount of *myo*-inositol, the internal standard. Standard sugars are not available for many of the LPS sugars. For these sugars, the correction factor of the standard sugar, with a retention time nearest to that of the unknown sugar, was used.

Two-keto-3-deoxyoctonate was quantitatively assayed by the thiobarbituric acid colorimetric method (33). Uronic acids were quantitatively assayed by the *m*-hydroxydiphenyl method (5).

Bacteriophage Isolation. Phage were isolated from a mixture of midwestern soils by the procedure of Vincent (30).

Determination of Lipid A Content of LPS. One-mg/ml solutions of LPS were made 1% in acetic acid, sealed, and heated at 100 C for 90 min. This procedure specifically hydrolyzes the ketosidic bonds of 2-keto-3-deoxyoctonate and results in a separation of the lipid A and the polysaccharide portions of the LPS (20, 23). The lipid A, which is precipitated, was removed by three separate extractions with 1 volume of $CHCl_3$. The $CHCl_3$ layers were combined, placed on a tared cup-shaped piece of aluminum foil, and allowed to dry. The aluminum foil was weighed on a microbalance and the net weight determined. The amount of $CHCl_3$-extractable material in the various LPS preparations, before the acetic acid hydrolysis, was negligible.

RESULTS

Purification of *Rhizobium* LPS. All of the LPS which have been characterized contain 2-keto-3-deoxyoctonate and lipid. We have used these constituents as criteria to define *Rhizobium* LPS.

Many *Rhizobium* LPS purified by the modified phenol-water extraction procedure are contaminated with a glucose-rich polysaccharide. The existence of the glucose-rich contaminant was demonstrated by binding the LPS to hydroxylapatite. The material which does not bind to the hydroxylapatite was rich in glucose and deficient in 2-keto-3-deoxyoctonate. A solution of 1% TEA eluted the material which had bound to the hydroxylapatite. This solubilized material was enriched in 2-keto-3-deoxyoctonate and depleted in glucose. These results suggested that the hydroxylapatite preferentially binds LPS. Purification of the LPS preparations on hydroxylapatite columns proved not to be feasible because separation of the glucose-rich contaminant from the LPS was incomplete.

It was found that the LPS can be cleanly separated from the glucose-rich contaminants by gel filtration on Sepharose 4B by eluting with an EDTA- and TEA-containing buffer. The LPS to be purified by this procedure (up to 10 mg) is dissolved in 0.5 ml of a solution of 100 mM EDTA and this solution is titrated to pH 7.0 with TEA. The final concentration of TEA is about 300 mM. The solubilized LPS is applied to a Sepharose 4B column (1.5 × 80 cm) which had been equilibrated with a 10 mM EDTA-300 mM TEA (pH 7.0) solution. The eluted fractions were assayed for hexose and 2-keto-3-deoxyoctonate. In this chromatographic system, the 2-keto-3-deoxyoctonate-rich material present in the phenol-water-extracted LPS preparations forms a partially included symmetrical peak. The ratio of 2-keto-3-deoxyoctonate to hexose is constant across this peak, suggesting homogeneity of composition. The 2-keto-3-deoxyoctonate-deficient, glucose-rich material present in the crude LPS preparations voids this column (Fig. 1). The amount of this void peak varies with the *Rhizobium* LPS being examined and is absent in the LPS isolated from *R. phaseolus* 127K17, *R. leguminosarum* 128C63, and *R. trifolii* 2S.

Contaminating polysaccharides are separated from the LPS at three different stages during the purification. One polysaccharide-containing fraction is removed from the pelleted *Rhizobium* prior to the phenol-water extraction by the saline washes. Another polysaccharide-containing fraction is separated from the LPS by ultracentrifugation or by agarose-1.5m column chromatography. A third polysaccharide-containing fraction is usually separated from the LPS by Sepharose 4B EDTA-TEA column chromatog-

raphy. All three of these polysaccharide-containing fractions obtained from *R. leguminosarum* 128C53 are rich in glucose, galactose, and uronic acids.

LPS Composition. Composition studies of LPS from Gram-negative bacteria have revealed much about the differences and the similarities which exist among different strains and species of these bacteria (18). The sugar compositions of three strains each of *R. phaseoli, R. leguminosarum,* and *R. trifolii* are given in Table I. Typical gas chromatograms of the alditol acetates of several *Rhizobium* LPS are compared in Figure 2. The LPS from all of the *Rhizobium* strains examined contain galactose, glucose, mannose, glucosamine, uronic acids, fucose, and 2-keto-3-deoxyoctonate. The LPS from each of the strains contain sugars which are not present in the LPS of any of the other strains of *Rhizobium* which we have studied with one exception. *R. leguminosarum* 3HOQ1 and *R. leguminosarum* 128C53 LPS have the same sugar compositions, but in different proportions (Table I). With one exception, all of the *Rhizobium* strains examined represent different chemotypes (*i.e.* qualitatively different compositions).

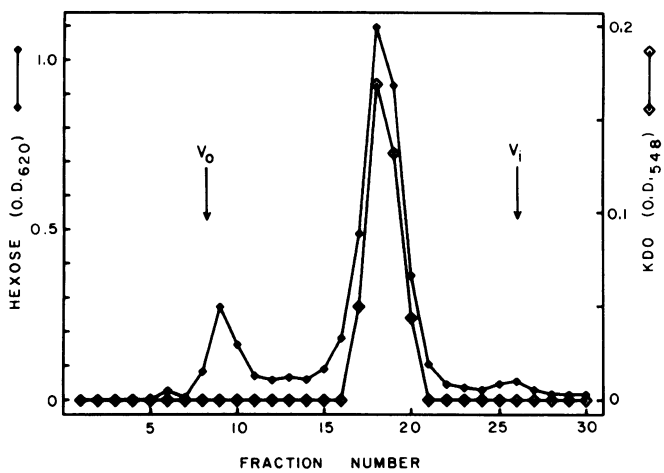

FIG. 1. Sepharose 4B EDTA-TEA gel filtration column of *R. trifolii* 0403 LPS. Fractions (8.4 ml) were assayed for hexose by the anthrone assay, and for KDO by the thiobarbituric assay.

Antigenic Specificity of *Rhizobium* LPS. The LPS of Gram-negative bacterial species have been shown to be structurally similar when the antiserum raised against one species reacts strongly with the LPS from another species (18, 19). Therefore, the ability of antiserum raised against the LPS of one strain of *Rhizobium* to react with the LPS of other *Rhizobium* strains can be another measure of the structural relatedness of the LPS from the various strains of *Rhizobium.*

The reaction between LPS antisera and either lyophilized *Rhizobium* cells or isolated LPS was detected by the formation of precipitin bands on double diffusion gels. The antiserum raised against *R. phaseoli* 127K17 LPS reacts (Fig. 3) with the cells or LPS of *R. phaseoli* 127K17 to form a precipitin band, but the antiserum does not react with the cells of other strains of *R. phaseoli* nor with the LPS from other *Rhizobium* species. *R. phaseoli* 127K17 LPS antiserum does not react with the LPS from any of the *Rhizobium* strains listed in Table I. Other double diffusion tests show that antiserum raised against *R. leguminosarum* 128C53 reacts strongly with both *R. leguminosarum* 128C53 and *R. leguminosarum* 3HOQ1 LPS (the strain with similar chemotype), while a light precipitin band is formed with *R. phaseoli* 127K17 LPS. *R. leguminosarum* 128C53 LPS antiserum does not react with any of the other *Rhizobium* strains listed in Table I.

***Rhizobium* Phage Patterns.** The LPS are frequently the receptors for the bacteriophage of Gram-negative bacteria (32). Often, an observed change in the ability of a phage to lyse a bacterium is due to a change in the structure of the LPS of that bacterium (6, 18, 32).

Bacteriophage capable of lysing particular strains of *Rhizobium* were isolated and then were tested for their ability to lyse other *Rhizobium* strains. The results of these studies are summarized in Table II. There is extensive cross-lysis between most of the phage isolated by their ability to lyse *R. leguminosarum, R. phaseoli,* or *R. trifolii.* For example, phage isolated for their ability to lyse *R. leguminosarum* 128C53 also lyse some *R. phaseoli* and *R. trifolii* strains. Also, phage which lyse *R. phaseoli* 127K17 lyse some *R. leguminosarum* and *R. trifolii* strains, while phage which lyse *R.*

Table I. Composition of Rhizobium Lipopolysaccharides

	R. leguminosarum strain					R. phaseoli strain				R. trifolii strain			
LPS constituents	128C53			3HOQ1	128C63	127K17		127K24	127K14	0403	162S7	2S	
	(1)*	(2)	(3)			(1)	(2)					(1)	(2)
Mannose	6.9	5.3	10.7	11.0	2.8	1.9	1.8	1.7	+	2.4	1.9	1.7	2.3
Galactose	2.9	1.5	2.7	1.1	2.7	2.1	3.1	1.2	+	2.5	1.1	2.2	2.5
Glucose	1.5	1.6	1.1	13.4	2.3	1.3	2.1	2.4	+	3.1	0.9	1.0	0.4
Glucosamine*	1.6	1.0	1.9	2.2	2.6	2.1	-	Tr	-	2.7	1.5	1.6	2.2
2-keto-3-deoxyoctanoate	9.2	-	6.1	3.2	2.9	3.4	5.6	2.9	+	2.4	7.2	2.7	2.2
Uronic acids	6.2	-	7.4	4.7	7.3	6.2	10.8	+	+	8.8	2.2	7.0	7.6
Fucose	9.1	8.3	17.8	10.4	4.1	2.4	1.7	3.7	+	2.5	4.0	7.5	8.2
Rhamnose	5.9	5.3	12.1	7.4	0	0	0	0.7	0	0	0.6	7.5	8.9
3-O-methylhexose	0	0	0	0	0	0	0	0	0	0	0	1.1	1.0
2-O-methyl-6-deoxyhexose	0	0	0	0	0	0	0	4.2	0	2.9(1)	4.5(2)	0	0
3-O-methyl-6-deoxyhexose	0	0	0	0	0	0	0	0	0	0	0	3.6	3.7
2,3-di-O-methyl-6-deoxyhexose	0	0	0	0	1.3	0	0	0	+	0	0	0	0
3,4-di-O-methyl-6-deoxyhexose	0	0	0	0	0	0	0	0	0	0	0.7	0	0
N-methyl-3-amino-3,6-dideoxyhexose	0	0	0	0	2.8	1.5	1.9	0	0	3.7	0	0	0
2-amino-2,6-dideoxyhexose	0	0	0	0	0	0.5	1.3	0	+	0	0	0	0
Heptose	0	0	0	0	2.4	0	0	0	0	3.2	0	0	0
Lipid A	-	-	42.0	-	48.0	-	-	-	-	32.0	24.0	25.0	-

Numerical values are percent of dry weight of LPS.
+ = component present but not quantitated; 0 = component not present; - = not determined; Tr = trace amounts detected.
* No special hydrolysis procedures were used to determine glucosamine. Therefore, the amounts of glucosamine reported here are probably low.

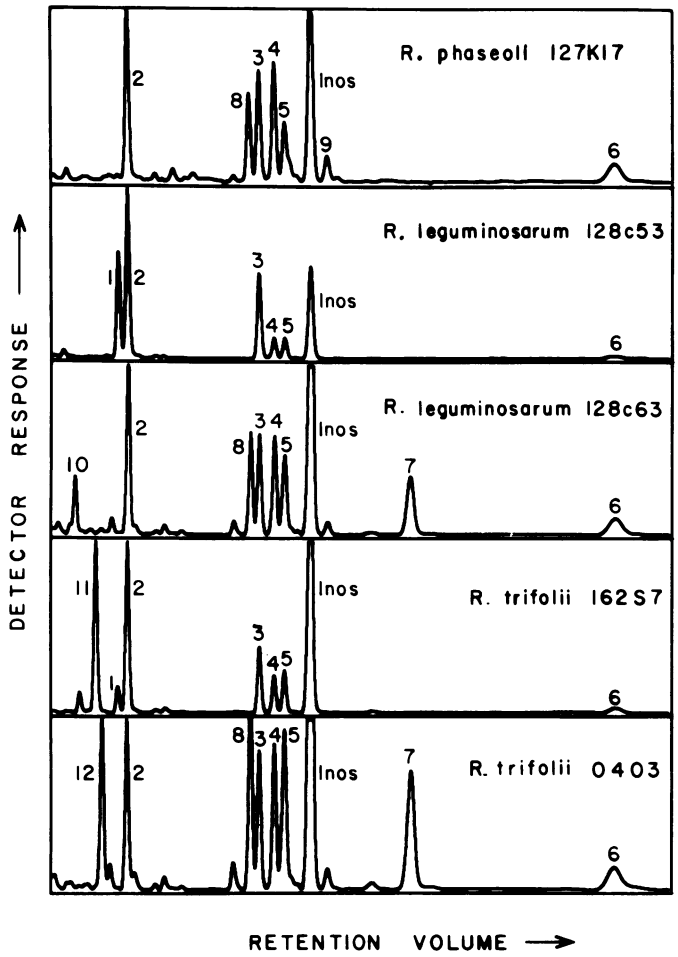

FIG. 2. Comparison of gas chromatograms of alditol acetates derived from sugars of five different LPS. Sugars are numbered as follows: 1 = rhamnose, 2 = fucose, 3 = mannose, 4 = galactose, 5 = glucose, 6 = 2-glucosamine, 7 = heptose, 8 = N-methyl-3-amino-3,6-dideoxyhexose, 9 = 2-amino-2,6-dideoxyhexose, 10 = 2,3-di-O-methyl-6-deoxyhexose, 11 = 2-O-methyl-6-deoxyhexose (1), and 12 = 2-O-methyl-6-deoxyhexose (2), INOS = *myo*-inositol (added as an internal standard).

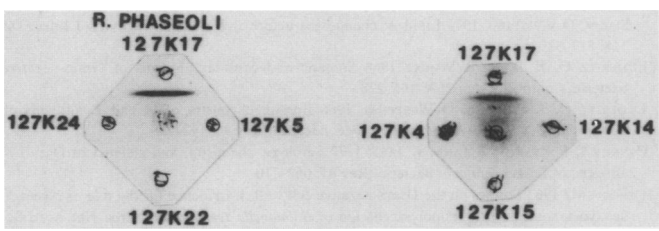

FIG. 3. Microdouble diffusion gel. Undiluted rabbit antiserum (center wells), formed against the LPS purified from *R. phaseoli* strain 127K17, was diffused against lyophilized cells of each of seven *R. phaseoli* strains (outer wells). Suspensions of lyophilized cells, 100 mg/ml in physiological buffered saline, of *R. phaseoli* strains 127K17, 127K22, and 127K4 and 50 mg/ml of *R. phaseoli* strains 127K24, 127K5, 127K15, and 127K14 were prepared. Each of the outer wells contains about 20 μl of the cell suspension of the indicated *R. phaseoli* strain. The center well contains 20 μl of antiserum.

trifolii 162S7 also lyse some *R. phaseoli* and *R. leguminosarum* strains.

DISCUSSION

Purification of *Rhizobium* LPS. The purity of a LPS preparation is difficult to determine since there is no easily assayed biological activity to monitor. Other criteria of purity, such as the symmetry of a peak eluting from a gel filtration column, have traditionally been difficult to obtain for LPS because LPS exist in varying aggregation states (11, 18). When *R. leguminosarum* 3HOQ1 LPS is applied to a Sepharose 2B column using a variety of conditions, the LPS elutes in the void volume of the column; Sepharose 2B has a molecular exclusion limit of 20×10^6 for polysaccharides.

Table II. Ability of Phage Isolates to Lyse a Variety of *Rhizobium* Strains.

Rhizobium strain used to isolate phage	*R. leguminosarum*			*R. phaseoli*			*R. trifolii*
	+128C53	-3HOQ1	-128C63	+127K17	+127K14	-127K24	+162S7
R. leguminosarum 128C53							
phage 1	+	-	-	+	+	-	+
phage 2	+	-	+	+	-	+	+
phage 3	+	-	-	+	+	+	+
phage 4	+	-	-	+	-	+	+
phage 5	+	-	-	+	+	+	+
R. phaseoli 127K17							
phage 1	-	-	-	+	-	-	+
phage 2	+	-	+	+	-	-	+
phage 3	+	-	-	+	+	-	+
phage 4	+	-	+	+	-	-	+
phage 5	+	-	-	+	-	-	+
R. trifolii 162S7							
phage 1	+	-	+	+	-	+	+
phage 2	+	-	-	+	+	+	+
phage 3	+	-	+	+	-	-	+

Galanos and Lüderitz (11) have shown that LPS aggregation is dependent on the ionic form of the LPS. When *Salmonella* LPS are partially converted to their free acid forms by electrodialysis and then titrated to pH 7 with TEA, the sedimentation coefficients of the LPS preparations are greatly reduced (11). Titration to pH 7 with Mg^{2+} or Ca^{2+} of a LPS which had largely converted to its acidic form results in highly aggregated LPS preparations (11). In fact, many LPS precipitate in the presence of Mg^{2+} or Ca^{2+}. These results suggest that the highly aggregated nature of LPS is due, in part, to the presence of Mg^{2+} and Ca^{2+} ions.

Exhaustive electrodialysis of *Rhizobium* LPS, followed by titration to pH 7.0 with TEA, results in LPS which still elute in the void volume of a Sepharose 2B column (this column is equilibrated and eluted with an imidazole buffer). There is substantial skewing of the LPS-containing peak into the sizing range of the column indicating that some decrease in aggregation of the LPS has occurred.

The apparent mol wt or degree of aggregation of *Rhizobium* LPS is greatly reduced when the LPS are dissolved and chromatographed in a solution of EDTA-TEA. *Rhizobium* LPS elute as partially included symmetrical peaks when chromatographed on a Sepharose 4B EDTA-TEA column (Fig. 1); Sepharose 4B has an exclusion limit of 5×10^6 for polysaccharides. This procedure separates the LPS from a glucose-rich polysaccharide fraction which elutes in the void volume. These results suggest, in agreement with the results of Galanos and Lüderitz (11) for *Salmonella* LPS, that highly aggregated forms of *Rhizobium* LPS require the presence of divalent metal ions such as Ca^{2+} or Mg^{2+}. The incomplete removal of Ca^{2+} and Mg^{2+} from *Salmonella* LPS by electrodialysis (11) appears also to be true for *Rhizobium* LPS. Purification of LPS by Sepharose 4B EDTA-TEA column chromatography is a procedure which can be usefully applied to the purification of the LPS from a variety of Gram-negative bacteria.

Several criteria suggest that *Rhizobium* LPS have now been purified to homogeneity. The LPS elute as symmetrical, compositionally homogeneous peaks upon Sepharose 4B EDTA-TEA

chromatography. All of the LPS preparations, except for *R. trifolii* 2S, lack an absorption maximum in the UV which suggests that the LPS preparations are free of DNA, RNA, and protein. None of the preparations contain ribose which confirms that the LPS preparations are free of RNA. The small amounts of glucose in these LPS preparations, and the separations of large amounts of glucose-rich polysaccharides from the LPS, suggest that the LPS are not significantly contaminated with a glucose-rich polysaccharide and that glucose is a constituent of *Rhizobium* LPS.

Composition of *Rhizobium* LPS. The *Rhizobium* LPS composition analyses show that the differences in the LPS structures are as great among strains of a single *Rhizobium* species as among the different species of *Rhizobium*. The LPS from each strain of *Rhizobium* has a unique sugar composition (Table I). Eight of the nine *Rhizobium* LPS examined have different sugar components or chemotypes, *i.e.* they differ from one another qualitatively. Only *R. leguminosarum* 3HOQ1 and *R. leguminosarum* 128C53 have LPS with the same sugar components. The sugars in the LPS of these two *R. leguminosarum* strains are present in different proportions, *i.e.* the two LPS are the same qualitatively but differ from one another quantitatively.

Immunochemical Specificity of *Rhizobium* LPS. The diversity in the structures of the *Rhizobium* LPS revealed by the composition analyses is confirmed by the immunochemical studies. Antisera raised against purified LPS preparations show either strict strain specificity (*e.g. R. phaseoli* 127K17) or limited interstrain specificities (*e.g.* the cross-reaction of antisera to *R. leguminosarum* 128C53 LPS with *R. leguminosarum* 3HOQ1 and *R. phaseoli* 127K24 but not with *R. leguminosarum* 128C63 or with other *Rhizobium*).

***Rhizobium* Phage Patterns.** Since many phage receptors of enteric bacteria reside in the LPS (6, 18, 32, 38, 39), phage lysis of *Rhizobium* was studied with the idea that this may be an additional probe for examining the similarity of the LPS of the various *Rhizobium* strains. However, there is no obvious correlation between phage lysis pattern and LPS composition or immunochemistry (see Tables I and II, and Fig. 2). The extensive cross-lysis by some phage of *R. trifolii, R. leguminosarum,* and *R. phaseoli* suggests that the receptors for these phage reside either in non-LPS structures or in common structural regions within the LPSs which are not detected by compositional or immunochemical analyses.

***Rhizobium* Strain Identification.** The phage lysis patterns in conjunction with LPS composition and immunochemical data provide a powerful technique for identifying strains of *Rhizobium*. The data reported in this paper show that composition and immunochemical analyses will generally distinguish one strain of *Rhizobium* from other *Rhizobium* strains. However, this is not always the case. The composition and immunochemical studies of the LPS from *R. leguminosarum* 3HOQ1 and *R. leguminosarum* 128C53 show that these two strains have the same LPS components and that the LPS from *R. leguminosarum* 3HOQ1 cross-reacts with the antiserum to the LPS from *R. leguminosarum* 128C53. In this case, the LPS composition and immunochemical studies did not determine whether these two *R. leguminosarum* strains are, in fact, different. However, the phage lysis studies reveal that five phage which lyse *R. leguminosarum* 128C53 do not lyse *R. leguminosarum* 3HOQ1, confirming that these strains are not the same.

Role of *Rhizobium* LPS in Symbiosis. The data presented in this paper suggest that *Rhizobium* LPS are structurally diverse and that there is no obvious correlation between LPS structure and nodulation groups. Phage lysis patterns also do not correlate with nodulation groups. The data show that differences in *Rhizobium* LPS compositions and immunodominant sites are as great among strains of a single *Rhizobium* species as they are among different species of *Rhizobium*. The data do not rule out the possibility that structural regions in the LPS other than the immunodominant sites or the possible LPS phage receptor sites could be involved in determining the specificity of *Rhizobium*-legume interactions. More structural studies, *i.e.* glycosyl linkage and glycosyl sequence analyses, are necessary in order to determine if the *Rhizobium* LPS could have a role in determining the specificity of host-symbiont interactions. It remains possible that a portion of the polysaccharide is removed from the LPS during symbiosis, as suggested by the data of Van Brussel (29), and that this alteration in LPS structure is necessary for an effective symbiosis to take place.

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LITERATURE CITED

1. ALBERSHEIM P, AJ ANDERSON-PROUTY 1975 Carbohydrates, proteins, cell surfaces, and the biochemistry of pathogenesis. Annu Rev Plant Physiol 26: 31–52
2. ALBERSHEIM P, DJ NEVINS, PD ENGLISH, A KARR 1967 A method for the analysis of sugars in plant cell-wall polysaccharides by gas-liquid chromatography. Carbohydr Res 5: 340–345
3. BISHOP PE, JG GUEVARA, JA ENGELKE, HJ EVANS 1976 Relation between glutamine synthesis and nitrogenase activities in the symbiotic association between *Rhizobium japonicum* and *Glycine max*. Plant Physiol 57: 542–546
4. BJÖRNDAL H, CB HELLERQUIST, B LINDBERG, S SVENSSON 1970 Gas-liquid chromatography and mass spectrometry in methylation analysis of polysaccharides. Angew Chem Int Ed 9: 610
5. BLUMENKRANTZ N, G ASBOE-HANSEN 1973 New method for quantitative determination of uronic acid. Anal Biochem 54: 484–489
6. BRAY D, PW ROBBINS 1967 Mechanism of ϵ^{15} conversion studies with bacteriophage mutants. J Mol Biol 30: 457–475
7. CROWLE AJ 1973 Double diffusion tests. *In* Immunodiffusion. Academic Press, New York, pp 286–295
8. DART PJ 1974 Infection and development of leguminous nodules. *In* A Quispel, ed, The Biology of Nitrogen Fixation. North Holland Publishing Co, Amersterdam, pp 381–429
9. DART PJ 1977 Infection and development of leguminous nodules. *In* R Hardy, W Silver, eds, A Treatise on Dinitrogen Fixation, Section III. John Wiley & Sons, New York, pp 367–472
10. DUDMAN WF 1977 The role of surface polysaccharides in natural environments. *In* IW Sutherland, ed, Surface Carbohydrates of the Prokaryotic Cell. Academic Press, New York, pp 357–414
11. GALANOS C, O LÜDERITZ 1975 Electrodialysis of lipopolysaccharides and their conversion to uniform salt forms. Eur J Biochem 54: 603–610
12. GRAHAM TL, L SEQUEIRA, TR HUANG 1977 Bacterial lipopolysaccharides as inducers of disease resistance in tobacco. Appl Environ Microbiol 34: 424–432
13. HUMPHREY BA, JM VINCENT 1965 The effect of calcium nutrition on the production of diffusible antigens by *Rhizobium trifolii*. J Gen Microbiol 41: 109–118
14. HUMPHREY BA, JM VINCENT 1969 The somatic antigens of two strains of *Rhizobium trifolii*. J Gen Microbiol 59: 411–425
15. LORKIEWICZ Z, R RUSSA 1971 Immunochemical studies on *Rhizobium* mutants. Plant Soil, Special Vol 1971: 105–109
16. LÜDERITZ O, C GALANOS, V LEHMANN, M NURIMINEN, ET RIETSCHEL, G ROSENFELDER, M SIMON, O WESTPHAL 1973 Lipid A: chemical structure and biological activity. J Infect Dis 128: 517–529
17. LÜDERITZ O, K JANN, R WHEAT 1968 Somatic and capsular antigens of Gram-negative bacteria. Comp Biochem 26A: 105–228
18. LÜDERITZ O, AM STAUB, O WESTPHAL 1966 Immunochemistry of O and R antigens of *Salmonella* and related *Enterobacteriaceae*. Bacteriol Rev 30: 192–255
19. ØRSKOV I, F ØRSKOV, B JANN, K JANN 1977 Serology, chemistry, and genetics of O and K antigens of *Escherichia coli*. Bacteriol Rev 41: 667–710
20. OSBORN MJ 1963 Studies on the Gram-negative cell wall. I. Evidence for the role of 2-keto-3-deoxyoctanoate in the lipopolysaccharide of *Salmonella typhimurium*. Proc Nat Acad Sci USA 50: 499–506
21. PLANQUÉ K, JW KIJNE 1977 Binding of pea lectins to a glucan type polysaccharide in the cell walls of *Rhizobium leguminosarum*. FEBS Lett 73: 64–66
22. RUSSA R, Z LORKIEWICZ 1974 Fatty acids present in the lipopolysaccharides of *Rhizobium trifolii*. J Bacteriol 119: 771–775
23. RYAN JM, HE CONRAD 1974 Structural heterogeneity in the lipopolysaccharide of *Salmonella newington*. Arch Biochem Biophys 162: 530–535
24. SMITH RW, H KOFFLER 1971 Bacterial flagella. Adv Microbial Physiol 6: 219–330
25. STEWART WDP 1967 Nitrogen Fixation in Plants. University of London, Athlone Press, London
26. SWEET DP, RH SHAPIRO, P ALBERSHEIM 1975 Quantitative analysis by various GLC response-factor theories for partially methylated and partially ethylated alditol acetates. Carbohydr Res 40: 217–225
27. TALMADGE KW, K KEEGSTRA, WD BAUER, P ALBERSHEIM 1973 Structure of plant cell walls. I. Macromolecular components of the walls of suspension-cultured sycamore cells with a detailed analysis of the pectic polysaccharides. Plant Physiol 51: 158–173
28. THOMAS L 1954 The physiological disturbances produced by endotoxins. Annu Rev Physiol 16: 467–490
29. VAN BRUSSEL AAN, K PLANQUÉ, A QUISPEL 1977 The wall of *Rhizobium leguminosarum* in

bacteroid and free-living forms. J Gen Microbiol 101: 51–56
30. VINCENT JM 1970 A Manual for the Practical Study of Root-Nodule Bacteria. Blackwell, Oxford
31. VINCENT JM 1974 Root-nodule symbioses with *Rhizobium*. *In* A Quispel, ed, The Biology of Nitrogen Fixation. North Holland Publ Co, Amsterdam, pp 265–341
32. WEIDEL W 1958 Bacterial viruses (with particular reference to adsorption/penetration). Annu Rev Microbiol 12: 27–48
33. WEISSBACH A, J HURWITZ 1958 The formation of 2-keto-3-deoxyheptonic acid in extracts of *Escherichia coli* B. J Biol Chem 234: 705–709
34. WESTPHAL O, K JANN 1965 Bacterial lipopolysaccharides. *In* RL Whistler, ed, Methods in Carbohydr Chem, Vol 5. Academic Press, New York, pp 83–91
35. WHATLEY MH, JS BODWIN, BB LIPPINCOTT, JA LIPPINCOTT 1976 Role for *Agrobacterium* cell envelope lipopolysaccharide in infection site attachment. Infect Immun 13: 1080–1083
36. WOLPERT J, P ALBERSHEIM 1976 Host symbiont interactions. I. The lectins of legumes interact with the O-antigen-containing lipopolysaccharides of their symbiont *Rhizobia*. Biochem Biophys Res Commun 70: 729–737
37. WRIGHT A, N BARZILAI 1971 Isolation and characterization of nonconverting mutants of bacteriophage e^{34}. J Bacteriol 105: 937–939
38. ZAJAC E, R RUSSA, Z LORKIEWICZ 1975 Lipopolysaccharide as receptor for *Rhizobium* phage 1P. J Gen Microbiol 90: 365–367
39. ZAJAC E, R RUSSA, Z LORKIEWICZ 1975 Studies on phage 1P receptors in *Rhizobium trifolii* and *Rhizobium leguminosarum*. Acta Microbiol Polan Ser A, 7: 181–188