The Presence of Acidic Polysaccharides and Muramic Acid Phosphate in the Walls of *Corynebacterium poinsettiae* and *Corynebacterium betae*

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SUMMARY

Wall preparations from *Corynebacterium poinsettiae* and *Corynebacterium betae* contained no teichoic acids but phosphate-free polysaccharides were present and were extractable with trichloroacetic acid at 35 °C. The substance from *C. poinsettiae* contained rhamnose, glucuronic acid, galactose, mannose and pyruvic acid (molecular proportions 1:2:1:0.33:0.35) and that from *C. betae* contained glucuronic acid, rhamnose, fucose and mannose (molecular proportions 1:2:0.5:0.3). In the former polymer, pyruvic acid appeared to be linked to galactose which was in turn linked to rhamnose. The residual peptidoglycans of these species contained much phosphorus. Some of this was present as muramic acid phosphate.

INTRODUCTION

The wall compositions of species of *Corynebacterium* have been studied in connexion with taxonomic significance (Cummins, 1962). Several plant-pathogenic species did not conform to the arabinose–galactose pattern of animal pathogens, and later the amino acid constituents of the peptidoglycan component were also found to be abnormal (Perkins & Cummins, 1964; Perkins, 1965). We have now studied in greater detail the non-peptidoglycan components of the isolated walls of two species.

METHODS

Preparation of the wall. *Corynebacterium poinsettiae* NCPP 177 and *Corynebacterium betae* NCPP 373 were grown at 30 °C in the casein hydrolysate medium CY (Novick, 1963) supplemented with 1 % (w/v) of glucose. Bacteria were harvested at 2 °C and suspended in distilled water, and the walls were prepared by differential centrifuging after disruption of the organisms by shaking with glass beads (Ballotini no. 12) in a cell homogenizer (Model MSK, B. Braun, Melsungen, Germany). The autolytic enzymes were inactivated by heating the suspension at 60 °C for 5 to 10 min. The walls were suspended in 0.05 M-sodium phosphate buffer, pH 7.6, digested with trypsin (1 mg/ml) at 37 °C overnight and then washed several times with distilled water. The trypsin-digested walls were subsequently treated with pepsin (0.5 mg/ml) in 0.05 M-HCl at 37 °C overnight, then washed twice with 1.0 M-NaCl, twice with distilled water and freeze-dried.

Isolation of wall polysaccharide. The following procedures were tried (though only method (iv) proved satisfactory): (i) Walls were mixed with 10 to 25 % (w/v) trichloroacetic acid at 0 °C and shaken for two or three days. After centrifuging, the supernatant liquid was treated with 6 vol. of ethanol or acetone (Armstrong *et al.* 1958). (ii) Walls were hydrolysed

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with 0.05 M-H₂SO₄ at 60 °C for 3 h or at 100 °C for 1 h (Knox & Hall, 1965). After neutralization with saturated aqueous Ba(OH)₂ and centrifuging, the supernatant liquid was freeze-dried. (iii) Walls were extracted with aqueous phenol (45 %, w/v) at 60 °C for 5 min (Hungerer *et al.* 1967). After centrifuging, both the aqueous layer and the sediment were dialysed against distilled water and freeze-dried. (iv) Walls were treated with 10 % (w/v) trichloroacetic acid at 35 °C overnight. After centrifuging, the supernatant liquid was extracted with ether to remove trichloroacetic acid and the aqueous layer was freeze-dried.

Analytical methods. Glucuronic acid was determined with carbazole (Davidson, 1966) and methylpentoses with cysteine-sulphuric acid (Dische & Shettles, 1947). Neutral sugars were estimated after acid hydrolysis (2 M-HCl, 3 h, 100 °C) by microchromatography on Eastman Kodak film 511V developed with triphenyltetrazolium as described by Moczar, Schilling & Robert (1967); the intensities of the spots were measured in the Chromoscan apparatus (M.K.2 Joyce, Loebel & Co. Ltd, Gateshead, County Durham). *N*-acetylglucosamine was determined as glucosamine by the method of Rondle & Morgan (1955) after hydrolysis with 4 M-HCl for 4 h at 100 °C, paper electrophoresis in 0.25 M-formic acid and elution of the glucosamine from the paper. Pyruvic acid was determined with *o*-phenylenediamine in 0.5 M-HCl (Harbon, Herman & Clauser, 1968). Phosphorus was measured as described by Umbreit, Burris & Stauffer (1957). Glucosamine and muramic acid were separated by adsorption of muramic acid to charcoal (Hughes, 1968) and measured by the method of Rondle & Morgan (1955).

Chromatography and electrophoresis. The paper used was Whatman no. 3 or 3MM. Paper chromatography was done in the following solvents: 1. *n*-butanol-acetic acid-H₂O (67:10:23, by vol.); 2. *n*-butanol-acetic acid-H₂O (4:1:5, by vol.); 3. *n*-butanol-pyridine-H₂O (6:4:3, by vol.); 4. ethyl acetate-acetic acid-H₂O (9:2:2, by vol.); 5. *n*-propanol-acetic acid-H₂O (5:1:1, by vol.). Paper electrophoresis was at 10 V/cm for 2 to 3 h in the following electrolytes: I. 0.25 M-formic acid, pH 1.9; II. acetic acid-collidine-H₂O (2.65:9.10:1000, by vol.), pH 7; III. 0.01 M-sodium phosphate buffer, pH 6.8; IV. 0.01 M-sodium borate buffer, pH 9.2.

The papers were developed with alkaline AgNO₃ for detection of sugars (Trevelyan, Procter & Harrison, 1950), ninhydrin for amino acids, Schiff reagent for glycols (Buchanan, Dekker & Long, 1950), the reagent of Hanes & Isherwood (1949) for phosphate, and that of Rydon & Smith (1952) for acetamido groups. Electrophoresis of the undegraded wall polysaccharide was carried out on strips of cellulose acetate (Oxoid, London) in 0.05 M-sodium phosphate buffer, pH 6.9, at 200 V for 20 min. The strips were developed with 1 % (w/v) alcian blue in 5 % (v/v) acetic acid (Herd, 1968).

Column chromatography. Undegraded polysaccharide was applied to a column (39 × 1 cm) of Sephadex G100 (Pharmacia, Uppsala, Sweden), which had been previously equilibrated with 1.0 M-NaCl, and was eluted with the same solution. Fractions (3 ml) were collected and uronic acids were determined.

Ultracentrifugation. Ultracentrifugation was carried out in a model E Spinco analytical ultracentrifuge. Polysaccharide (10 mg/ml) was dissolved in 0.02 M-citrate buffer (pH 4.25) containing 0.1 M-NaCl. Sedimentation velocity was measured in a capillary-type synthetic boundary cell. Molecular weights were determined by the method of Yphantis (1960).

Action of phosphatase on the wall products. The presence of a phosphomonoester in wall products was detected by incubation with a solution of alkaline phosphomonoesterase (enzyme from *Escherichia coli*, Sigma (London) Ltd, Kingston-upon-Thames, Surrey, 10 mg/ml) in 0.2 M-ammonium carbonate (pH 8.4) for 16 h at room temperature, followed by measurement of inorganic phosphate.

RESULTS

Composition of the walls of Corynebacterium poinsettiae and Corynebacterium betae

Treatment of the wall of *Corynebacterium poinsettiae* and *C. betae* with aqueous trichloroacetic acid at 0 °C did not solubilize any polymer. Hydrolysis with dilute sulphuric acid or treatment with phenol–water extracted from the wall only a small amount of polymer, which was contaminated with amino acids and amino sugars from the peptidoglycan. By extraction of the walls of both species with 10 % (w/v) trichloroacetic acid at 35 °C polysaccharides were obtained practically free of the peptidoglycan components. They accounted for 50 % of the weight of the wall and contained no detectable phosphate. Neither micro-organism contained wall teichoic acids, since no glycerol or ribitol could be detected in hydrolysates of the walls.

Homogeneity of the polysaccharide from Corynebacterium poinsettiae

The polysaccharide appeared to be homogeneous by the following criteria:

(i) *Ultracentrifugation*. The material gave a sedimentation coefficient ($s_{20,w}$) of 1.28 S when the partial specific volume (v) was taken as 0.63, and was monodisperse after 3.5 h centrifuging at 59 700 rev./min. The apparent molecular weights at concentrations of 0.64, 0.42 and 0.21 % were measured at two speeds (20 410 and 27 690 rev./min) at 32 min intervals, beginning 90 min after reaching speed. Five photographs were taken at different times. The results at any particular concentration showed no significant dependence on time or speed and were therefore averaged. This procedure gave the apparent molecular weights as 10 000, 10 900 and 12 300 at 0.64, 0.42 and 0.21 % respectively, i.e. there was some dependence on concentration.

(ii) *Gel filtration*. Only one component was detected after filtration through Sephadex G100, with a peak at 19 ml of eluent. The void volume of the column was 12 ml. This component contained all the chemical components of the untreated polysaccharide.

(iii) *Electrophoresis*. A single anionic band was detected with alcian blue when the polysaccharide was run at pH 6.9 on cellulose acetate electrophoresis.

Composition of the polysaccharide from Corynebacterium poinsettiae

By hydrolysis of the polysaccharide with 2 M-HCl at 100 °C for 3 h followed by chromatography in solvents 1, 2, 3 and 4 and electrophoresis in buffers I and II, the following sugars were identified as main components: rhamnose, glucuronic acid, galactose and mannose. Traces of glucose were also found. By quantitative microchromatography in solvent 5 the proportions of rhamnose–glucuronic acid–galactose–mannose were 1:2:1:0.33. The amount of glucuronic acid relative to the other sugars was also determined with the carbazole reagent with the same result.

After, but not before, partial hydrolysis of the polysaccharide with 0.5 M-HCl at 100 °C for 15 min, material giving the Elson–Morgan reaction for *N*-acetylhexosamines was released. Paper chromatography in solvents 2 and 3 yielded a single spot giving a positive reaction for sugar and acetamido groups. In both solvents it coincided with *N*-acetylglucosamine and upon isolation from the chromatogram and reaction with the Ehrlich reagent (Reissig, Strominger & Leloir, 1955) gave a chromogen with the same absorption spectrum as that given by *N*-acetylglucosamine under the same conditions. The *N*-acetylglucosamine released was measured and gave a ratio to total rhamnose of 0.1:1. This represented virtually the whole of the glucosamine that could be found in a total acid hydrolysate. Thus glucosamine was at most a minor component or could conceivably have been a contaminant.

After hydrolysis with 1.0 M-HCl at 100 °C for 3 h and electrophoresis in buffer 2, a silver nitrate-positive spot was found that moved further towards the anode than glucuronic acid or phosphoric acid and that coincided with pyruvic acid. On chromatography in solvents 2 and 3 the R_f value of this component also coincided with pyruvic acid. When the compound was eluted and allowed to react with 2,4-dinitrophenylhydrazine it gave the characteristic absorption spectrum of the 2,4-dinitrophenylhydrazone of pyruvic acid. The extracellular polysaccharide from *Corynebacterium insidiosum* is known to contain a substituted monosaccharide identified as 4,6-*O*-(1'-carboxyethylidene)-D-galactose, which on hydrolysis produced pyruvic acid and galactose (Gorin & Spencer, 1964). When walls of *C. insidiosum* were suspended in water and heated at 100 °C overnight in a sealed tube they gave the same silver nitrate-positive spot on electrophoresis in buffer 2 that was observed with the polysaccharide hydrolysate from *C. poinsettiae*. The amount of pyruvic acid residues in the polysaccharide from *C. poinsettiae* was 2.4 %, the molar ratio to rhamnose being 0.35:1. The known components described here accounted for 88 % by weight of the dry polysaccharide.

Composition of the polysaccharide from Corynebacterium betae

The same methods of identification and quantitative estimation described for the polysaccharide from *Corynebacterium poinsettiae* were followed with the polysaccharide from *C. betae*, which also appeared to be a single polymer as judged by gel filtration (where it eluted in the same position as the polysaccharide from *C. poinsettiae*) and electrophoresis on cellulose acetate. The main components were glucuronic acid, rhamnose, fucose and mannose in the proportions 1:2.0:5:0.3 respectively. Traces of glucose and pyruvic acid were also detected.

Identification of muramic acid phosphate

The walls of both *Corynebacterium poinsettiae* and *C. betae* contained phosphate. The peptidoglycan that remained after extraction of the wall with 10 % (w/v) trichloroacetic acid also contained phosphate, but the polysaccharide did not.

To identify the phosphorylated component, peptidoglycan was hydrolysed in 2 M-HCl for 3 h at 100 °C. By electrophoresis of the hydrolysate in buffer I two components containing phosphate were identified. One coincided with phosphoric acid and the other was ninhydrin-positive and gave the Elson-Morgan reaction for amino sugar (Partridge, 1948). This compound was isolated by preparative electrophoresis and incubated with alkaline phosphomonoesterase; the products were inorganic phosphate and a hexosamine that coincided with muramic acid both in electrophoresis in buffers I and II and in chromatography in solvents 1 and 3. The eluate from the chromatogram after reaction with the Elson-Morgan reagent (Rondle & Morgan, 1955) gave a chromogen with the same absorption spectrum as that of muramic acid (i.e. the maximum was at 510 nm and the extinction increased after 24 h).

The proportions of muramic acid, glucosamine and phosphate in the wall and peptidoglycan of *Corynebacterium poinsettiae* and *C. betae* are shown in Table 1. The treatment of either the wall or the purified peptidoglycan of *C. poinsettiae* and *C. betae* with alkaline phosphomonoesterase did not release any inorganic phosphate.

Partial acid hydrolysis of the polysaccharide from Corynebacterium poinsettiae

The isolated polysaccharide was hydrolysed in dilute hydrochloric acid for periods of up to 1 h. The yield of oligosaccharides appeared to be best when 0.5 M-HCl was used at 100 °C for 15 min. A preliminary separation of the product of this hydrolysis by electrophoresis

Table 1. *The hexosamine and phosphate contents of walls and peptidoglycan*

The values are given in $\mu\text{mol/mg}$ dry wt and show the range of 3 or 4 estimations.

Organism	Fraction	Glucosamine	Muramic acid	Phosphate
<i>Corynebacterium poinsettiae</i>	Wall	0.35	0.38 to 0.39	0.14 to 0.17
	Peptidoglycan	0.64	0.42	0.30 to 0.41
<i>Corynebacterium betae</i>	Wall	0.28	0.32 to 0.34	0.26 to 0.31
	Peptidoglycan	0.68 to 0.74	0.40	0.58 to 0.70

in buffer II yielded neutral and acidic components. The neutral material was fractionated by chromatography in solvent 3 and in addition to free rhamnose it contained galactose, mannose and *N*-acetylglucosamine, and an oligosaccharide that ran with *N*-acetylglucosamine in solvent 3 but separated from it in solvent 1. On further hydrolysis the oligosaccharide gave rhamnose and galactose in equimolecular proportions. The acidic material isolated after electrophoresis gave three components during chromatography for 48 h in solvent 3. The slowest hardly moved from the origin and contained all the sugars of the complete polysaccharide. The second component yielded only galactose and pyruvic acid on further hydrolysis. The fastest-moving component contained galactose, pyruvic acid, rhamnose and glucuronic acid. The results indicate that, as in other organisms, at least some of the pyruvic acid is linked to galactose, which in turn is joined to rhamnose.

In some experiments aqueous solutions of the acidic polysaccharide were allowed to auto-hydrolyse at 100 °C for periods of 1 to 3 h. In each case the only products of small molecular weight were pyruvic acid and galactose. This result implies that at least some of the galactose residues were present as non-reducing terminals.

DISCUSSION

The extracellular acidic polysaccharides of some plant-pathogenic species of *Corynebacterium* contain galactose, glucose and fucose and acidic groups attributable to pyruvic acid present as a carboxyethylidene group (Gorin & Spencer, 1961, 1964). The present work shows that the walls of *Corynebacterium poinsettiae* contain a somewhat similar polysaccharide, which is covalently linked to the peptidoglycan component. The latter is remarkable for its high phosphorus content, some of which could be isolated as muramic acid phosphate. None of the phosphorus was released as teichoic acid by acid extraction and no evidence for the presence of glycerol or ribitol could be obtained. It seems probable that the polysaccharide was linked glycosidically to some of the phosphate residues, as proposed for *Lactobacillus casei* (Knox & Hall, 1965). Whereas the polysaccharide from *L. casei* was released by hydrolysis in 0.05 M-sulphuric acid at 60 °C within a few hours, this treatment had little effect on the walls of *C. poinsettiae*. However, the linking sugar in *L. casei* was a hexosamine, as also in the rapidly released teichuronic acid of *Bacillus licheniformis* (Hughes, 1970), whereas in the polysaccharide from the walls of *C. poinsettiae* only a little hexosamine was present, none of which gave Elson-Morgan chromogen in unhydrolysed samples.

Since their original description in seaweed polysaccharide (Hirase, 1957), carboxyethylidene and other alkylidene groups have been observed in many exocellular microbial polysaccharides, for example: *Xanthomonas campestris*, Sloneker & Orentas (1962); *Klebsiella rhinoscleromatis*, Wheat, Dorsch & Godoy (1965); and *Salmonella typhimurium*, *Escherichia coli* and *Aerobacter cloacae*, Garegg, Lindberg, Onn & Sutherland (1971). The proportion of pyruvic acid found in the wall polysaccharide of *Corynebacterium poin-*

settiae (2.4 %, w/w) was comparable with the amounts found in other bacterial products. However, under similar growth conditions *C. betae* elaborated a related wall polysaccharide that contained only traces of pyruvic acid.

The preliminary results on the structure of the polysaccharide from *Corynebacterium poinsettiae* suggest that, as in the products of other organisms, the pyruvic acid is linked to galactose, and that some of the galactose is present as non-reducing terminals. This resembles the structure proposed for a mucoid from *Salmonella typhimurium* (Garegg, Lindberg, Onn & Holme, 1971).

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