The Lipopolysaccharide of Free-living and Bacteroid Forms of *Rhizobium leguminosarum*

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The lipopolysaccharide of free-living *Rhizobium leguminosarum* was isolated and purified, and its homogeneity was determined by gel electrophoresis and, after mild acid degradation, by gel filtration. On electrophoresis, two molecular species were observed. After acid degradation and gel filtration, three components could be isolated: one was very rich in glucose, one contained 2-*O*-methylfucose, fucose, mannose, galactose, glucose, L-glycero-D-manno-heptose, 2-keto-3-deoxyoctonic acid, L-alanine, quinovosamine and uronic acids, and the third component consisted of low molecular weight material. In the lipid A fraction, D-glucosamine, β -hydroxymyristic acid, β -hydroxypalmitic acid and β -hydroxystearic acid were detected as major components. The phosphorus content was low. No major chemical differences were observed in the neutral sugar and fatty acid compositions of the lipopolysaccharides isolated from bacteria and bacteroids. The lipopolysaccharide of bacteroids was rapidly lost during isolation from the nodules and on dialysis, and behaved anomalously during ultracentrifugation.

INTRODUCTION

Bacteria of the genus *Rhizobium* are able to establish a symbiosis with Leguminosae. The bacteria are restricted to the root nodule during this symbiosis and are characterized by their ability to reduce atmospheric nitrogen to ammonia. Inside the root nodules of most plants, the bacteria undergo morphological changes: they swell, elongate and branch, resulting in a great number of different shapes – the so-called bacteroids.

Van Brussel *et al.* (1977) observed that bacteroids of *R. leguminosarum* were more sensitive to the action of lysozyme, several detergents and alkaline conditions in osmotic lysis experiments than were free-growing bacteria. Fractionation of bacterial and bacteroidal walls by phenol/water extraction showed the polysaccharide content of the bacteroidal walls to be much smaller (Van Brussel *et al.*, 1977). Recent findings suggest that carbohydrate-binding proteins (phytohaemagglutinins or lectins) of the host plants may play a role in the recognition of invading rhizobia (for a review, see Bauer, 1977). Thus a thorough study and characterization of the lipopolysaccharide (LPS) and other surface polysaccharides of *Rhizobium* was desirable. Only partial analyses of LPS of *Rhizobium* have been reported previously (Humphrey & Vincent, 1969; Graham & O'Brien, 1968; Russa & Lorkiewicz, 1974; Oparin, 1976). This paper describes the chemical composition of LPS of bacteria and bacteroids of *Rhizobium leguminosarum* strain A171.

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METHODS

Growth of bacteria and bacteroids. *Rhizobium leguminosarum* strain A171 was maintained on glucose/mannitol/yeast extract agar. Bacteria were grown in mannitol/yeast extract broth; growth conditions, harvesting and storage were as described previously (Van Brussel *et al.*, 1977).

Bacteroids were isolated as described by Planqué & van Brussel (1976) except that after excision the nodulated roots were frozen in liquid nitrogen, stored at -20°C and bacteroids were isolated in distilled water instead of sucrose/phosphate buffer. Purified freeze-dried bacteroids were stored at -20°C . The degree of purity of the bacteroids has been reported (Planqué *et al.*, 1977).

Definition of fractions. The observation that the wall polysaccharides of *R. leguminosarum* could be fractionated into several molecular species, one of which contained none and another which contained all of the characteristic components of LPS (Planqué & Kijne, 1977), prevented the description of the material isolated by phenol/water extraction as LPS. The fractions were therefore named as follows: (1) crude aqueous-phase material (cAPM), material obtained by phenol/water extraction and not further purified; (2) aqueous-phase material (APM), nucleic acid-free material obtained from cAPM; (3) PS, the soluble fraction remaining after treatment of APM with acetic acid at 100°C for 1.5 h; (4) PS I, PS II and PS III, fractions obtained by gel chromatography of PS on Sephadex G-50.

Isolation of APM. Bacterial cAPM was prepared by extracting walls, isolated as described by Van Brussel *et al.* (1977), with phenol/water (Westphal & Jann, 1965). Nucleic acids were removed by repeated ultracentrifugation for 4 h at 100000g, leaving APM in the pellet. Bacteroidal cAPM was isolated from intact cells by phenol extraction. This cAPM differed from that of bacteria. It was not possible to purify it by ultracentrifugation as the pellets became enriched in nucleic acids. However, bacteroidal cAPM could be purified, with considerable losses, by incubation with ribonuclease A (Sigma) and T_1 (Merck) followed by dialysis. No significant amounts of polysaccharide-containing material could be isolated either by cold trichloroacetic acid extraction (Boivin & Mesrobian, 1935) or by phenol/chloroform/petroleum ether extraction (Galanos *et al.*, 1969). The APMs were shown to be free from nucleic acids by gas-liquid chromatography (absence of ribose) and spectrophotometrically (absence of absorption at 260 nm; 0.1 mg APM ml^{-1} in 0.01 M-NaOH). The protein content (Lowry *et al.*, 1951) was 3.5 to 3.7%. However, except for L-alanine, no significant amounts of amino acids were revealed by autoanalysis.

Analytical methods. Chromatography of neutral sugars was carried out on Whatman no. 1 and Schleicher and Schüll 2043a paper. Chromatograms were developed for 5 and 16 h for fast and slow moving components respectively, using ethyl acetate/pyridine/water (8:2:1, by vol.; solvent A) and 1-butanol/ethanol/water (5:1:2, by vol.; solvent B). Neutral sugars were visualized with alkaline silver nitrate (Trevelyan *et al.*, 1950) or aniline hydrogen phthalate (Partridge & Westall, 1948), and deoxysugars and O-methyl sugars with piperazine nitroprusside (Edwards & Waldron, 1952).

Uronic acids were fractionated by chromatography on Whatman no. 1 paper with pyridine/ethyl acetate/acetic acid/water (5:5:1:3, by vol.; solvent D). The relative proportions of the two compounds found were determined from fractions isolated by preparative chromatography. Corrections were made by determining the recoveries of co-chromatographed standards. Uronic acids were visualized with naphthoresorcinol, amino groups with ninhydrin, and amino sugars by the Morgan-Elson procedure (Bryson & Mitchell, 1951; Brossmer, 1962; Aminoff & Morgan, 1948). Amino sugars and uronic acid components were separated by high voltage electrophoresis (h.v.e.) on Whatman no. 1 paper using pyridine/acetic acid/water, pH 5.3 (10:4:86, by vol.; solvent C) as buffer. 2-Keto-3-deoxyoctonic acid (KDO) was identified by h.v.e. and by comparison with its authentic ammonium salt (a kind gift from Dr C. Galanos, Freiburg, F.R.G.). All samples analysed by paper chromatography and h.v.e. were defatted after hydrolysis (Folch *et al.*, 1957).

Neutral sugars were determined as their alditol acetate derivatives (Sawardekar *et al.*, 1965; Schmidt *et al.*, 1970) using gas-liquid chromatography (g.l.c.) on glass columns (150 $\times$ 0.3 cm) packed with 3% ECNSS-M on Chromosorb Q (Applied Science Laboratories) at 165 to 195°C and a carrier gas flow rate of 40 ml min^{-1} . Hydrolysis for 16 h at 105°C in 0.5 M- H_2SO_4 provided optimal yields of neutral sugars while the values for heptose and pentoses were corrected for breakdown during hydrolysis according to Schmidt *et al.* (1970). Inositol was added as the internal standard after hydrolysis.

One compound, which could not initially be identified by paper chromatography or by g.l.c., was isolated by preparative chromatography on Whatman no. 3 paper using solvent A for 5 h. The compound was eluted from the paper and identified after reduction with NaBD_4 as its alditol acetate derivative by combined g.l.c.-mass spectrometry carried out by Drs J. Weckesser and H. Mayer of the Max-Planck-Institut für Immunbiologie, Freiburg, F.R.G., under the conditions previously described (Weckesser *et al.*, 1975). Final identification was by g.l.c. after demethylation with BBr_3 (McOmie *et al.*, 1968).

Fatty acids were liberated by hydrolysis for 4.5 h at 100°C in 6 M-HCl and extracted with petroleum ether. They were identified as their methyl esters by g.l.c. on glass columns (150 $\times$ 0.3 cm) packed with 15% EGSS-X on Chromosorb P (Applied Science Laboratories) at 160°C at 40 ml min^{-1} , by comparison with

authentic standards, by combined g.l.c.-mass spectrometry, and by thin-layer chromatography on silica gel G with dichloromethane as the solvent and iodine as the detection reagent.

Hexuronic acid was determined with the carbazole reagent using glucuronic acid as the standard (Dische, 1947).

KDO was determined according to Osborn *et al.* (1972) except that mild hydrolysis for 30 min in 0.01 M-H₂SO₄ at 100 °C was employed to optimize the release of KDO. Neuraminic acid (Sigma) was used as a standard. Amino groups were determined with 2% ninhydrin after hydrolysis in 1 M-HCl at 100 °C for 8 h (the optimal period) and removal of the acid by evaporation. Glucosamine was used as the standard.

Amino sugars were determined according to Randle & Morgan (1955) using glucosamine as the standard and also by autoanalysis, after hydrolysis in 6 M-HCl for 4 and 16 h at 100 °C, using norleucine as the internal standard (Wilkinson, 1976).

The configuration of alanine was confirmed enzymically on a sample which had been purified by h.v.e. (Yoshida, 1965). Glucosamine liberated by hydrolysis in 6.1 M-HCl at 105 °C for 4 h was isolated by column chromatography on Dowex 50 (H⁺) (Crumpton 1959), the optical rotation being determined polarimetrically (Wilkinson, 1976).

Phosphorus was determined according to Lowry *et al.* (1954).

Homogeneity studies. Bacterial APM was degraded to lipid A and polysaccharide (PS) fractions by mild acid hydrolysis in 1 M-acetic acid at 100 °C for 1.5 h. The PS was fractionated by gel filtration on Sephadex G-50 as described previously (Planqué & Kijne, 1977).

The homogeneity of LPS was studied by SDS-polyacrylamide gel electrophoresis as described by Jann *et al.* (1975). Gels were stained with the periodate-Schiff reagents. The LPS of *Escherichia coli* 08:K273:H- strain F492 and strain F470, *Salmonella typhimurium* SR strain F971 and *Citrobacter* strain 396 (F7165) (gifts from Dr K. Jann of the Max-Planck-Institut für Immunbiologie, Freiburg, F.R.G.) were used as markers. Molecular weights of LPS were calculated by plotting the mobilities of the bands against the percentage of lipid A in the LPS, using the data available for the marker LPS (Jann *et al.*, 1975).

RESULTS

Bacteria

Rhizobium leguminosarum A171 grew on mannitol/yeast extract broth as a mixture of motile and non-motile rods. Nigrosin staining and phase contrast microscopy showed the absence of capsular material. On glucose/mannitol/yeast extract agar, shiny white colonies formed without the production of the large amounts of slime found with some other *R. leguminosarum* strains.

Analysis of APM

Neutral sugars. Fucose, mannose, galactose and glucose were identified by paper chromatography in solvents A and B and by g.l.c. The first compound appearing on g.l.c. (retention time relative to glucitol hexaacetate, 0.146) was not detected by paper chromatography in solvent B, but could be isolated by developing the chromatogram with solvent A for 5 h (R_{ribose} , 1.34; R_{glucose} , 2.58). The spot stained brown with aniline hydrogen phthalate and blue with piperazine nitroprusside. Preparative paper chromatography followed by combined g.l.c.-mass spectrometry indicated that the compound was a 2-*O*-methyl-deoxyhexose (m/e of the main fragments, 43, 118 and 275). The material had a retention time identical to the alditol acetate derivative of 2-*O*-methylfucose. Treatment with BBr₃, followed by g.l.c. of the demethylated product, conclusively identified the original compound as a 2-*O*-methylfucose (2-*O*-MF). We were not able to determine a g.l.c. response factor for 2-*O*-MF. However, the ratio of the peak areas for fucose and 2-*O*-MF derivatives was close to 1:1 and the response factor for fucose was used for 2-*O*-MF. Quantitative data on the composition of APM are included in Table 1.

On g.l.c., a heptose derivative with the same retention time as the alditol acetate from L-glycero-D-manno-heptose was detected. However, the breakdown product expected from L-glycero-D-manno-heptose was not detected (Schmidt *et al.*, 1970).

Quantitatively, the composition of APM varied slightly from batch to batch. The ratio heptose:fucose:2-*O*-MF was constantly 3:2:2, but the mannose, galactose and glucose

Table 1. *Quantitative analyses of APM, PS, PS I, PS II and PS III from bacteria and of APM from bacteroids*All results are expressed as nmol mg⁻¹.

	APM		PS	PS I	PS II	PS III
	Bacterial	Bacteroidal				
Neutral sugars*						
2- <i>O</i> -Methylfucose	140–210	20	140	0	420	40
Fucose	150–200	20	140	0	320	30
Mannose	140–110	50	90	80	30	670
Galactose	100–110	80	110	30	40	680
Glucose	90–110	110	210	990	60	270
<i>L</i> -glycero- <i>D</i> -manno-Heptose	210–290	35	290	0	650	130
Amino compounds†						
<i>L</i> -Alanine	90	ND	50	tr	80	tr
<i>D</i> -Glucosamine	100	ND	tr	tr	tr	tr
Quinovosamine	40	ND	90	tr	140	tr
Unknown X ₂	20	ND	50	tr	110	tr
Other components‡						
Total hexuronic acid	760	ND	850	50	610	510
2-Keto-3-deoxyoctonic acid	90–100	0	100	tr	20	100
Phosphorus	35	ND	20	ND	ND	ND

tr, Traces detected; ND, not determined.

* Determined by g.l.c.

† Determined by autoanalysis.

‡ Determined colorimetrically.

ratios were variable. All the neutral sugars detected in bacterial APM were identified in APM of bacteroids and the molar ratios of heptose, fucose and 2-*O*-MF were similar in the two types of APM (Table 1). No significant difference in fatty acid composition was observed (Table 2).

Comparison of the heptose contents determined by g.l.c. analysis of cAPM from the supernatants of the root nodule homogenate and the first washing, and of hydrolysates of the walls, showed that much of the heptose was lost during isolation of bacteroids and walls. The lack of an adequate purification procedure and the low recovery of APM from bacteroids prevented a complete study of the non-neutral compounds.

Acidic compounds. Colorimetric assay of KDO showed the presence of 90 to 100 nmol mg⁻¹ in bacterial APM. The thiobarbituric acid assay for KDO was inconclusive for bacteroidal APM. The difference in absorption between 552 and 502 nm was negative, and the spectrum indicated the presence of another compound which presumably masked any absorption due to KDO.

H.v.e. followed by naphthoresorcinol staining revealed the presence of galacturonic acid and a minor compound, which was identified by preparative h.v.e. and paper chromatography in solvent D as glucuronic acid. The ratio galacturonic acid:glucuronic acid was approximately 3:1. The total uronic acid content of APM was 760 nmol mg⁻¹.

Ninhydrin-positive components. H.v.e. of hydrolysates revealed five ninhydrin-positive spots of which only one (glucosamine) reacted with *p*-*N,N*-dimethylaminobenzaldehyde after *N*-acetylation. One very basic compound (X₁), presumably a polyamine, was not investigated further.

Autoanalysis (Table 1) resulted in the detection of glucosamine, quinovosamine, alanine and another unidentified component (X₂) which was eluted after ammonia. X₂ was not detected in the neutral or hexosamine fractions isolated by h.v.e., and it was not destroyed

Table 2. *Analysis of compounds present in lipid A*

Fatty acid methyl esters were determined in APM from bacteria and bacteroids, and the results are expressed as relative peak areas; retention times (R_t) are expressed relative to $C_{21:0}$. D-Glucosamine was determined in lipid A and the results are expressed as nmol mg⁻¹.

Fatty acid	R_t	Bacterial APM	Bacteroidal APM
Unknown component	0.11	0.14	0.12
β -OH-myristic ($C_{14:0}$)	0.74	1.00	1.00
Unknown component	0.93	0.18	0.26
β -OH-palmitic ($C_{16:0}$)	1.38	0.19	0.23
β -OH-stearic ($C_{18:0}$)	2.50	0.17	0.17
D-Glucosamine		Lipid A	
Determined by autoanalysis		350	
Determined by ninhydrin reaction		1000	
Determined by Rondle & Morgan (1955) method		450–700	

by more drastic hydrolysis (6 M-HCl, 100 °C, 16 h), indicating that it was not a product of partial hydrolysis.

Alanine was found to have the L-configuration, indicating that it was not a contaminant of mucopeptide origin. The specific optical rotation of glucosamine was calculated as $[\alpha]_D + 68.5^\circ$ (c 0.07 in water), close to the value for D-glucosamine hydrochloride of $+72.5^\circ$. Insufficient quinovosamine was available to determine its configuration. Determination of total hexosamine in APM by the Rondle & Morgan (1955) method gave 4% compared with 2.8% by autoanalysis.

Phosphorus. APM of *R. leguminosarum* contained little phosphorus. After degradation of APM with 1 M-acetic acid, 20 nmol mg⁻¹ were found in the PS moiety, whereas the lipid A contained 100 nmol mg⁻¹.

Composition of lipid A. Lipid A isolated as described in Methods contained 5% of the total neutral sugars present in APM and made up 33 to 40% by weight of the total APM. The composition of lipid A from bacteria and bacteroids is shown in Table 2.

Homogeneity of APM. As reported previously (Planqué & Kijne, 1977), the polysaccharides in APM, degraded by mild acid hydrolysis with 1 M-acetic acid, could be separated into three fractions by gel filtration on Sephadex G-50. The three fractions, designated PS I, PS II and PS III, differed in sugar composition: PS I represented a glucan-type polysaccharide, being rich in glucose and poor in heptose, fucose, 2-O-MF, KDO and hexuronic acid; PS II contained all the typical LPS constituents (heptose, fucose, 2-O-MF, KDO) and all the amino components of APM except glucosamine; PS III consisted of low molecular weight material. Table 1 gives the quantitative sugar compositions of these fractions. The data on neutral sugars and amino compounds are derived from different batches of PS which may explain the different proportions of sugars in PS and PS II. The apparent heterogeneity of APM was also indicated by the variations in neutral sugar composition of the material at different stages during ultracentrifugal purification. The ratio of heptose to glucose was 1.0:1.55 in cAPM and decreased during purification from 1.0:0.86 in the first pellet to 1.0:0.65 in the APM.

SDS-polyacrylamide gel electrophoresis of APM revealed two bands (Fig. 1) of high mobility compared with the marker LPS samples of *E. coli*, *S. typhimurium* and *Citrobacter*. PS I did not migrate in the gel. The two migrating bands might indicate the presence of two forms of LPS differing in their degree of substitution by O-antigenic side-chains. Polyacrylamide gel electrophoresis of bacteroidal APM in the presence of 0.1% SDS gave two bands with relative mobilities identical to those for bacterial APM (Fig. 1), although the faster band of bacteroidal APM was weaker. Controls, including the addition of bacterial APM to that from bacteroids, increasing the amount of bacteroidal APM, the use of an

Fig. 1. SDS-polyacrylamide gel electrophoresis of APM from *R. leguminosarum* and of marker LPS. Each gel contained 100 μ g. (1) Bacteroidal APM. (2) Bacterial APM. (3) *Citrobacter* 396 LPS. (4) *Escherichia coli* F492 (smooth) LPS. (5) *Escherichia coli* F470 LPS. (6) *Salmonella typhimurium* F971 LPS.

Fig. 2. SDS-polyacrylamide gel electrophoresis of APM from *R. leguminosarum*. (1) Bacterial APM (500 μ g). (2) Bacterial APM (500 μ g) plus bacteroidal APM (500 μ g). (3) Bacteroidal APM (2500 μ g). (4) and (5) As (1) and (3), but treated with RNAase. (6) and (7) As (1) and (3), but run for only 1 h to detect fast-moving polysaccharides.

RNAase treatment and short running time to check on fast-moving components, did not affect the electrophoresis pattern (Fig. 2).

DISCUSSION

The material obtained by standard phenol/water extraction of intact *Rhizobium* or its isolated walls does not consist merely of LPS, as shown in an earlier study (Planqué & Kijne, 1977). In addition to the presence of a high molecular weight polysaccharide (PS I), we report here evidence (based on polyacrylamide gel electrophoresis) for further heterogeneity of the LPS moiety itself.

The lack of application of homogeneity criteria in earlier published analyses of rhizobial LPS makes comparison with this study difficult. However, all *Rhizobium* wall polysaccharide preparations contain at least fucose, mannose and KDO (Humphrey & Vincent, 1969, for *R. trifolii*; Oparin, 1976, for *R. lupini*; Sanders *et al.*, 1978, for *R. leguminosarum*; Planqué & Kunz, unpublished results, for *R. japonicum*). The variable occurrence of a heptose in *R. trifolii* and *R. leguminosarum* remains a problem yet to be solved [see

Humphrey & Vincent (1969) for differences between two *R. trifolii* strains, and Sanders *et al.* (1978) and this paper for differences between strains of *R. leguminosarum*]. The composition of the LPS from the related genus *Agrobacterium* is also largely unknown. However, a recent analysis showed the presence of only 3-hydroxy fatty acids, as in rhizobial LPS (Salkinoja-Salonen & Boeck, 1978; Russa & Lorkiewicz, 1974).

Three polysaccharide species can be found in preparations from *R. leguminosarum* A171: one, called extracellular polysaccharide (XPS), is precipitable from the culture medium, and two high molecular weight species, PS I and PS II, can be isolated after mild acid hydrolysis of APM. The determination of the origin and significance of PS I and PS II is, therefore, a central problem emerging from this study. From the data presented here and that reported by Planqué & Kijne (1977), three conclusions can be drawn. Firstly, XPS is not identical with PS I. This conclusion is based on their different contents of hexuronic acids, their different lectin-binding capacities and their different hexose compositions. Secondly, PS I probably does not form a part of the LPS molecule. The ratio of glucose (the major sugar in PS I) to heptose decreases in the pellets obtained during ultracentrifugal purification of APM and all 'typical' LPS constituents can only be found in PS II. Moreover, the LPS from *R. leguminosarum* 128c53 is virtually free from glucose (Sanders *et al.*, 1978). Thirdly, LPS is present in two forms. By comparing the mobilities of the bands of rhizobial LPS with those of the markers, using the known lipid A content of these markers, and assuming the molecular weight of rhizobial LPS to be equal to that from Enterobacteriaceae, we calculated apparent molecular weights of 7400 and 6350 (Jann *et al.*, 1975). In this calculation no correction was made for differences in fatty acid substitution. Evidence suggests there is a broad resemblance between the lipid A backbones of Gram-negative bacteria (Hase & Rietschel, 1976) but the difference in fatty acid substitution of the rhizobial lipid A compared with that of, for instance, *Salmonella*, would require corrections to be made to the molecular weight. Moreover, it is not known whether rhizobial LPS behaves like enterobacterial LPS on SDS gels. However, the lipid A content calculated by electrophoresis was in reasonable agreement with the yield obtained in the preparation of lipid A; 38 and 44% calculated, compared with 33 to 40% recovery. More definite proof for the existence of the different molecular species demands a genetic approach, which is beyond the scope of this paper.

Comparison of the APM of bacteria and bacteroids after isolation and purification showed differences in physical characteristics and in some aspects of chemical composition. No differences were apparent on polyacrylamide gel electrophoresis, indicating that a smooth-rough conversion did not occur.

Further comparison between the respective polysaccharides is hampered by the lack of data on amino sugars and hexuronic acids in bacteroids and by the gross loss of LPS during its isolation and purification from bacteroids. Indeed, the material finally isolated may be non-representative, thus preventing a meaningful comparison with material isolated from the parent bacteria.

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