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XANTHOMONAS POLYSACCHARIDES – IMPROVED METHODS FOR THEIR COMPARISON

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ABSTRACT

The extracellular polysaccharides from a number of Xanthomonas *strains, together with preparations from strains grown under different conditions, have been examined by conventional biochemical methods. The carbohydrate composition showed little variation – D-glucose, D-mannose and D-glucuronic acid being present in the approximate molar ratio 2:2:1 – but considerable differences were noted in the acetyl and pyruvyl contents. Two different enzyme preparations have been used to digest the polysaccharides and the resultant products have been examined. Determination of the ratio of certain fragments from enzyme degradation under standard conditions provides an accurate indication of the proportion of pyruvylated to non-pyruvylated side-chains and may also indicate the presence of side-chains lacking glucuronic acid.*

INTRODUCTION

The exopolysaccharides of bacteria may either be distinct chemotypes from different strains of the same species or they may represent a common chemotype secreted by bacteria of varying diversity. The polymers from different *Klebsiella (Enterobacter) aerogenes* strains conform to the first pattern, comprising a large group of differing chemotypes with distinct structural units, many being acetylated and pyruvylated (Sutherland, 1977). Colanic acid, the polysaccharide composed of hexasaccharide repeating units, which is synthesised by *Escherichia coli*, *Salmonella* species and *Aerobacter (Enterobacter) cloacae* strains represents an example of the second pattern (Lawson *et al.*, 1969), as does bacterial alginate isolated from *Azotobacter vinelandii* or from *Pseudomonas aeruginosa* (Gorin & Spencer, 1966; Linker & Jones,

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1966). Exopolysaccharides formed of repeating units can, however, show considerable variation in the nature and extent of their acylation despite apparent constant carbohydrate composition (Garegg *et al.*, 1971b).

Attention has focused on exopolysaccharides from *Xanthomonas* species because of their commercial potential as rheological agents (Morris, 1977). Structural studies have revealed the polysaccharide of *Xanthomonas campestris* as having a cellulose backbone with attached acylated trisaccharides (Jansson *et al.*, 1975; Melton *et al.*, 1976). Similarities have been noted between the physical properties of *X. campestris* exopolysaccharide and preparations from *Xanthomonas phaseoli* and other *Xanthomonas* species (Morris *et al.*, 1977). As only four species of *Xanthomonas* are now recognised (Buchanan & Gibbons, 1974), it was of interest to determine whether the four species and isolates of the same species from different plant hosts and geographical locations produced the same polymer. Thus, improved and rapid methods of polymer 'definition' were required. The published analyses also indicated the presence of pyruvate in a molar ratio less than one per pentasaccharide repeating unit; the acetate content also appears to vary. This could be due to several possible distributions of the pyruvate ketal: (i) it could occur regularly on every second or third repeating unit; (ii) it could be distributed randomly; or (iii) some polysaccharide chains might be completely pyruvylated while others were pyruvate-free or contained very much less pyruvate. Most of the bacterial exopolysaccharides examined so far, contain acyl or ketal groups in a molar ratio of one per repeating unit. However, a strain of *Klebsiella aerogenes* type 54 was unusual in that acetyl groups were detected regularly on every alternate repeating unit (Sutherland & Wilkinson, 1968). This was recognised through digestion of the polymer with two different enzymes, both endofucosidases, one of which yielded an acetylated octasaccharide while the other produced equal quantities of an acetylated and a non-acetylated tetrasaccharide.

The present paper describes an investigation of the composition of a number of preparations of *Xanthomonas* exopolysaccharides using analysis and enzyme hydrolysis.

METHODS

Micro-organisms

The strains listed in Table 1 were obtained from the National Collection of Plant Pathogenic Bacteria, Harpenden, Hertfordshire, England. *X. campestris* 6 was a laboratory strain. Cultures were maintained in screw-capped vials on a synthetic medium (Sutherland & Wilkinson, 1965) supplemented with 1% (w/v) casamino acids.

Polysaccharide Production

The synthetic medium without amino acid supplementation was used for routine preparation of most polysaccharides. A few strains required the supplemented medium

TABLE 1
Percentage Composition (Dry Weight Basis) of *Xanthomonas* Polysaccharides

Strain	*Host*	*Glucose*	*Mannose*	*Glucuronic acid*	*Acetate*	*Pyruvate*
X. campestris	Brassicas	30·1	27·3	14·9	6·5	7·1
X. albilineans 1738[a]	Sugar cane	30·2	27·5	13·9	5·8	5·1
X. albilineans 2257[a]	Sugar cane	25·5	26·1	14·2	5·1	5·8
X. axonopodis 457		30·6	28·6	13·7	5·5	7·2
X. fragaria 1469	Strawberry	32·5	28·5	15·1	3·9	7·0
X. fragaria 1822[a]	Strawberry	24·6	26·1	14·0	5·5	4·9
X. gummisudans 2182	Gladiolus	34·8	30·7	16·5	10·0	4·7
X. juglandis 411	Walnut	33·2	30·2	16·8	6·4	6·9
X. manihotis 1159	Tapioca	33·3	28·7	16·7	6·8	7·6
X. phaseoli 556	Bean	29·1	34·2	17·8	1·8	7·7
X. phaseoli 1128	French bean	30·9	28·6	15·3	6·4	1·8
X. vasculorum 702	Sugar cane	34·9	30·2	17·9	6·3	6·6
X. vasculorum 796	Sugar cane	33·2	26·2	15·3	8·8	5·4

[a] These three preparations also contained D-galactose.

for satisfactory growth. The medium was dispensed in 1 litre amounts into 2-litre Erlenmeyer flasks. To determine the effect of various carbon sources on polysaccharide composition, glucose was replaced in the medium by the carbon source at 1% (v/v) concentration. All cultures were incubated at 30°C and harvested after 36 h on a rotary shaker. The supernatant fluids were recovered after centrifugation at 200 000 g for 1 h in a preparative ultracentrifuge, dialysed overnight against cold distilled water and poured into two volumes of cold (−20°C) acetone. The precipitated material was washed with acetone and dried under vacuum at 30°C. Purification was effected by redissolving the polymers, further dialysis against distilled water, ultracentrifugation and reprecipitation with acetone.

Enzyme Preparation

The two enzyme-producing cultures used were grown in shake flasks of synthetic medium at 30°C; details of the cultures and of the mode of enzyme preparation and specificity will be published later. Enzymes were obtained from the cultures following 96 h growth in medium with commercial gum xanthan as the sole carbon source. The enzyme preparations were tested for glycosidase activities using nitrophenyl-glycosides as substrates. Enzyme action on the polysaccharides was followed by release of oligosaccharides and by measurement of reducing values by the technique of Park & Johnson (1949).

Substrate Modification

Polysaccharide from the laboratory strain of *X. campestris* was deacetylated by treatment with 0·1 M NH_4OH at 40°C for 60 min, dialysed against tap water, then

distilled water and lyophilised. Pyruvate groups were removed from the polysaccharide by the method of Dudman & Heidelberger (1969) and the polymer similarly recovered and isolated. The carboxyl-reduced polymer was prepared essentially according to Hungerer *et al.* (1967).

Hydrolysis and Analysis

The polysaccharides were hydrolysed in 0·5 M trifluoroacetic acid for 16 h; hydrolysates were evaporated to dryness, redissolved in a small volume of H_2O and again evaporated under vacuum. The methods of microanalysis for monosaccharides, acyl and ketal groups have been used in previous studies (Sutherland, 1971). Total carbohydrate was determined by a micromodification of the phenol/H_2SO_4 reaction (Dubois *et al.*, 1956) using 700 µl total volume. All analyses were performed in duplicate on at least two preparations of polysaccharide. Alditol acetates were prepared and examined essentially as described by Laine *et al.* (1972).

Paper Chromatography and Electrophoresis

Whatman No. 1 paper was used for descending paper chromatography in the following solvents: (A) butan-1-ol-pyridine-water (6:4:3); (B) ethyl acetate-acetic acid-formic acid-water (18:3:1:4); (C) methanol-acetic acid-water (16:1:3); (D) butan-1-ol-acetic acid-water (4:1:5); (E) butan-1-ol-ethanol-water (4:1:5). Paper electrophoresis on Whatman 3 mm paper was performed in a Locarte (London) equipment with 80 x 20 cm cooled plate area. Pyridinium acetate buffer pH 5·3 (pyridine-acetic acid-water 5:2:43) (solvent F) was used with a current of approximately 100 mA for 3–4 h.

RESULTS

Polysaccharides of Xanthomonas *Strains*

All the preparations listed in Table 1 contained the same major monosaccharides when hydrolysed and subjected to paper chromatography. Mannose, glucose, glucuronic acid and glucuronic lactone were recognised by comparison with standards. Preparative paper chromatography was used to isolate samples of these components (solvent A) and confirm the identity of D-mannose and D-glucose by specific enzymic micromethods. The uronic acid was tested by the carbazole reaction which was strongly positive and was clearly different in its chromatographic mobility on DEAE paper (solvent C) from mannuronic, galacturonic and guluronic acids. Its identity as the D-isomer was confirmed by the increase in D-glucose following carboxyl reduction. Pyruvate and acetate were detectable by the dinitrophenylhydrazine and hydroxamic acid reactions, respectively. Derivatives prepared and chromatographed in solvents A and E respectively were detected in the same position as the authentic compounds.

Quantitative analysis showed that, while the carbohydrate composition remained relatively constant in the different preparations, variations in acetate and pyruvate

content did occur. Two strains, both originally classified as *X. phaseoli*, appeared to produce polymers differing considerably from the other preparations in their very low content of acetate and pyruvate, respectively. In neither case was sufficient of the respective acyl or ketal derivative available to be recognisable in chromatograms as was the case for other polysaccharides tested.

Polysaccharides were also prepared from a number of mutants recognisable either by their altered colonial appearance or colony viscosity. Products from the laboratory strain grown on different carbon sources were also prepared as were deacetylated, depyruvylated and carboxyl-reduced polysaccharides from this strain. In addition, polymer was isolated during mid-logarithmic phase of growth and from a culture grown in sub-inhibitory concentrations of fosfomycin (an antibiotic analogue of phosphoenolpyruvate). Analysis of these products are listed in Table 2.

TABLE 2
Percentage Composition (Dry Weight Basis) of Mutant and Derived Polysaccharides

X. campestris	*Glucose*	*Mannose*	*Glucuronic acid*	*Acetate*	*Pyruvate*
Culture, 20 h	30·1	27·3	14·9	6·5	7·1
Culture, 6 h	27·8	26·9	17·5	2·1	5·8
Mutant 5	34·1	26·1	15·3	4·3	8·2
Mutant 6	37·0	28·5	15·8	3·6	6·1
Grown on:					
Alanine	29·1	30·2	14·3	6·0	5·0
Glutamate	34·0	29·6	15·7	5·4	8·6
Glycerol	30·0	28·2	14·3	4·2	8·0
Pyruvate	29·6	29·8	13·8	6·4	10·6
Fosfomycin medium	30·4	27·7	15·7	4·7	6·2
Deacetylated	36·5	37·1	16·8	0	7·0
Depyruvylated	42·6	33·1	19·9	0	0·4
Carboxyl-reduced	57·3	38·1	1·3	0	–

Enzyme Reactions

The two enzymes were tested on all the polysaccharide preparations listed in Tables 1 and 2 using a standard incubation time of 16 h at 30°C. In the majority of substrates tested, the reducing value of the solutions increased greatly and two fractions were obtained, along with varying amounts of neutral material, on paper electrophoresis. The fragments from treatment with enzyme A – designated A_1 and A_2 – had M_{GlcA} 0·70 and 0·39, respectively in solvent E; the products from enzyme B, B_1 and B_2 had different mobilities – M_{GlcA} 1·03 and 0·45, respectively. The presence or absence of these fractions and the ratio of fraction 1 to fraction 2 for enzyme A is shown in Table 3. Similar results were obtained using enzyme B, but as the enzyme preparations contained less activity, smaller yields of oligosaccharides were obtained and conse-

TABLE 3
Fractions Obtained from *Xanthomonas* Polysaccharides by Enzyme Treatment

Strain	*Fraction* A_1	*Fraction* A_{21}	*Fraction* A_{22}	*Ratio* A_1/A_2
X. campestris 6	+	+	+	1:2·7
glutamate grown	+	+	+	1:1·5
alanine grown	+	+	+	1:2·1
glycerol grown	+	+	+	1:1·1
pyruvate grown	+	+	+	1:0·5
mutant 5	+	+	+	1:3·0
mutant 6	+	+	+	1:4·8
X. axonopodis 457	+	+	+	1:3·3
X. fragaria 1469	+	+	+	1:1·9
X. gummisudans 2182	+	+	+	1:0·5
X. mannihotis 1159	+	+	+	1:2·3
X. phaseoli 556	+	–	+	1:4·7
X. phaseoli 1128	–	+	+	–
X. juglandis 411	+	+	+	1:3·7
X. vascolorum 702	+	+	+	1:1·4
X. vascolorum 796	+	+	+	1:5·0
X. campestris				
deacetylated	+	–	+	1:2·5
depyruvylated[a]	–	–	+	–
carboxyl reduced	–	–	–	–

[a] Acetyl groups are also removed.

quently the results were less accurate. Although the chemical structures of the oligosaccharide have not yet been completely elucidated, the electrophoretically faster-moving fractions from both enzymes appear to be pyruvylated while the slower moving material is non-pyruvylated. No material corresponding to free pyruvate or to free glucuronic acid was detected. As was expected, the acid-depyruvylated polysaccharide lacked electrophoretic fraction 1 and this was also the case for polysaccharide from *X. phaseoli* strain 1128 which had a very low pyruvate content. Attempts to resolve the pyruvylated fragments by paper chromatography have so far not been entirely satisfactory, due to lack of suitable solvents. However, the non-pyruvylated material from enzyme A could be resolved in solvent B into two fragments A_{21} and A_{22} (R_{Glc} 0·46 and 0·19, respectively) in approximately equal amounts. The presence of these is listed in Table 3. All the A_{21} and A_{22} fractions tested contained 2 moles of D-glucose per mole of uronic acid. One mole of glucose was reduced by borohydride treatment, but no glucose was released by β-glucosidase preparations. Thus the enzyme appears to cleave the main chain between the unsubstituted glucose residues and those carrying the trisaccharide side chains. All the A_{21} fractions tested contained acetate; it was absent from the A_{22} fractions. No A_{21} fraction was detected from enzyme hydrolysates of *X. phaseoli* 556 nor from the deaceylated and depyruvylated polymers.

Although the enzyme preparations did not appear to contain an esterase when tested with nitrophenylacetate as substrate, the presence of an enzyme removing the acetyl or pyruvyl groups during prolonged incubation could not be discounted. Accordingly, enzyme-hydrolysed polymer from a large incubation mixture was sampled over 16 h at 30 min intervals and subjected to paper electrophoresis and paper chromatography. The pattern of oligosaccharides remained the same throughout the course of incubation. The small amounts of neutral material detected on paper electrophoresis were eluted and subjected to paper chromatography in solvent A. They comprised a mixture of mannose and glucose, the proportions depending on the polysaccharide substrate. Smaller amounts of a faster-moving fragment found in some preparations were probably acetylmannose as they contained equal amounts of acetate and hexose; on acid or alkaline hydrolysis, mannose was the sole monosaccharide detected. On borohydride reduction, all the mannose was reduced to mannitol.

While the deacetylated and depyruvylated polysaccharides were good substrates for both enzyme systems, the carboxyl-reduced polymer was hydrolysed, albeit poorly, by enzyme B, but not at all by the enzyme A system. Of the natural *Xanthomonas* polysaccharides treated, only that from strain S19, two from *X. albilineans* strains 1738 and 2257 and one from a strain of *X. fragariae* 1822 were resistant to enzymic hydrolysis and obviously different from the *X. campestris* type. A number of other bacterial exopolysaccharides containing glucose, glucuronic acid and mannose or other monosaccharides were tested, but were not hydrolysed by either enzyme system.

DISCUSSION

The exopolysaccharides from *Xanthomonas campestris* strains are of particular interest because of their carbohydrate structure and consequent physical characteristics; they are essentially cellulose molecules substituted on alternate glucosyl residues (Jansson *et al.*, 1975; Melton *et al.*, 1976). A rapid and accurate method of determining the structural characteristics is required both for the study of new polymers, which may be related to the structure already characterised and for rapid screening of material grown under different cultural conditions. The use of the enzymes described in this paper provides the basis for a rapid screening technique which can be used for both purposes. It is clear that the *Xanthomonas* strains examined, with the exception of *Xanthomonas* S19 (Fareed & Percival, 1976) and three other strains representing the distinct species *X. albilineans* and *X. fragariae*, produce polysaccharides differing little, if at all, in their carbohydrate structure from *X. campestris*. Thus the combination of chemical analysis and enzyme digestions confirm that most of the *Xanthomonas* 'species' earlier listed represent *X. campestris* isolates from a wide range of plant hosts. The similarity of the chemical composition of polysaccharides from *X. phaseoli* and *X. campestris* had already been observed (Misaki *et al.*, 1962), while poly-

saccharides from these two sources behaved similarly during studies of the synergistic interaction between different polysaccharides in solution (Dea *et al.*, 1977). One might, therefore, expect that all the polysaccharides tested in the present study, which were similar in their chemical composition to that of *X. campestris* and susceptible to enzymic hydrolysis, would behave similarly in this type of test for synergistic gelation with locust bean or tara gum.

The major differences in the *Xanthomonas* polysaccharides tested here are to be found in the degree of acylation and ketalation. There appears to be considerable latitude in the extent of these substituents. This appears strange when compared to results found for most other bacterial exopolysaccharides (Sutherland, 1972) where there appears to be uniform substitution, usually to the extent of one acyl or ketal group per repeating unit of the polymer. However, as has been mentioned, one polymer possesses acetyl groups on every alternate repeating unit (Sutherland & Wilkinson, 1968). The published figures for *X. campestris* polysaccharide structure indicate one mole of pyruvate per 2·7 repeating units and one mole of acetate per repeating unit (Jansson *et al.*, 1975; Melton *et al.*, 1976) and these appear to be fairly typical for the range of polysaccharides examined in the present study. In terms of the biosynthesis of the polysaccharides, the control over non-carbohydrate substituents would be relatively relaxed if these represent typical polysaccharide chains. It was earlier shown that mutants lacking acetyl groups can be obtained in colanic acid-synthesising systems (Garegg *et al.*, 1971a). However, it has so far not proved possible to isolate, for example, non-pyruvylated colanic acid polysaccharides from strains which originally contained the ketal groups. Sandford *et al.* (1977) obtained xanthan preparations with 25 g and 4·4 g pyruvate per 100 g, respectively. These would correspond *theoretically* to pyruvate on alternate terminal D-mannose residues and on every fourth such residue, respectively. However, no proof was presented that such distributions of pyruvate were actually present. An alternative explanation for the lack of a 1 : 1 pyruvate : glucuronic acid ratio could be that all the preparations examined represent a mixture of polysaccharide strand types, some perhaps being fully pyruvylated while others are effectively lacking in pyruvate. It is possible that, under certain conditions of growth, the presumed pyruvyl donor phosphoenol-pyruvate is not present in sufficient concentration to permit ketalation of the polymer and formation of a non-ketalated polymer results. Experiments to test this hypothesis are proceeding.

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