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Quantitative Assay of Polysaccharide Components Obtained from Cell Wall Lipopolysaccharides of *Xanthomonas* Species

WESLEY A. VOLK

Department of Microbiology, School of Medicine, University of Virginia, Charlottesville, Virginia 22903

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The cell wall lipopolysaccharides from 17 species belonging to the genus *Xanthomonas* were extracted from the cells with hot 45% phenol. After purification, the components of the polysaccharide were obtained by acid hydrolysis of the lipopolysaccharide and were quantitatively assayed. Data obtained show that all preparations contained uronic acid, phosphate, and mannose in molar ratios of approximately 1:2:1, and glucose and rhamnose in more variable concentrations. Most lipopolysaccharides contained either xylose or fucose, but those extracted from *X. sinensis* and *X. campestris* contained neither xylose nor fucose.

The demonstration that D-galacturonic acid-1-phosphate occurs as a product of the partial hydrolysis of the cell wall lipopolysaccharide (LPS) of *Xanthomonas campestris* (11), together with the qualitative analysis of the LPS from a number of *Xanthomonas* species (10), have indicated that the heteropolysaccharide portion of the LPS has a composition and structure unlike those reported to occur in the cell walls of enteric organisms.

The present investigation was undertaken to determine whether a quantitative determination of the constituents of the cell wall heteropolysaccharides from a number of species of *Xanthomonas* would yield information which could be used to postulate a basic molecular structure. The data obtained show that the heteropolysaccharide portion of the cell wall LPS from 17 species of *Xanthomonas* do contain a number of common components, and, by assuming that a common basic structure exists among these very closely related species, several generalizations concerning their structure can be postulated.

MATERIALS AND METHODS

The 17 species of *Xanthomonas* used in this study were obtained either from the International Collection of Phytopathogenic Bacteria through the generosity of Mortimer Starr or from the American Type Culture Collection.

The organisms were grown either in Fernbach flasks on a rotary shaker, or in 12-liter VIRTIS fermentor flasks by use of a medium containing 0.5% peptone (Difco), 0.3% yeast extract (Difco), and 0.3% malt extract (Difco) in distilled water. Cells were harvested by centrifugation after 36 to 48 hr of growth at room temperature, and were washed twice with distilled water. Growth beyond 48 hr resulted in the formation of large quantities of extracellular polysaccharide. Even at the shorter growth time, some polysaccharide was almost always present, but this was discarded in the distilled water wash.

Acetone powders were prepared from all washed cells prior to extraction of the cell wall LPS. The method of extraction and purification of the cell wall LPS was performed as previously described (11). All species except *X. sinensis* formed a glucan which was eliminated from the LPS preparation by treatment with β-glucosidase (11).

Protein was determined by the method of Lowry et al. (9), and inorganic phosphate was assayed by the method of Ames and Dubin (1) after hydrolysis of the LPS in 0.2 N HCl for 60 min at 100 C. The identification of 2-keto-3-deoxy octonic acid (KDO) as a component of these LPS preparations was based only on the presence of a component which migrated identically to authentic 3-deoxy-D-manno-octulosonic acid on paper chromatograms developed in a solvent containing *n*-butyl alcohol-pyridine-0.1 N HCl (5:3:2). Authentic 3-deoxy-D-manno-octulosonate was obtained from E. C. Heath as the pentaacetyl methyl ester and was converted to free KDO by treatment with 0.2 N NaOH according to the procedure of Ghalambor et al. (5). The KDO was made visible on paper by use of the thiobarbituric acid spray reagents described by Warren (12), and was assayed by the procedure of Aminoff (2) after hydrolysis of each LPS in 0.02 N H_2SO_4 for 2 hr at 100 C. All other assays were done as previously described (11).

Isolation and quantitation of hydrolysis products. The heteropolysaccharide portion of the LPS was obtained by suspending the purified LPS in 0.2 N HCl at a concentration of 5.0 mg per ml, and placing

the suspension in a boiling-water bath for 1 hr. The hydrolyzed LPS was centrifuged, and the clear supernatant fluid was separated from the sedimented material. The sediment was washed twice, and the washings were pooled with the original supernatant fluid; the volume was then adjusted with distilled water so that 1 ml was equivalent to 2.5 mg of the original LPS. The sedimented material containing lipid, glucosamine, and phosphate was dried under vacuum and weighed.

To hydrolyze the heteropolysaccharide material into its component monosaccharides, a portion of the 0.2 N HCl hydrolysate was made 2.0 N with HCl, and the material was placed in a boiling-water bath for 2 hr. At this time, the material was quantiatively washed onto a column containing 1.5 cm^3 of Dowex 1-acetate for each milliequivalent of chloride present. After passing through the resin, the column was washed with three bed volumes of distilled water. The eluate and washings were taken to dryness under reduced pressure at 50 C, and the resulting chloride-free syrup was dissolved in water so that the concentration of the hydrolyzed material was equivalent to 5.0 mg per ml of the original LPS.

Paper chromatography was performed on Whatman no. 1 paper which had been previously washed with water. A sample of the hydrolyzed material which contained the sugars originally present in 0.75 mg of the intact LPS was streaked over a 16-cm width of the paper. Guide strips 4 cm wide on each side of the paper received an amount equivalent to the sugars derived from 0.15 mg of the original LPS. The descending chromatogram was developed at room temperature in pyridine-ethyl acetate-water (20:72:23) for 16 hr. After drying, the guide strips were cut off and the sugars were observed by use of a $AgNO_3$ dip (3). With the developed guide strips as markers, the sugars in the central part of the chromatogram were quantitatively eluted. The eluted material was taken to dryness at 50 C by blowing a stream of air over the surface of the material. It was then dissolved in 0.1 ml of distilled water.

Glucose oxidase in the form of the Glucostat reagent (Worthington Biochemical Corp., Freehold, N. J.) was used to determine glucose in the hydrolyzed mixture before chromatography and for the glucose eluted from the chromatograms. This provided a control for the effectiveness of the elution technique. Mannose was assayed on the eluted material by the method of Dische and Shettles (4), and the pentose corresponding to xylose was assayed by the orcinol method (8). Those LPS preparations which did not contain fucose were assayed for rhamnose in the 0.2 N HCl hydrolysate without prior chromatography by use of the method of Dische and Shettles (4). In those cases where both fucose and rhamnose were present, it was necessary to separate them by chromatography, elute them from the chromatograms, and then assay them individually. Galacturonic acid was routinely determined on the 0.2 N HCl hydrolyzed material by the borate carbazole assay (6), with authentic galacturonic acid as a standard.

RESULTS

The quantitative data obtained in the analysis of the cell wall lipopolysaccharides from 17 species of *Xanthomonas* are tabulated in Table 1. All LPS

TABLE 1. *Quantitation of lipopolysaccharide components*[a]

Organism	Insoluble material	Inorganic PO_4	Total PO_4	Uronic Acid	Mannose	Glucose	Rhamnose	KDO	Fucose	Xylose	Recovered
	μg										μg
Xanthomonas alfalfae	224	0.32	0.66	0.24	0.40	0.16	1.88	0.06		0.67	824
X. begonie	374	0.41	0.57	0.30	0.44	0.16	0.19	0.005		0.48	851
X. carotae	338	0.38	0.78	0.33	0.28	0.18	1.48	0.04		0.30	808
X. corylinia	299	0.34	0.62	0.31	0.39	0.53	1.09	0.045		0.32	779
X. juglandis	352	0.41	0.76	0.31	0.36	0.20	1.23	0.04		0.33	813
X. maculifoligardeniae	290	0.38	0.68	0.31	0.38	0.19	1.10	0.055		0.38	726
X. phaseoli	246	0.32	0.57	0.28	0.33	0.15	1.81	0.05		0.72	803
X. pruni	341	0.34	0.67	0.30	0.22	0.50	1.14	0.04		0.32	795
X. ricinicola	320	0.35	0.60	0.24	0.41	0.23	1.40	0.055		0.41	816
X. translucens	470	0.41	0.71	0.25	0.39	0.13	1.15	0.03		0.53	910
X. uppalli	273	0.32	0.62	0.26	0.50	0.17	1.65	0.04		0.56	829
X. clerodendri	330	0.40	0.76	0.27	0.46	0.25	1.33	0.045	0.30		824
X. gerani	295	0.28	0.58	0.21	0.30	0.13	1.87	0.05	0.92		904
X. hyacinthia	321	0.33	0.55	0.25	0.40	0.28	1.49	0.03	0.43		829
X. malvacaerum	425	0.42	0.74	0.30	0.43	0.17	1.21	0.045	0.37		885
X. sinensis	420	0.40	0.80	0.33	0.28	0.17	1.40	0.035			731
X. campestris	471	0.52	0.81	0.47	0.44	0.26	0.07	0.032			748

[a] Procedures and assays are as described in text. All concentrations are expressed as micromoles per milligram of starting lipopolysaccharide, and those assays performed after the 2 N HCl hydrolysis are corrected for 10% destruction. This value was arrived at by the use of known mixtures of sugars hydrolyzed under identical conditions. "Insoluble material" represents the amount obtained after 0.2 N hydrolysis for 60 min at 100 C. This seems to be analogous to the "Lipid A" described in lipopolysaccharides from enteric organisms.

were found to contain similar quantities of uronic acid (chromatographically identified as galacturonic acid), phosphate, and mannose, and somewhat more variable amounts of glucose and rhamnose. Chemotypes among these species varied only as to whether the cell wall LPS contained xylose or fucose or neither.

DISCUSSION

The genus *Xanthomonas* contains a large number of species that are metabolically very much alike and differ primarily in their ability to infect different plants. Because of this close relationship, it seemed reasonable to assume that a study of the cell wall LPS from a number of species would be analogous to a study of a number of mutants of a single species, and, as a result, might show some common characteristics in their structure.

The results of this study demonstrate that most of these cell wall LPS have quite similar amounts of uronic acid, phosphate, and mannose, and somewhat variable amounts of glucose and rhamnose. If, however, one calculates the ratio of uronic acid to mannose, there appear to be two different groups of LPS. The first group, containing *X. carotae*, *X. pruni*, *X. sinensis*, and *X. campestris*, has an average uronic acid-mannose ratio of 1.2, whereas the second group, containing the remaining organisms listed in Table 1, has an average uronic acid-mannose ratio of 0.66, or approximately one-half that of the former group.

The data also show that, although most of the LPS preparations contain either xylose or fucose, *X. sinensis* and *X. campestris* contain neither of these sugar components. On the basis of those species of *Xanthomonas* studied, these two sugars seem to be mutually exclusive. The extremely small amount of rhamnose found in the LPS from *X. campestris* is not consistent with the amount found in all other species. It is interesting that this same organism possesses a second LPS which is phenol-soluble and which contains 3.6 μmoles of D-rhamnose per mg of LPS (7). The water-soluble LPS from *X. campestris* described in this paper contains L-rhamnose (J. Hickman, *personal communication*).

Based on the uniform presence of uronic acid, phosphate, and mannose in these LPS preparations, one could theorize that this closely related group of organisms does possess a common basic structure in the heteropolysaccharide portion of its cell wall LPS. Furthermore, it would appear that neither fucose nor xylose is a necessary component of any minimal common structure.

The acid lability of part of the total phosphate of all *Xanthomonas* species is in agreement with the proposal that the uronic acid may be attached to the cell wall through an acid-labile phosphate linkage, as has been proposed for *X. campestris* (11).

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