JOURNAL OF BACTERIOLOGY, Mar. 1968, p. 782-786
Copyright © 1968 American Society for Microbiology
Vol. 95, No. 3
Printed in U.S.A.

Isolation of D-Galacturonic Acid 1-Phosphate from Hydrolysates of Cell Wall Lipopolysaccharide Extracted from *Xanthomonas campestris*

WESLEY A. VOLK
Department of Microbiology, School of Medicine, University of Virginia, Charlottesville, Virginia 22903

Received for publication 2 October 1967

D-Galacturonic acid 1-phosphate was found to be one of the products formed during hydrolysis of the cell wall lipopolysaccharide of *Xanthomonas campestris* in 0.01 N acetic acid at *p*H 3.3. The molecule was shown to consist of equimolar amounts of D-galacturonic acid and phosphate. Resistance to borohydride reduction before, but not after, treatment with *Escherichia coli* alkaline phosphatase indicated that the phosphate group is attached to carbon-1 of the galacturonic acid. The presence of an additional phosphate group in the heteropolysaccharide of the cell wall lipopolysaccharide was also demonstrated. This phosphate group was considerably more resistant to acid hydrolysis than was the phosphate associated with the galacturonic acid. It is suggested that the more resistant phosphate is attached to either mannose or glucose, or to both.

Volk (14) hypothesized that the structure of the heteropolysaccharide portion of *Xanthomonas* endotoxin is different from the structure of the polysaccharides occurring in members of the *Enterobacteriaceae* in that the *Xanthomonas* polysaccharide does not contain heptose. Heptose phosphate has been shown to provide the basic core of the lipopolysaccharide (LPS) occurring in the cell walls of the enteric bacteria, and it is from this core that the nonphosphorylated sugars branch off to form the remainder of the complex heteropolysaccharide which is responsible for the serological type of a bacterium (9, 10, 11, 12, 15). It is generally assumed that all gram-negative bacteria possess this very toxic lipopolysaccharide as a component of their cell walls, but no extensive study has been carried out on organisms not containing heptose in this polysaccharide.

The present report provides data which support the proposal that galacturonic acid is bound to the LPS of *Xanthomonas campestris* through an acid-labile phosphate ester. The isolation and identification of D-galacturonic acid 1-phosphate from the partial hydrolysis of the LPS provide support for this theory.

Materials and Methods

Xanthomonas campestris was either grown as described elsewhere (14) or was obtained from the Grain Processing Company, Muscatine, Iowa. Acetone powders were prepared from washed cells, and, prior to the extraction of the cell wall LPS, the acetone powders were suspended in distilled water (1% by weight) and stirred for at least 1 hr. After centrifugation, this step was repeated, resulting in the liberation of considerable protein. Electron micrographs showed that essentially all of the cells were ruptured.

Descending chromatography was done on Whatman #1 paper by use of the following solvent systems: A, pyridine-ethyl acetate-acetic acid-water (5:5:1:3); B, ethyl acetate-acetic acid-water (3:1:3); C, amyl acetate-acetic acid-water (3:3:1); D, pyridine-ethyl acetate-water (20:72:23); and E, *n*-butanol-pyridine-0.1 N HCl (5:3:2). Paper electrophoresis was performed in pyridine-acetic acid-water (5:2:43), *p*H 5.3, at 50 v cm^{-1} by use of a model D high-voltage electrophorator (Gilson Medical Electronics, Middleton, Wis.). Free sugars were visualized with a $AgNO_3$ dip (3), and phosphorylated compounds were visualized by use of the technique of Runecles and Krotkov (13).

Inorganic phosphate was determined by the method of Ames and Dubin (1), and total phosphate was determined by the same method after ashing. Galacturonic acid was assayed by the borate-carbazole assay (8). In cases where it was necessary to determine free galacturonic acid in the presence of bound galacturonic acid, the mixture was chromatographed on paper using solvent C, and the free galacturonic acid was eluted from the paper after overnight development. Authentic 3 - deoxy - D - mannooctulosonate (KDO) was obtained from Dr. Heath as a pentacetyl methyl ester. This ester was converted to free KDO

by treatment with 0.2 N NaOH, according to the procedure of Ghalambor et al. (7). KDO was assayed by Aminoff's thiobarbituric acid assay (2) and was identified by the fact that its migration on paper chromatography was identical to that of authentic KDO when solvent E was used. Authentic D-galacturonic acid 1-phosphate was obtained from Gilbert Ashwell. Chromatographically pure *Escherichia coli* alkaline phosphatase was purchased as an ammonium sulfate suspension (Worthington Biochemical Corp., Freehold, N.J.). This suspension contained 200 units/ml, and each unit was defined as liberating 1 μmole of phosphate per min from *p*-nitrophenyl phosphate at 25 C.

Quantitative assays for glucose and mannose were carried out after the LPS was hydrolyzed in 2 N HCl for 2 hr at 100 C. The hydrolysate was deionized by passage through a Dowex-1 acetate column, concentrated to dryness under reduced pressure, and dissolved in a known volume of water. A sample of this material was chromatographed overnight by use of solvent D. Glucose was determined enzymatically with a glucose oxidase which was purchased as a glucostat reagent (Worthington Biochemical Corp., Freehold, N.J.). The glucose assay was carried out on the hydrolyzed mixture before chromatography and on the glucose eluted from the chromatogram to provide a measure for the effectiveness of the elution technique. Mannose was determined on the material eluted from the paper chromatogram, according to the method of Dische and Shettles (5).

Isolation of the lipopolysaccharides. The washed acetone powders were extracted with hot 45% phenol by the method of Westphal et al. (16). The aqueous phase was dialyzed for 72 hr against running tap water, partially concentrated under reduced pressure, and lyophilized. The crude lyophilized LPS contained a glucan which could be eliminated by enzymatic hydrolysis with a β-glucosidase. Glucose was the only sugar solubilized by this treatment. The cellulase (Worthington Biochemical Corp., Freehold, N.J.) was prepared by dissolving the commercial preparation in water (2.5 mg/ml). Before use, the cellulase preparation was centrifuged at 37,000 × *g* for 15 min, and the supernatant solution was passed through a 0.45-μ Millipore filter to rid the preparation of any suspended solids. The LPS was suspended at 5 mg/ml in 0.05 M citrate buffer, *p*H 4.0, and the cellulase was added to yield a final concentration of 0.25 mg of crude enzyme preparation per mg of LPS. After 8 to 10 hr at room temperature, a second amount of cellulase, equal to the first addition, was added, and the reaction mixture was allowed to set overnight. At this time, the reaction mixture was again dialyzed against running tap water for 48 hr. Because of the low *p*H optimum of the cellulase, no difficulty with bacterial growth was experienced during the prolonged cellulase treatment.

After dialysis, the material was partially concentrated under reduced pressure and was then centrifuged for 60 min at 96,000 × *g* in a model L-2 65 centrifuge (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) having a type 65 rotor. The resulting supernatant fluid was discarded, and the sedimented LPS was resuspended in distilled water and recentrifuged. After the second washing and recentrifugation, the material was suspended in water and lyophilized to yield the final purified LPS.

Hydrolysis of lipopolysaccharide. A 1-g amount of LPS from *X. campestris* was suspended in 100 ml of 0.01 N acetic acid, and the *p*H was adjusted to 3.3 by the addition of 2 N acetic acid. The material was placed in a boiling-water bath for 30 min and was then cooled in a cold-water bath. The hydrolyzed material was centrifuged for 60 min at 78,000 × *g* in a Spinco model L-2 65 centrifuge having a type 30 angle-head rotor. The clear supernatant fluid was decanted and was labeled hydrolysate 1. The pellet was resuspended in 100 ml of 0.01 N acetic acid, *p*H 3.3, rehydrolyzed for 30 min at 100 C, and centrifuged. The supernatant fluid from the centrifuged material was decanted and was labeled hydrolysate 2. The pellet was suspended in 75 ml of 0.01 N acetic acid, *p*H 3.3, and the hydrolysis step was repeated for a total of four additional times. The supernatant fluid obtained by centrifugation after each hydrolysis was assayed for uronic acid to measure cleavage from the insoluble lipid-glucosamine portion of the molecule. These results are listed in Table 1.

The supernatant solutions from all six hydrolyses were pooled, adjusted to *p*H 8.2 by the addition of 3.0 N KOH, and diluted to a total volume of 1 liter with distilled water.

Chromatography. Diethylaminoethyl (DEAE) cellulose chromatography was carried out as described by Osborn (10). The pooled hydrolysates were adsorbed onto a DEAE-cellulose column (5.6 cm × 35 cm) which was previously equilibrated with 0.01 M tris-(hydroxymethyl)aminomethane (Tris) acetate, *p*H 8.2. The flow rate was 1.5 to 1.7 ml/min, and 17-ml fractions were collected at 2 C with an automatic fraction collector. The column was washed with 600 ml of distilled water before starting the gradient elution. Six liters of a linear gradient (0.02 to 0.60 M) of pyridinium acetate, *p*H 5.3, were passed through the column, and fractions were collected as before. The fractions were assayed for total carbohydrate content by the phenol-sulfuric acid assay (6). The results are illustrated in Fig. 1.

The fractions from each of the eight peaks were pooled, evaporated to dryness under reduced pressure

TABLE 1. *Release of galacturonic acid by repeated hydrolysis of 1.0 g of LPS at pH 3.3 (see text for details)*

Hydrolysis	Total μmoles of galacturonic acid in the centrifuged supernatant fluid
#1	108
#2	83
#3	70
#4	43
#5	13
#6	3

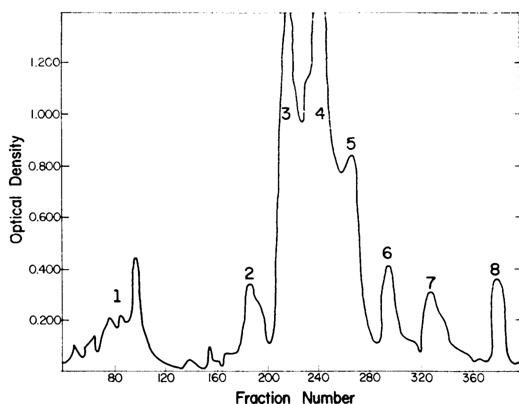

FIG. 1. *Phenol-sulfuric acid assays (6) of 1.0-ml samples of the hydrolysis (at pH 3.3) of 1.0 g of X. campestris LPS after absorption and elution from a DEAE-cellulose column (see text for details).*

at 40 C, and dissolved in a known volume of distilled water.

RESULTS

Table 2 lists the results of the quantitative assays of each peak collected from the DEAE cellulose column. As shown, all peaks contained all of the components of the heteropolysaccharide, i.e., uronic acid, glucose, mannose, and phosphate. In addition, traces of rhamnose, which were demonstrated to occur in the complete LPS at a level of 0.07 μmole/mg, were seen in peaks 3, 4, and 5.

All of the uronic acid in peaks 1 and 2 existed as free unbound uronic acid. Peak 1 contained a material which reacted with the thiobarbituric acid assay for KDO and migrated on solvent E in the same way that authentic 3-deoxy-D-manno-octulosonic acid migrates with this solvent. On this basis, it would appear that peak 1 contained free unbound KDO. No other free monosaccharide was found following hydrolysis at *p*H 3.3.

Study of the material in peak 7 was of particular interest because the large amounts of bound uronic acid and phosphate, as compared to the much smaller amounts of glucose and mannose, seemed to indicate a possible covalent linkage between uronic acid and phosphate.

Evidence for a 1-phospho-galacturonic acid linkage. The data presented in Table 3 show that phosphate can be liberated by treatment of peak 7 with alkaline phosphatase. Chromatography of the reaction mixture on solvents A, B, and C and high-voltage paper electrophoresis demonstrated that free galacturonic acid was also liberated by the phosphatase treatment. Neither free glucose nor free mannose was detected following the alkaline phosphatase treatment of peak 7.

The data in Table 4 demonstrate that galacturonic acid cannot be reduced with borohydride until the phosphate group is removed, thus providing evidence that the phosphate group is attached to carbon 1 of the galacturonic acid. Linkage to the anomeric carbon would account for the marked acid lability of this ester.

The identification of galacturonic acid was established by use of a crude uronic acid isomerase made available by Jean Hickman. This enzyme converted D-glucuronic acid to D-fructuronic acid and D-galacturonic acid to D-tagaturonic acid (4). All other known uronic acids were reported to be inactive. The formation of tagaturonic acid from the uronic acid present in the LPS of *X. campestris* is shown in Table 5, and this con-

TABLE 2. *Quantitative composition of DEAE-cellulose peaks*[a]

Peak	Uronic acid	Organic phosphate	Inorganic phosphate	Glucose	Mannose	KDO
1	100	12	—	6.0	**[b]	0.7
2	110	13.2	—	3.5	6.8	4.9
3	20.1	—	404	32.8	80	3.5
4	36.4	67	22.6	65.6	84	5.2
5	17.9	43.8	—	42.6	36	2.8
6	3.4	10.9	—	11.2	10	0.7
7	48.2	47.5	—	4.6	7.4	1.1
8	4.1	6.9	—	2.8	5.6	0.5

[a] Since only trace amounts of rhamnose were present in peaks 3, 4, and 5, quantitation of this sugar was not carried out. All values are expressed as total micromoles present in the peak.

[b] Not assayed.

TABLE 3. *Release of inorganic phosphate by treatment of peak 7 with Escherichia coli alkaline phosphatase*[a]

Galacturonate phosphate present (μmoles)	Alkaline phosphatase (units)	Inorganic phosphate released (μmoles)	Free galacturonic acid recovered (μmoles)
4.8	2.0	4.6	4.0
2.4	none	none	none

[a] A sample of peak 7 was brought to a concentration of 0.04 M by the addition of Tris buffer and was adjusted to a *p*H of 8.2 by the addition of 3.0 N KOH. To one sample, 2 units of alkaline phosphatase was added. After 60 min at room temperature, inorganic phosphate was assayed on one sample treated with phosphatase and on one sample not treated with phosphatase. Free galacturonic acid was assayed by chromatography of a sample of the reaction mixture on solvent C for 16 hr. The galacturonic acid was located by developing guide strips containing known galacturonic acid. It was eluted from the paper and assayed.

TABLE 4. *Sodium borohydride treatment of peak 7 with and without prior incubation with alkaline phosphatase*[a]

Galacturonate phosphate present (μmoles)	Alkaline phosphatase (units)	Galacturonic acid remaining after borohydride reduction (μmoles)
4.8	2.0	0.27
2.4	none	2.35

[a] Reaction with alkaline phosphatase was the same as in Table 3. Subsequent borohydride reduction was accomplished by three additions of 0.1 ml of 5% sodium borohydride at 20-min intervals. After 60 min at room temperature, excess borohydride was destroyed by acidification with acetic acid.

TABLE 5. *Enzymatic isomerization of the galacturonic acid present in peak 7*[a]

Compound	Solvent A (cm)	Solvent B (cm)
Galacturonic acid	24	18
Isomerase products	24	18
	28	27.5
Tagaturonic acid	28	27.5
Fructuronic acid	31	27.5

[a] The galacturonate phosphate (2.4 μmoles) present in peak 7 was brought to a *p*H of 2.0 by the addition of 1.0 N HCl. The material was placed in a boiling-water bath for 30 min, cooled, and concentrated at 50 C. Sodium borate buffer (0.1 ml of a 0.1 M solution, *p*H 8.5) was added, and the *p*H adjusted to 8.5. A 50-μliter amount of crude uronic acid isomerase was added, and the tubes were incubated at 37 C for 60 min. The reaction was stopped by acidification with Dowex-50 (H^+), and boric acid was removed by distillation as methyl borate. The resulting syrup was dissolved in a small amount of water, and was subjected to paper chromatography in solvents A and B.

version established the isolated uronic acid as D-galacturonic acid.

After completing the experiments which characterized the 1-phospho-galacturonic acid present in peak 7, it was found that high-voltage paper electrophoresis could separate the galacturonic acid phosphate from all of the other components in peak 7. The galacturonic acid phosphate in peak 7 moved in a manner identical to that of authentic 1-phospho-galacturonic acid and showed an R_M of 1.7 when compared to authentic mannose 6-phosphate. Because of the small amounts of material available and the fact that the glucose-mannose moiety present in peak 7 was not visualized by either the $AgNO_3$ dip (3) or the phosphate dip (13), information was not available concerning the migration of the other components of peak 7 in the high-voltage electrophoresis experiment.

Supporting evidence for the possibility that galacturonic acid is attached to the LPS through an acid-labile phosphate ester is shown in Table 6. These data demonstrate that very mild acid hydrolysis (0.01 N HCl) will liberate an amount of phosphate equivalent to the amount of uronic acid present in the LPS. Other experiments have demonstrated that, under similar conditions, 0.01 N HCl hydrolysis will liberate essentially all of the galacturonic acid from the LPS (0.34 μmole from a total of 0.39 μmole). Since chromatography and elution of known amounts of galacturonic acid gave low recoveries, it is believed that these values represent complete liberation of uronic acid from the LPS.

DISCUSSION

Galacturonic acid is probably attached to the cell wall lipopolysaccharide of *X. campestris* through a phosphate ester. This conclusion is based on (i) the isolation of D-galacturonic acid 1-phosphate from a mild acid hydrolysis (*p*H 3.3, 100 C, 60 min) of the intact LPS, and (ii) the stoichiometric liberation of galacturonic acid and inorganic phosphate by hydrolysis of the LPS in 0.01 N HCl at 100 C for 10 min.

Additional evidence for the location of the phosphate on carbon 1 of the galacturonic acid was provided by the sodium borohydride reduc-

TABLE 6. *Release of inorganic phosphate by hydrolysis of the intact LPS from Xanthomonas campestris in various concentrations of HCl*[a]

Normality of HCl	Inorganic phosphate in hydrolysate (μmoles/mg of LPS)	Total phosphate in hydrolysate (μmoles/mg of LPS)
0.01	0.48	0.80
0.05	0.52	0.81
0.10	0.52	0.81
0.20	0.52	0.81
0.50	0.49	0.91
1.0	0.52	0.96

[a] The LPS (1.0 mg/ml) was suspended in various normalities of HCl and was hydrolyzed at 100 C for 60 min. Total and inorganic phosphate of the supernatant fluid were determined after discarding the insoluble lipid-containing material. Total uronic acid was determined to be 0.47 μmoles per mg of LPS after the 0.2 N HCl hydrolysis. The increase in total phosphate after 0.5 N and 1.0 N HCl hydrolysis is believed to be a result of partial solubilization of the lipid moiety of the LPS.

tion studies with and without prior treatment with alkaline phosphatase.

The results of total and inorganic phosphate analyses after hydrolysis of the LPS with various concentrations of HCl demonstrate that there are two different phosphate linkages in this heteropolysaccharide. The more acid-labile phosphate seems to be linked to the anomeric carbon of galacturonic acid, thus forming a phosphodiester linkage with the polysaccharide molecule. The remaining phosphate seems to be in a phosphodiester linkage with mannose or glucose, or both, since treatment of the intact LPS with alkaline phosphatase did not liberate any phosphate. This latter phosphate linkage may well be analogous to the phosphate linked to heptose to form the core structure of the LPS from enteric organisms.

The observation that neither free glucose nor free mannose was liberated by the alkaline phosphatase treatment of peak 7 indicates that these sugars were not present as simple sugar monophosphates. Since the thiobarbituric acid-reacting material present in peak 7 did not migrate on paper chromatography with either authentic KDO or the KDO-like material in peak 1, it seems probable that it is bound to a larger molecule, i.e., a glucose-mannose complex.

Acknowledgments

This investigation was supported by Public Health Service research grant AM 08338 from the National Institute of Arthritis and Metabolic Diseases.

The author is indebted to Gilbert Ashwell for his valuable suggestions and Nancy Salomonsky for her technical assistance.

Literature Cited

1. Ames, B. N., and D. L. Dubin. 1960. The role of polysamines in the neutralization of bacteriophage deoxyribonucleic acid. J. Biol. Chem. **235**:769–775.
2. Aminoff, D. 1961. Methods for the quantitative estimation of N-acetylneuraminic acid and their application to hydrolysates of sialomucoids. Biochem. J. **81**:384.
3. Anet, E. F. L. G., and T. M. Reynolds. 1954. Isolation of mucic acid from fruits. Nature **174**:930.
4. Ashwell, G., A. J. Wahba, and J. Hickman. 1960. Uronic acid metabolism in bacteria. I. Purification and properties of uronic acid isomerase in *Escherichia coli*. J. Biol. Chem. **235**:1559–1565.
5. Dische, Z., and L. B. Shettles. 1948. A specific color reaction of methyl pentoses and a spectrophotometric micromethod for their determination. J. Biol. Chem. **175**:595–603.
6. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
7. Ghalambor, M. A., E. M. Levine, and E. C. Heath. 1966. The biosynthesis of cell wall lipopolysaccharide in *Escherichia coli*. III. The isolation and characterization of 3-deoxyoctulosonic acid. J. Biol. Chem. **241**:3207–3215.
8. Gregory, J. D. 1960. The effect of borate on the carbazole reaction. Arch. Biochem. Biophys. **89**:157–160.
9. Naide, Y., H. Nikaido, H. Makela, R. G. Wilkinson, and B. A. D. Stocker. 1965. Semirough strains of *Salmonella*. Proc. Natl. Acad. Sci. U.S. **53**:147–153.
10. Osborn, M. J. 1963. Studies on the gram negative cell wall. I. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. U.S. **50**:499–506.
11. Osborn, M. J., S. M. Rosen, L. Rothfield, and B. L. Horecker. 1962. Biosynthesis of bacterial lipopolysaccharide. I. Enzymatic incorporation of galactose in a mutant strain of *Salmonella*. Proc. Natl. Acad. Sci. U.S. **48**: 1831–1838.
12. Robbins, P. W., A. Wright, and J. L. Bellows. 1964. Enzymatic synthesis of the *Salmonella* O antigen. Proc. Natl. Acad. Sci. U.S. **52**: 1302–1309.
13. Runecles, V. C., and G. Krotkov. 1957. The separation of phosphate esters and other metabolites by ionophoresis and chromatography on paper. Arch. Biochem. Biophys. **70**:442–453.
14. Volk, W. A. 1966. Cell wall lipopolysaccharides from *Xanthomonas* species. J. Bacteriol. **91**: 39–42.
15. Westphal, O., I. Beckman, U. Hammerling, K. Jann, and O. Luderitz, 1964. *In* M. Landy and W. Braun [ed.], Bacterial endotoxins. Rutgers Univ. Press, New Brunswick, N.J.
16. Westphal, O., O. Luderitz, and Z. Bister. 1952. Uber die Extraktion von Bacterien mit Phenol/Wasser. Z. Naturforsch. **7b**:148.