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Chemical Characterization of the Lipopolysaccharide of *Pseudomonas solanacearum*

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The carbohydrates present in lipopolysaccharide (LPS) from *Pseudomonas solanacearum* are rhamnose, xylose, 2-amino-2-deoxyglucose, glucose, heptose, and 2-keto-3-deoxyoctonate. LPS extracted from cultures grown on either glycerol or glucose (as the major source of carbon) and extracted after various incubation periods had similar compositions. The LPS from several strains of the bacterium contained the same component sugars, but the amounts of each sugar varied considerably. It was observed, however, that xylose and 2-amino-2-deoxyglucose increased proportionately with rhamnose, the major component. Phenol-water-extracted LPS contained measurable amounts of nucleic acid, protein, and arabinan, but none of these polymers were detected in LPS extracted with phenol-chloroform-petroleum ether. Polysaccharides liberated from LPS by mild acid hydrolysis were purified by gel filtration. Carbohydrate analysis of the LPS from a virulent, fluidal strain (K60) showed that the O-specific antigen consisted of rhamnose, xylose, and 2-amino-2-deoxyglucose in the proportions 4:1:1. The LPS of an avirulent, afluidal strain (B1) lacked the O-specific antigen; the R-core region consisted of rhamnose, glucose, heptose, and 2-keto-3-deoxyoctonate. Methylation analysis indicated that the K60 O-specific antigen was composed of a hexasaccharide repeating unit containing 3-, 2-, and 3,4-substituted rhamnopyranosyl residues, 3-substituted 2-amino-2-deoxyglucose, and terminal xylopyranose in the molar ratios 2:1:1:1:1.

Pseudomonas solanacearum is the causal agent of a vascular wilt disease that affects a wide range of hosts in the warm temperate and tropical regions of the world. The ability to cause a hypersensitive reaction on tobacco leaves has been correlated with structural variations in the lipopolysaccharides (LPS) of certain strains. It was determined that hypersensitive reaction-inducing strains lack the O-specific antigen (R-type LPS) and do not produce extracellular polysaccharide (13). The LPS of *P. solanacearum* also has been reported to induce resistance to various pathogens (5). These results suggested that LPS may be bound to specific receptors on the plant cell and that it may be involved in recognition phenomena.

These findings indicated that a more detailed analysis of the LPS structure was warranted. Other than preliminary reports from this laboratory (13; C. J. Baker and L. Sequeira, Phytopathology **71**:201, 1981) and a short article by Drigues et al. (2), very little information is available on the structure of the LPS of *P. solanacearum*. Here we report some general structural information on the polysaccharide portion of the LPS of this bacterium.

MATERIALS AND METHODS

Bacterial cultures. Two isolates of *P. solanacearum* were used for the major portion of this study: K60, a fluidal, compatible strain, highly pathogenic on tobacco (*Nicotiana tabacum* L.); and B1, an afluidal, avirulent form of isolate K60. Other cultural variants of strain K60 (13) were also used in some experiments. The cultures were obtained from the culture collection maintained at the Department of Plant Pathology, University of Wisconsin, Madison. Cultures were stored in sterile distilled water at 20°C. Before use, isolates were grown on tetrazolium agar medium (7) for 48 h at 30°C. Sterile aqueous suspensions of approximately 1.5×10^8 CFU ml^{-1} were prepared from the tetrazolium agar medium plates and used to start broth cultures (1 ml of suspension $liter^{-1}$). The broth contained 10 g of peptone (Difco Laboratories, Detroit, Mich.), 10 g of glucose, and 1.0 g of Casamino Acids (Difco) in 1 liter of distilled water. Cultures were incubated at 30°C on a rotary shaker at 150 rpm for 48 h. In some experiments, as indicated, the culture medium was made with an equivalent amount of glycerol instead of glucose or the length of the incubation period was changed or both.

Purification of LPS. (i) Phenol-water (PW) extraction. One liter of broth culture was centrifuged at 10,000 × *g* for 30 min to collect bacterial cells. The bacterial pellet was washed with 100 ml of 0.85% NaCl, yielding about 16 g (wet weight). Then the LPS was extracted by a modification of the procedures of Westphal and Jann (12). The bacteria were suspended in 80 ml of water preheated to 68°C; 80 ml of 90% aqueous phenol at 68°C was added, and the mixture was shaken vigorously for 20 min at 68°C. The mixture was then cooled to 10°C and centrifuged at 10,000 × *g* for 30 min. The water phase was aspirated off and saved. The phenol layer was extracted a second time with 80 ml of water at 68°C as described above. The water extracts were combined, dialyzed for 3 to 4 days to remove all traces of phenol, and then lyophilized. The crude LPS was resuspended in 50 ml of water at 50°C and centrifuged at 5,000 × *g* to remove any insoluble material. The supernatant, which contained the LPS, was then centrifuged at 80,000 × *g* for 6 h. The pellet was resuspended and recentrifuged several times until the supernatant reached an optical density at 260 nm of 0.05 or less.

The pellet was suspended in 2 ml of 0.1 M Tris, pH 8.6, and applied to a column (2.5 by 95 cm) of Sepharose 4B, equilibrated with the same buffer plus 0.02% sodium azide. The column was eluted at 25°C at a flow rate of 30 ml h^{-1}; 3-ml fractions were collected. Fractions were assayed for total carbohydrate (3) and 2-keto-3-deoxyoctonate (6). Subfrac-

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tions containing LPS were combined, dialyzed, and lyophilized.

(ii) PCP extraction. LPS also was extracted as described by Galanos et al. (4). Bacterial cells were obtained from broth cultures and washed with saline as described previously. Then the cells were washed successively with ethanol, acetone, and ethyl ether. The dry bacteria (5 to 10 g) then were extracted with 50 ml of a solution containing 90% aqueous phenol-chloroform-petroleum ether (PCP; 2:5:8, by volume). After centrifugation at 10,000 × *g* for 15 min, the pellet was again extracted with the above mixture. The supernatants from the extractions were combined and filtered, and the chloroform and petroleum ether were removed by rotary evaporation. Water was added dropwise to the phenol phase until LPS precipitated from the solution as described by Galanos et al. (4). In one experiment, bacterial cells used for this PCP extraction were subsequently extracted by the PW procedure to determine whether additional LPS or LPS of different composition could be obtained.

GLC. Neutral sugars were converted to their alditol acetate derivatives (1) and analyzed by gas-liquid chromatography (GLC) on a Varian gas chromatograph, model 3700, equipped with a flame ionization detector and a model CDS-111 integrator. Peaks were identified by co-chromatography with authentic compounds. The alditol acetates were separated on glass columns (1.83 m by 2 mm) containing 3% SP-2340 on 100-120 mesh Supelcoport, with a temperature program of 155 + 2°C min^{-1} to 220°C. Derivatives of amino sugars were separated with 3% OV-275 on 100-120 mesh Gas-Chrom Q with a temperature program of 175 + 2°C min^{-1} to 230°C; the final temperature was maintained for 15 min. Partially methylated alditol acetates were analyzed on a Hewlett-Packard 5992B GLC-mass spectrometry instrument, with 3% OV-17 on 100-120 mesh Gas-Chrom Q. The temperature was held at 120°C for 5 min and then increased at 4°C min^{-1} to 280°C and held at this temperature for 32 min.

Hydrolysis, reduction, and derivatization of carbohydrates were carried out in 1-dram (ca. 1.2-g) screw-capped vials by a procedure modified from Albersheim et al. (1). A sample (1 to 2 mg) of the polysaccharide was weighed in a vial; 1 ml of 2 M trifluoroacetic acid, containing 200 μg of inositol as the internal standard, was added; and the vial was sealed with a Teflon-lined cap. The sample was hydrolyzed at 121°C for 1 h, allowed to cool, placed in a water bath at 40°C, and evaporated to dryness under a stream of air. One milliliter of 0.5 M NH_4OH containing 1.5 mg of sodium borohydride was added, and after 1 h at 22°C the solutions were acidified by the dropwise addition of glacial acetic acid. The sample was dried as described above except that, during the final stages of drying, five 1-ml amounts of methanol were added and evaporated successively. Acetylation was completed by adding 200 μl each of pyridine and acetic anhydride, sealing the vials, and heating to 100°C for 45 min. After cooling, the reagents were evaporated in an air stream. Two 1-ml additions of toluene were used to aid in the evaporation.

Alditol acetates were extracted from the residue by addition of 1 ml each of water and ethyl acetate. After mixing thoroughly, the upper organic phase was removed and the extraction was repeated twice. The combined ethyl acetate solution was evaporated, and the residue was dissolved in 50 μl of acetone for analysis by GLC.

For methylation analysis, the methods of Sanford and Conrad (8) and Talmadge et al. (11) were modified as follows. Dry samples, 2 to 3 mg, were placed in 1-dram screw-capped vials fitted with a Teflon septum. The sample was dissolved in 1.5 ml of dry dimethyl sulfoxide; the solution was stirred constantly, and a steady flow of nitrogen was maintained through two 18-gauge hypodermic needles inserted through the septum. Dimethyl sulfoxide anion solution was added, usually about 0.5 ml or less, to give a final concentration of about 0.5 M anion. The dimethyl sulfoxide anion was prepared by slowly adding 5 to 8 ml of dimethyl sulfoxide to 2 ml of a 35% suspension of potassium hydride in mineral oil (Aldrich Chemical Co., Milwaukee, Wis.). The final anion concentration was determined by titration with HCl. The sample reaction mixture was stirred under nitrogen for 2 h at 50°C.

The samples were cooled in a water bath to 15 to 20°C, and 20 μl of methyl iodide was added. After 5 to 10 min, additional amounts of methyl iodide were added until the solution cleared and changed color to pale yellow. The permethylated polysaccharide was isolated by chromatography on a Sephadex LH-20 column (1 by 30 cm) eluted with chloroform-methanol (1:1) (11). The methylated sample then was hydrolyzed with trifluoroacetic acid and derivatized for analysis by GLC as described above.

Mild acid hydrolysis of LPS. A suspension of 150 mg of LPS in 15 ml of 1% acetic acid was heated to 100°C for 1.5 h (9). After cooling, the suspension was extracted with an equal volume of chloroform. The aqueous phase was removed and lyophilized. The recovered material (40 mg, K60; 16 mg, Bl) was fractionated by gel filtration. Columns (2.5 by 95 cm) containing Sephadex G-75 (for K60 polysaccharides) and Sephadex G-25 (for B1 polysaccharides) were eluted with water-pyridine-acetic acid (986:10:4, by volume). Samples were dissolved in 2 ml of this solution before application to the column. Columns were monitored for carbohydrate by the phenol-sulfuric acid method (3). Dextran standards were run independently to aid molecular weight estimates.

RESULTS

Effect of culture conditions on LPS composition. Preliminary experiments were carried out to determine whether the carbon source would have a signficant effect on the sugar constituents of LPS. Cultures of *P. solanacearum* strains K60 and B1, grown on either glycerol or glucose as the major source of carbon were in late log phase after 24 to 30 h and well in stationary phase after 72 h. When LPS from these cultures was extracted by the PW method and analyzed, the composition did not change significantly with culture age or with change in carbon source (Table 1). Rhamnose, xylose,

TABLE 1. Effect of carbon source and culture age on composition of LPS extracted from cultures of *P. solanacearum* by the PW method

Strain	Carbon source	Culture age (h)	Major sugar composition (%)[a]		
			Rhamnose	Xylose	Heptose
K60	Glycerol	30	67	15	17
	Glucose	30	69	15	16
	Glucose	72	72	14	14
B1	Glycerol	30	61	0	39
	Glucose	30	65	0	35
	Glucose	72	65	0	35

[a] Glucose, a major constituent of B1 LPS, was not included due to its presence in contaminating polysaccharides in these preparations. Data are expressed as percentage (weight/weight) of sugars reported.

TABLE 2. Sugar composition of PW-extracted LPS from several strains of *P. solanacearum*

Strain	% Composition (wt/LPS wt)				
	Rhamnose	Xylose	Glucose	Heptose	Glucosamine
B1	4.1	0.0	5.7	6.8	2.0
K60	19.5	5.1	3.1	4.6	8.6
B1-3	41.3	7.1	5.1	2.7	10.5
B1-6	30.1	5.9	3.0	2.7	11.8
M	40.1	9.4	3.3	2.7	12.4
S	29.4	1.0	2.9	2.6	14.1

and heptose were the only sugars used in this comparison because they were not present in contaminating polysaccharides. The LPS from cultural variants of *P. solanacearum* were also analyzed to determine the diversity in sugar composition that might be expected (Table 2). The major component sugars were detected in all variants except strain B1, which was devoid of xylose. Although the amounts of sugars were variable, the overall results show that xylose and 2-amino-2-deoxyglucose increase with the major component rhamnose, except in variant S, in which the relative amount of xylose is significantly lower.

Purification of LPS. When LPS from *P. solanacearum* was extracted by the PW procedure, the preparation required several high-speed centrifugations to remove traces of nucleic acid (Table 3). In addition, the preparations from both bacterial strains contained variable amounts of arabinose, ranging from 0 to 5% (dry weight). The arabinose was considered a contaminant because amounts varied in different preparations and it could be diminished by repeated ultracentrifugation or by gel filtration on Sepharose 4B (Fig. 1). In the latter procedure, LPS, probably in the form of micelles, eluted at the void volume with an aggregate molecular weight of $>5 \times 10^6$. With the same column, arabinose eluted in different fractions not associated with specific carbohydrate peaks.

The PCP procedure was used to extract R-type LPS from strain B1 and S-type as well as R-type LPS from strain K60. The LPS preparations obtained by this procedure did not contain detectable arabinose, nucleic acid, or protein (Table 3). Bacterial cells extracted by the PCP procedure were extracted subsequently by the PW procedure to determine whether additional or different LPS could be obtained. Additional LPS was obtained only from strain K60; this LPS contained low levels of arabinose and ribose (Table 3).

Mild acid hydrolysis of LPS obtained by the PCP procedure yielded water-soluble products which were fractionated by gel filtration. The K60 material eluted as two major peaks, K60-I and -II, with estimated molecular weights of about 25×10^3 and 2.5×10^3 to 3.0×10^3, respectively (Fig. 2A). Analysis showed that K60-I was composed of S-type LPS sugar constituents such as xylose and glucosamine. The K60-II fraction resembled R-type LPS (Table 4) in that it had a lower molecular weight and contained less of these sugars, suggesting that it was incomplete and lacked a major portion of the O-specific antigen. The B1 material eluted as a single carbohydrate peak with an estimated molecular weight of about 2.5×10^3 (Fig. 2B).

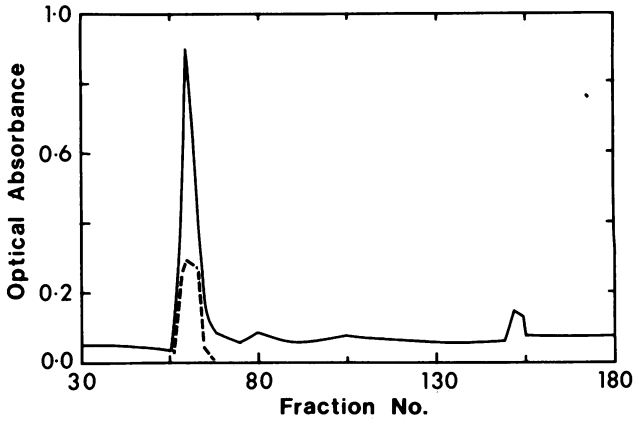

FIG. 1. Gel filtration of K60 PW-extracted LPS on a column (2.5 by 95 cm) of Sepharose 4B, eluted with 0.1 M Tris buffer (pH 8.6). Fractions were analyzed for total carbohydrate (absorbance at 485 nm; ———) and 2-keto-3-deoxyoctonate (absorbance at 548 nm; - - - -).

The methylation analysis of fraction K60-I is shown in Table 5. Partially methylated alditol acetates were unequivocally identified by GLC-mass spectrometry. Rhamnose, the major constituent of LPS, was identified as terminal, 2-substituted, 3-substituted, and 3,4-disubstituted pyranosyl residues. The substituted residues were in the molar proportions 19:37:24. Xylose occurred as a terminal pyranosyl residue in approximately the same amount as the disubstituted rhamnose. The 2-amino-2-deoxyglucose was substituted at O-3; the mass spectrum of this derivative contained characteristic fragments at *m/e* 274, 161, 158, and 116 (10). Difficulty in obtaining reproducible response factors by GLC of this latter derivative prevented its accurate quantification.

TABLE 3. Sugar composition of *P. solanacearum* LPS isolated by PW and PCP extraction procedures

Component	% Composition (wt/LPS wt)				
	PW		PCP		PWI (K60)[a]
	K60	B1	K60	B1	
Rhamnose	19.5	4.1	29.8	4.2	28.9
Xylose	5.1	0.0	6.0	0.0	5.3
Glucose	3.1	5.7	2.4	5.3	2.7
Heptose	4.6	6.8	3.6	6.4	4.0
Glucosamine	8.6	2.0	17.6	5.1	15.6
Arabinose	0.2	4.6	0.0	0.0	1.7
Ribose	0.2[b]	0.0	0.0	0.0	0.6[b]

[a] PWI, PW extraction of PCP-extracted bacterial cells.
[b] Derived from residual nucleic acid after three to six centrifugations at 80,000 × *g* for 6 h.

DISCUSSION

The sugar composition of LPS from two strains of *P. solanacearum* did not appear to change significantly under different cultural conditions. The main constituents of LPS

TABLE 4. Sugar composition of polysaccharides obtained after mild acid hydrolysis of PCP-extracted LPS from *P. solanacearum*

Component sugar	Polysaccharide fraction (μmol mg^{-1})[a]		
	B1	K60-I	K60-II
Rhamnose	0.79	2.92	2.52
Xylose	0.00	0.71	0.09
Glucose	0.93	0.12	0.46
Heptose	1.06	0.12	0.56
Glucosamine	0.00	0.77	0.34

[a] Fractions obtained after gel filtration of soluble products of mild acid hydrolysis (see Fig. 2A and B).

of several cultural variants, with the exception of xylose, appeared to be the same, but relative amounts of the sugars varied. Whatley et al. (13) and Drigues et al. (2) reported the same constituents in their preparations of *P. solanacearum* LPS. Assuming heptose is present in the R-core in a constant amount, the rhamnose/heptose ratio would indicate the relative size of the polysaccharide side chain. Apart from B1, the variants listed in Table 2 apparently contained side chains larger than that of K60.

Extraction of S-type LPS from isolate K60 by the PCP procedure was unexpected since this procedure is reportedly specific for R-type LPS (4). The rhamnose/heptose ratios of K60 LPS extracted by PCP and PWI (PW extraction of PCP-extracted bacterial cells) were significantly higher than the corresponding ratios in PW-extracted LPS (Table 3). This result may reflect differences in workup procedures, since with the PW method extensive high-speed centrifugation was necessary to remove protein and nucleic acid.

Mild acid hydrolysis of the PCP-extracted LPS appeared to cleave the polysaccharide moiety from the lipid A portion of the molecule. Assuming that isolate B1 contains R-type LPS, the polysaccharide released by mild acid hydrolysis would consist of only R-core. Sugar analysis showed that the B1 polysaccharide was composed of residues of rhamnose, glucose, and heptose in approximately equal amounts. By substracting equimolar amounts of these three R-core constituents present in K60 (Table 4) from the total K60-I polysaccharide residues, the composition of the O-specific side chain can be deduced. By this procedure, the components appear to be rhamnose, xylose, and 2-amino-2-deoxyglucose in the molar ratio 4:1:1. Since these ratios represent a repeating unit with a molecular weight of about 10^3, the number of these units per side chain would be about 20, based on the molecular weight of K60-I less the R-core portion (approximately 20×10^3). The methylation analysis of the K60-I polysaccharide indicated a hexasaccharide repeating unit composed of two 3-substituted, one 2-substituted, and one 3,4-disubstituted rhamnopyranosyl residue and one residue each of terminal xylopyranose and 3-substituted 2-amino-2-deoxyglucose (Table 5). The presence of similar amounts of 2-rhamnose and 2,3,4-xylopyranose suggests that terminal xylose is present in a side chain.

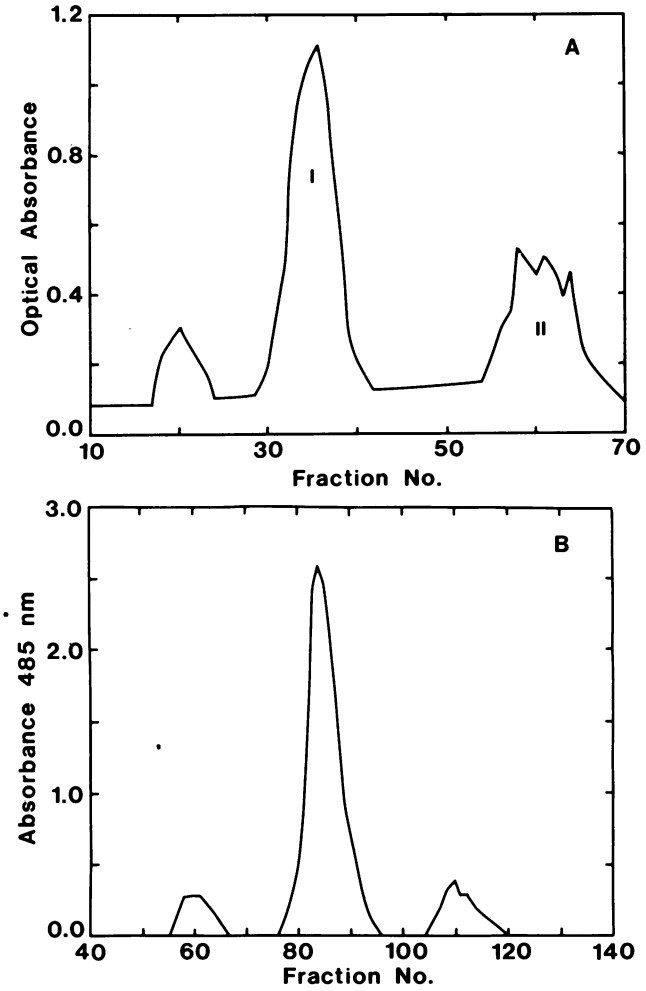

FIG. 2. Gel filtration of the water-soluble products obtained after mild acid hydrolysis of PCP-extracted LPS from (A) K60 (column Sephadex G-75) and (B) B1 (column Sephadex G-25). The eluant was water-pyridine-acetic acid (986:10:4, vol/vol/vol). Fractions were analyzed for total carbohydrate (absorbance at 485 nm).

TABLE 5. Methylation analysis of K60-I polysaccharide (Fig. 2A) obtained from PCP-extracted LPS from *P. solanacearum*

Derivative[a]	RT[b]	Mol%
2,3,4-Rha	0.75	Tr
2,3,4-Xyl	0.79	20
3,4-Rha	0.92	19
2,4-Rha	0.97	37
2,3,4,6-Glc	1.00	Tr
2-Rha	1.07	24
4,6-GlcNAc		+[c]

[a] 2,3,4-Rha, 2,3,4-tri-*O*-methylrhamnose, etc.
[b] RT, Retention time of derived alditol acetate on an OV-17 column relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.
[c] +, Derivative unable to be quantitated accurately.

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