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Cell Wall Lipopolysaccharides from *Xanthomonas* Species

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ABSTRACT

VOLK, WESLEY A. (University of Virginia, Charlottesville). Cell wall lipopolysaccharides from *Xanthomonas* species. J. Bacteriol. **91**:39–42. 1966.—The lipopolysaccharides from 20 species of *Xanthomonas* were extracted and purified. Biological studies suggest that these lipopolysaccharides are analogous to the endotoxins extracted from enteric organisms, as judged by their mouse lethality and their ability to provoke the local Shwartzman reaction in rabbits. Studies on the composition of the polysaccharides revealed that all contained uronic acid, glucose, mannose, and a compound apparently identical to the 2-keto-3-deoxyoctonate previously described in enteric organisms. The polysaccharide also contains organic phosphate, and additional carbohydrates such as rhamnose, xylose, fucose, and galactose are found in some, but not all, species. In contrast to the composition of the enteric lipopolysaccharides, heptose was not found in any of the lipopolysaccharides of the *Xanthomonas* species studied.

Recent interest in the mechanism of synthesis of heteropolysaccharides has resulted in a study of the polysaccharide portion of various cell wall bacterial lipopolysaccharides (LPS). Specific qualitative, quantitative, and structural studies have been directed primarily toward the gram-negative bacteria classified in the *Enterobacteriaceae* and more specifically to the genus *Salmonella* (6, 10–12, 14). These studies have demonstrated that the heteropolysaccharide portion of the cell wall LPS (also known as the endotoxin and as the somatic antigen) is composed of as many as seven different sugars, and that in all cases the polysaccharide apparently consists of a heptose phosphate core from which the other sugars branch off in a highly specific manner (18).

The objective of this study was to determine the composition of the polysaccharide portion of the LPS from a group of gram-negative organisms which are morphologically and taxonomically unrelated to the members of the *Enterobacteriaceae*. For this purpose, 20 species of the genus *Xanthomonas* were studied. This genus was selected because of the apparent homogeneity of its species; i.e., all are morphologically similar, all produce yellow pigment, and all are plant pathogens.

The results of our studies demonstrate that the LPS preparations from *Xanthomonas* consist of a glucosamine-lipid complex linked to a heteropolysaccharide. In addition, toxicity studies with mice and rabbits indicate that the *Xanthomonas* endotoxins are biologically similar to those from *Escherichia* and *Salmonella* species. Unlike the heteropolysaccharide portions of endotoxins from these latter genera, that isolated from *Xanthomonas* does not contain heptose.

MATERIALS AND METHODS

The following organisms were obtained from the International Collection of Phytopathogenic Bacteria through the generosity of Mortimer P. Starr: *X. begoniae, X. campestris, X. corylina, X. geranii, X. juglandis, X. phaseoli, X. pruni, X. sinensis*, and *X. vasculorum*. The remaining *Xanthomonas* species were all obtained from the American Type Culture Collection. This group consisted of the following species: *X. alfalfae, X. badrii, X. carotae, X. clerodendri, X. hyacinthi, X. maculifoliigardeniae, X. malvacearum, X. papavericola, X. ricinicola, X. translucens*, and *X. uppalii.*

The LPS were extracted from the intact washed organisms with hot 45% phenol by the method of Westphal et al. (17). The aqueous phase was dialyzed for 48 to 72 hr against running tap water, partially concentrated under reduced pressure, and lyophilized. The lyophilized material was suspended in 100 ml of water and centrifuged in a Spinco (model L) ultracentrifuge at 100,000 $\times$ g for 1 hr. The supernatant

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liquid was discarded, and the pellet was suspended in 100 ml of distilled water and centrifuged as before. The washings were discarded, and the pellets were dissolved in water and lyophilized. Yields varied from organism to organism, but an average of 250 to 300 mg of LPS was obtained from 50 g of wet cell paste. Glucose was determined with the Glucostat reagent (Worthington Biochemical Corp., Freehold, N.J.). Inorganic phosphate was determined by the method of Ames and Dubin (1), and total phosphate was measured by the same method after ashing. Total 6-deoxyhexoses were determined by the method of Dische and Shettles (5), and aldoheptoses by the cysteine sulfuric acid method of Dische (4). Keto-deoxyoctonate was located with the thiobarbituric acid spray of Warren (15). Pentoses were determined by the orcinol method (9), and uronic acids by the borate-carbazole technique (7).

Descending chromatography was carried out on Whatman no. 1 paper with the following solvents: (A) ethyl acetate-pyridine-water (72:20:23); (B) amyl acetate-acetic acid-water (30:30:10); (C) ethyl acetate-pyridine-*n*-butanol-butyric acid-water (10:10:5:1:5); (D) *n*-butanol-pyridine-0.1 N HCl (50:30:20).

RESULTS

Mouse lethality of LPS from Xanthomonas. A comparison study was conducted to determine whether the LPS isolated from *Xanthomonas* species was similar in its biological activity to that reported for LPS isolated from enteric organisms. The LPS from two arbitrarily chosen *Xanthomonas* species was tested in mice.

Three-week-old Swiss albino mice in groups of five were injected intraperitoneally with graded amounts of LPS dissolved in freshly prepared saline. The mice were observed for 3 days, and the number of deaths occurring during this period was recorded (Table 1). The LD_{50} as calculated by the method of Reed and Muench (13) was 500 μg for *X. ricinicola* and 750 μg for *X. maculifoliigardeniae*.

Shwartzman reaction. A general property of the Shwartzman reaction is the apparent broad specificity concerning the source of LPS used for the preparatory or the provocative dose. An experiment was carried out in which 10- and 25-μg amounts of LPS from *X. ricinicola* and 10- and 25-μg amounts of LPS from *S. enteriditis* (Difco) were injected intradermally into individual sites on two rabbits as a preparatory dose; 24 hr later, a provocative dose of 100 μg of LPS from *X. ricinicola* was injected intravenously into one rabbit, and 100 μg of LPS from *S. enteriditis* was injected intravenously into a second prepared rabbit; 3 hr later, all prepared sites demonstrated local areas of erythemia, induration, and varying amounts of central necrosis. Within 5 hr after the intravenous provocative injection, both rabbits died.

TABLE 1. *Mouse lethality following the intraperitoneal injection of graded amounts of LPS from Xanthomonas*

Amt of LPS injected	*X. ricinicola* deaths after 3 days	*X. maculifoliigardeniae* deaths after 3 days
μg		
100	2	1
200	0	0
300	3	1
400	2	1
500	2	2
600	2	1
700	4	1
800	3	3
900	4	3
1,000	5	4

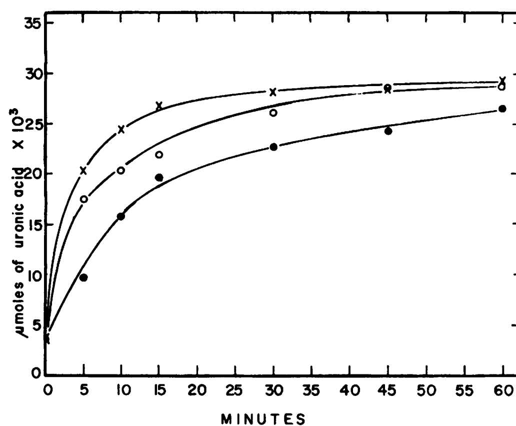

FIG. 1. *Hydrolysis of polysaccharide from the glucosamine-lipid portion of the LPS from Xanthomonas ricinicola in (×) 0.1 N HCl, pH 1.0; (○) 0.01 N HCl, pH 2.0; and (●) 0.1 N acetic acid, pH 3.0. For each pH value, seven 4.0-ml samples of LPS (1 mg/ml) in screw-cap tubes were immersed in boiling water. At the times indicated, a tube was withdrawn and immediately cooled in an ice bath. At the end of the experiment, 6.0 ml of water was added to each sample, and the mixture was then transferred to a centrifuge tube and centrifuged at 100,000 × g for 1 hr. Uronic acid was determined on a 0.7-ml sample of the supernatant fluid and compared with standard glucuronic acid.*

Stability of linkage between polysaccharide and lipid portion of LPS. Hydrolysis in dilute acid of the LPS from various *Xanthomonas* species resulted in the cleavage of the bond linking the polysaccharide and the lipid-glucosamine portions of the molecule. The intact LPS and the hydrolyzed lipid moiety were quantitatively precipitated by centrifugation in a model L Spinco centrifuge. The amount of released polysaccharide remaining in the supernatant liquid was estimated

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TABLE 2. *Qualitative components of the polysaccharide portion of Xanthomonas LPS**

Organism	Galactose	Fucose	Xylose	Rhamnose
X. alfalfae†	−	−	+	+
X. badrii†	−	−	−	+
X. begoniae†	−	−	+	+
X. campestris	+	−	−	+
X. carotae†	−	−	+	+
X. clerodendri	−	+	−	+
X. corylina†	−	−	+	+
X. geranii	−	+	−	+
X. hyacinthi	−	+	−	+
X. juglandis	−	−	+	+
X. maculifoliigardeniae	−	−	+	+
X. malvacearum	−	+	−	+
X. papavericola	−	−	+	+
X. phaseoli	−	−	+	+
X. pruni	−	−	+	+
X. ricinicola	−	−	+	+
X. sinensis	−	−	−	+
X. translucens	−	−	+	+
X. uppalii	−	−	+	+
X. vasculorum	−	+	−	+

* In addition to the components shown, all of the test organisms contained uronic acid, keto-deoxyoctonate, glucose, and mannose.

† LPS also had one or more unidentified slow-moving components on solvent A, which were possibly amino sugars. On some occasions, a spot corresponding to ribose was observed. However, since this did not seem to be consistent from one preparation to another, it was concluded that the ribose probably originated from contaminated nucleic acid.

by the determination of uronic acid in solution (7). Figure 1 illustrates the results with purified LPS from *X. ricinicola*.

Sugar components released on hydrolysis of Xanthomonas LPS. The component sugars composing the heteropolysaccharide of the LPS from *Xanthomonas* species were determined by hydrolysis of the LPS in 2 N H_2SO_4 for 2 hr at 100 C. Insoluble lipid material was filtered off and discarded. The supernatant solution was neutralized with $Ba(OH)_2$ to the phenolphthalein end point and, after discarding the precipitated $BaSO_4$, a sample was absorbed on Whatman no. 1 paper and developed by descending chromatography in solvents A, B, and C. The sugar spots were located by use of a $AgNO_3$ dip (2). In addition to their position on the respective chromatograms, spots identified as either rhamnose or fucose were eluted and shown to give the color reaction expected of a 6-deoxyhexose (5). Eluted xylose spots reacted with the orcinol reagent to give a typical pentose spectrum (9), and glucose was oxidized by the specific enzyme, glucose oxidase. The eluted mannose spot gave a typical reaction for hexoses in the PCyR3 test of Dische and Shettles, as well as a yellow color after 48 hr which is characteristic for mannose. Table 2 gives a summary of the results of the chromatograms. All 20 strains possessed a substance which seems to be analogous or identical to the keto-deoxyoctonate described by Heath and Galambor (8). This material was detected by the thiobarbituric acid spray, and in solvent D could not be separated from a similar spot obtained from the LPS of *S. enteriditis*. The presence of uronic acid was detected only as a color reaction.

Absence of heptose. All strains gave a negative color reaction for heptose. In addition, 2 N HCl hydrolysates of all species were chromatographed in 95% acetone and 5% water (3) alongside a similar hydrolysate from *S. enteriditis*. The heptose spot from the *S. enteriditis* was readily apparent, but no heptose from the *Xanthomonas* species was visible. To demonstrate further that the heptose color test was not being inhibited or masked by some constituent of the LPS, the hydrolyzed LPS from 10 different *Xanthomonas* species were chromatographed and the individual sugars were eluted and assayed for heptose. In no case was heptose found.

Phosphate as a constituent of the polysaccharide portion of the LPS. Hydrolysis of intact LPS in 0.1 N HCl for 15 min at 100 C cleaved essentially all of the polysaccharide from the glucosamine-lipid portion of the molecule. Centrifugation of the hydrolyzed mixture for 1 hr at 100,000 $\times$ *g* sedimented any unhydrolyzed portion of the LPS as well as the lipid portion of the molecule. The supernatant fluid contained the polysaccharide and phosphate, but no measurable glucosamine. Experiments with *X. ricinicola* demonstrated that the intact LPS contained 0.78 μmole of phosphate per mg of LPS. After hydrolysis for 15 min at 100 C in 0.1 N HCl, 0.28 μmole of phosphate per mg of LPS was liberated, and the polysaccharide portion contained 0.32 μmole of organic phosphate per mg of original LPS.

DISCUSSION

The results presented in this paper provide evidence that the lipopolysaccharides extracted from *Xanthomonas* species have very similar biological properties to those present in the enterics. This is supported by the mouse lethality of the LPS from two different *Xanthomonas* species and the results from the Shwartzman reaction.

However, the chemical composition of the

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polysaccharide portion of the LPS is considerably different in these two groups. Westphal et al. (16) reported that, of hundreds of enterobacterial lipopolysaccharides tested, all were found to contain heptose. Osborne et al. (11, 12) suggested that the enterobacterial polysaccharides possess the general structure of a heptose-phosphate core to which chains of as many as five additional sugars may be linked glycosidically. Osborne further postulated that 2-keto-3-deoxyoctonate may provide a link between the heptose phosphate core and the lipid-glucosamine moiety.

In the present studies with *Xanthomonas* species, heptose was not detected as a component of the polysaccharides. Our results demonstrate that the cleaved polysaccharide contains organically bound phosphate, and suggest that the backbone of these heteropolysaccharides may consist of a carbohydrate-phosphate chain analogous to the heptose-phosphate chain proposed for the enterics.

On the basis of our present data, it is not possible to speculate concerning the nature of the linkage between the polysaccharide and the glucosamine-lipid portion of the molecule. The acid lability of this linkage (Fig. 1) is very similar to that reported by Osborn (11) for the LPS isolated from *S. typhimurium*, and, since keto-deoxyoctonate also seems to be a constant component of these lipopolysaccharides, it is tempting to suggest that this linkage might be similar to that suggested by Osborne for *S. typhimurium*.

The absence of the 3,6-dideoxyhexoses from these lipopolysaccharides is based on the observation that in no case were unknown sugar spots found which migrated faster than rhamnose in solvent A. Since all of the known 3,6-dideoxyhexoses do migrate considerably faster than rhamnose on this solvent, any significant amount should have been readily apparent.

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