The Exopolysaccharides of Klebsiella Serotype 2 Strains as Substrates for Phage-induced Polysaccharide Depolymerases

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SUMMARY

A phage-induced enzyme has been used to hydrolyse the exopolysaccharides prepared from nine *Klebsiella* serotype 2 strains. In each case, the major product was a tetrasaccharide with chemical composition corresponding to the carbohydrate repeating unit of the polysaccharide. The tetrasaccharides also contained formate, sometimes with acetate or pyruvate. As the terminal reducing sugar in each tetrasaccharide was mannose, the enzyme is a mannosidase hydrolysing the D-mannosyl 1 $\rightarrow$ 4 D-glucose linkage.

The enzyme is highly specific, being inactive against carboxyl-reduced type 2 polysaccharide and against polysaccharides from a number of other *Klebsiella* strains of different serotype. In contrast, similar phage-induced enzymes from *Klebsiella aerogenes* serotype 54 strains hydrolyse both type 2 and type 54 polysaccharides, yielding the same products from type 2 material as does the homologous enzyme. No further polysaccharides among those currently tested acted as substrates for the phage-induced enzymes.

INTRODUCTION

Exopolysaccharides (capsules or slime) from several bacterial species are the substrates for soluble enzymes isolated from bacteriophage-infected bacterial cultures. In some such systems, despite a dramatic drop in the viscosity of the polysaccharide solutions following addition of enzyme, no hydrolysis products have been identified (Adams & Park, 1956; Eklund & Wyss, 1962). The enzymes induced by several phages in *Klebsiella aerogenes* type 54 cultures hydrolysed the polysaccharide from this strain, producing a tetrasaccharide corresponding to the repeating unit of the polymer (Sutherland, 1967). Subsequently the oligosaccharide was shown to contain acetate and formate (Sutherland & Wilkinson, 1968; Sutherland, 1970).

The isolation of a phage inducing polysaccharase formation in a *Klebsiella pneumoniae* type 2 strain was reported by Watson (1966). The enzyme had no effect on bacterial viability but removed the capsules and also affected the immunochemical reactions of the polysaccharide. Polysaccharides from four different *Klebsiella* type 2 strains all contained the same tetrasaccharide repeating unit (Fig. 1; Gahan, Sandford & Conrad, 1967). Results from a further strain indicated that some variation in polysaccharide structure might occur, although the monosaccharide composition remained constant (Park, Eriksen & Henriksen, 1967). Further indications that polysaccharides might have constant carbohydrate structure but variable acyl substituents came from a preliminary examination of a number of *Klebsiella* strains of different serotype (Sutherland, 1971). The aim of the present work was to determine the effect of a phage-induced enzyme on the capsular polysaccharides isolated from *Klebsiella* type 2 strains and on others known to possess structural similarities.

Fig. 1. The structure of the repeating unit of serotype 2 capsular polysaccharide of *Klebsiella aerogenes*. (Gahan *et al.* 1967).

METHODS

Bacterial strains. The four strains of *Klebsiella* type 2 for which the structure of the polysaccharide has already been established (Gahan, Sandford & Conrad, 1967) were obtained from the National Collection of Type Cultures, Colindale, London. These were strains 243, 418, 5505 and 9505. Strain 22 was provided by Dr D. G. McPhee, La Trobe University, Melbourne, Australia and strains 28, 2534, 2895 and 2930 came from Dr G. Hermann, Center for Disease Control, Atlanta, Georgia, U.S.A. Strains A1 and A3 were departmental cultures of serotypes 1 and 54 respectively.

Cultures were maintained on nutrient agar slopes in screw-capped vials. The polysaccharides were prepared from solid or liquid cultures in nitrogen-deficient medium (Sutherland & Wilkinson, 1965) and purified as described by Dudman & Wilkinson (1956). Some preparations were purified further by digestion with pronase.

Bacteriophage. Phage F40 (Watson, 1966) was routinely prepared using cultures of strain 243; enzyme was prepared from phage-infected cultures of strain 243 or 418 as described for other phage-induced polysaccharide depolymerases (Sutherland, 1967) and partially purified by ammonium sulphate precipitation and chromatography on DEAE-cellulose. Preparations from either host strain were identical in their activities. The preparation of polysaccharide-depolymerases (fucosidases) from phages F31, 34 and 39 cultured on strain A3 has already been described (Sutherland, 1967).

Analysis techniques. Monosaccharides were determined on unhydrolysed material by the cysteine/sulphuric acid reaction for hexoses (Dische, Shettles & Osnos, 1949) and the carbazole reaction for uronic acid (Bitter & Muir, 1962). Hydrolysates of polysaccharides or oligosaccharides ($N-H_2SO_4$ at 100° for 16 h) were neutralized with Amberlite IR410 resin (HCO_3 form) and used to determine glucose and galactose with the respective oxidase reagents. The mannose content was calculated by difference from the total hexose values. Reducing sugar in enzymatic hydrolysates was measured by the Somogyi (1945) method.

Acetyl and pyruvyl contents were determined by micro-modifications of the hydroxamic acid and dinitrophenylhydrazine methods respectively (Hestrin 1949; Sloneker & Orentas 1962). Formate was assayed enzymatically with formate dehydrogenase (Quayle, 1966) and also colorimetrically by the procedure of Grant (1948) if acetate was known to be absent. Acetate and formate derived from polysaccharides were characterized by the technique of Thomson (1951) involving preparation of hydroxamic acid derivatives and chromatography in solvent B. Chromatograms were developed with 10% (w/v) aqueous $FeCl_3$ solution. Pyruvate was identified by preparation of the dinitrophenylhydrazone and chromatography in solvent A along with authentic standards.

Partial acid hydrolysis. Approximately 40 mg. of each polysaccharide was dissolved in 20 ml. 0.5 $N-H_2SO_4$ in a tightly stoppered tube and heated at 100° for 30 min. The hydrolysates were neutralized with saturated $Ba(OH)_2$ solution, filtered and concentrated under reduced pressure. The syrups so obtained were applied to Whatman 3 MM paper and sub-

jected to paper electrophoresis. After staining of guide strips with alkaline silver nitrate solution, fractions were eluted and compared by paper chromatography (solvents B and C).

Enzymatic hydrolysis. Samples of each polysaccharide (10 mg) were dissolved in 1 ml of 0.001 M-phosphate buffer (pH 7.0) and sufficient depolymerase enzyme added to give maximal hydrolysis in 20 h at 20°. Microbial growth was inhibited by the addition of toluene. Hydrolysates were examined by paper chromatography (solvent D) or paper electrophoresis. When the enzymatic hydrolysis was complete, as determined by following release of reducing material, the solutions were dialysed against distilled water to separate the products from enzyme protein and undigested polymer. The diffusible material, comprising 80 to 90% of the original polysaccharide, was concentrated under reduced pressure at 40 to 50° and the resultant syrups subjected to paper electrophoresis. Guide strips were stained to locate oligosaccharides. Because of the presence of variable amounts of salts in polysaccharide preparations, preparative amounts of the oligosaccharides were always separated by electrophoresis then by paper chromatography. The reduction in viscosity was followed using an Ostwald-type viscometer at 30°.

Paper chromatography and paper electrophoresis. The following solvent systems were used for descending paper chromatography on Whatman no. 1 paper. Solvent A: butan-1-ol-pyridine-water (6:4:3, by vol.) (Whistler & Conrad, 1954); solvent B: butan-1-ol-acetic acid-water (4:1:5, by vol.) (Partridge, 1946); solvent C: ethyl acetate-acetic acid-formic acid-water (18:3:1:4, by vol.) (Feather & Whistler, 1962); solvent D: ethyl acetate-pyridine-acetic acid-water (5:5:1:3, by vol.) (Fischer & Dörfel, 1955). Paper electrophoresis was performed in pyridine-acetic acid buffer (pH 5.3). A current of 80 to 100 mA was applied at 3000 V using a Locarte (London) paper electrophoresis equipment with 70 × 20 cm cooled plate area, for 3 h to separate oligosaccharides, for 30 min to remove salts or enzyme proteins.

Borohydride reduction. The terminal reducing sugars of the oligosaccharides were determined by borohydride reduction (Sutherland, 1967). After removal of the salts by paper electrophoresis, material was eluted and hydrolysed (N-H₂SO₄ for 16 h). Sugar alcohols were identified by chromatography of hydrolysates in solvents A and C.

RESULTS

Composition of the exopolysaccharides. Acid hydrolysates from each polysaccharide were neutralized and examined by paper chromatography in solvents A and D. In all the preparations from type 2 strains, glucose, mannose and glucuronic acid were detected, the most intense spot being glucose. This agrees with the results of Gahan *et al.* (1967). Traces of galactose were also observed in a few hydrolysates. These were thought to originate from contaminating lipopolysaccharide which is known to contain considerable quantities of galactose (Koeltzow, Epley & Conrad, 1968). Galactose was not found in the oligosaccharides obtained by enzymatic hydrolysis. The polysaccharides of type 1 and type 54 both contained L-fucose, D-glucose and D-glucuronic acid. Analyses for acetate, formate and pyruvate were made on all the polysaccharides. These results, together with determinations for the sugars detected chromatographically are shown in Table 1. It is clear that all the type 2 polysaccharides contain the same monosaccharides – glucose, mannose and glucuronic acid – in the same approximate molar ratio of 2:1:1 respectively. Probably, all possessed the tetrasaccharide repeating unit described by Gahan *et al.* (1967) (Fig. 1). Further evidence was obtained from partial acid hydrolysates. Each yielded the same products, a series of charged oligosaccharides indistinguishable from those previously described by Gahan *et al.*

Table 1. *The composition of Klebsiella type 2 and other capsular polysaccharides (results as % dry weight)*

Type	Strain	D-Glucose	Mannose	Glucuronic acid	Pyruvate	Formate	Acetate
2	2S	41.4	19.9	26.4	0.1	4.1	0.1
	418	42.4	20.5	23.1	0	3.9	0
	5505	43.0	20.7	21.6	0.3	3.9	0
	9504	45.3	23.5	20.8	0.7	4.2	0
	2534	46.0	21.6	25.7	0.6	5.0	0
	2930	43.0	20.5	24.1	0	5.9	8.3
	2895	46.0	22.1	24.6	0.1	4.6	4.5
	243	46.3	21.9	22.9	0	4.8	8.1
	22	36.4	20.3	19.7	5.2	3.9	0
Fucose							
I	A 1	43.0	19.3	23.4	10.3	0	0
54	A 3	37.9	18.8	23.2	0	4.6	4.5

(1967). The carbohydrate composition of all the type 2 preparations was therefore similar if not identical.

When eight type 2 polysaccharide preparations were examined for non-carbohydrate constituents, much greater variation was noted. Although all the polymers contained formate, only one contained pyruvate. Acetate was found in three polysaccharides. These results were confirmed by the preparation and chromatography of the appropriate derivatives. The type 2 polysaccharides can therefore be separated into three groups: (i) those polymers which contain formate; (ii) the polysaccharides which contain formate and acetate; and (iii) the single preparation in which both formate and pyruvate are detected. There was no indication that any other non-carbohydrate residues were associated with the type 2 *Klebsiella* exopolysaccharides.

Although type 1 and type 54 material contained the same monosaccharides, confirming the earlier results of Dudman & Wilkinson (1956), significant differences were seen in their acyl substituents. The type 1 polymer contained pyruvate, while the type 54 polysaccharide was both acetylated and formylated.

Polysaccharides as phage enzyme substrates. All the type 2 *Klebsiella* strains were susceptible to infection with phage F40. When phage plaques or areas of confluent lysis were examined, they were surrounded by a zone which was partially cleared. These zones contained viable, non-capsulate bacteria. On subculture they proved to be identical with the original bacteria, being surrounded with capsules and free from phage. As phage F40 was known to produce a polysaccharide depolymerase (Watson, 1966) this indicated that all the capsular polysaccharides were probably substrates for the enzyme. When polysaccharide solutions were treated with the partially purified phage F40-induced depolymerase enzyme, there was a very marked reduction in the viscosity of the solution. This was accompanied by the release of reducing material. Typical results for the release of reducing material from solutions of exopolysaccharide (strain 418) on enzyme treatment are shown (Fig. 2). Similar results were obtained from all type 2 polysaccharide preparations, within the limits of experimental error, no differences were detected in the time required for 50% drop in solution viscosities. The type 2 strains and the type 1 strain were insensitive to the three phages (F31, 34, 39) having type 54 as their normal host. No hydrolytic activity was found using a number of other *Klebsiella* exopolysaccharides nor was any found using carboxyl-reduced

Fig. 2. The release of reducing material from *Klebsiella* polysaccharide solutions on treatment with phage enzymes. Polysaccharide solutions (4 mg/ml) were incubated at 30° with enzymes (0.1 mg protein/ml) and samples removed at intervals for determination of reducing sugars. ■, *Klebsiella aerogenes* type 54 polysaccharide and type 54 enzyme; ●, *Klebsiella* type 2 polysaccharide and type 54 enzyme; ▲, *Klebsiella* type 2 polysaccharide and type 2 enzyme.

Klebsiella material of serotype 2. As phages F31, 34 and 39, isolated using *Klebsiella aerogenes* type 54 as host bacteria, showed partial clearing of the type 2 strains on solid media, enzymes prepared from phage-infected type 54 cells were also tested. The effect was the same as that observed with the F40-induced enzyme. The viscosity of the solutions fell and reducing material was liberated (Fig. 2). No fall in viscosity of solutions of polysaccharides from the type 1 strain was seen despite the known chemical similarities and serological cross-reactions of this polymer. Attempts to isolate similar enzymes from non-infected bacteria were unsuccessful.

Isolation and characterization of enzyme products. The products from hydrolysis of strain 22 polysaccharide were separated into two fractions (P1 and P2) which could not be resolved further by paper chromatography. The strains containing formate as the sole acyl group produced a single fragment (P1) on electrophoresis and it, too, could not be resolved further by chromatography. The acetylated polymers yielded approximately equal amounts of two oligosaccharides (P1 and P3) which were separable by paper chromatography in solvent D. Exactly the same products were obtained from the respective polysaccharides when F31, F34 or F40-induced enzymes were used.

Examination of the oligosaccharides obtained by enzymatic hydrolysis in this way showed that the slower-moving (chromatographically) fractions from strain 22 and the acetylated strains were indistinguishable and also appeared to be identical with the single product from the strains containing formate as the sole acyl substituent. Hydrolysates of all three fragments contained glucose, mannose and glucuronic acid in the approximate molar ratio of 2:1:1. All the mannose in each oligosaccharide was reduced to mannitol by treatment with sodium borohydride, indicating that each was a tetrasaccharide in which mannose was the terminal reducing sugar. The properties of the three tetrasaccharides and the polymers from which they were produced are listed in Table 2. Attempts to identify the sites of acyl groups are continuing.

DISCUSSION

Capsular polysaccharides prepared from nine different strains of *Klebsiella* type 2 were all hydrolysed by a phage-induced enzyme. As judged by similar rates of release of reducing material and of reduction of solution viscosity, the differing acyl groups present on the polysaccharides did not affect the enzyme activity. Attempts to find other substrates for the

Table 2. *The properties of oligosaccharides isolated from enzymatic hydrolysates*

Oligosaccharide	Source	Components $\mu\text{moles}/100 \mu\text{l}$ solution*						$M_{\text{GlcA}}^\dagger$	$R_{\text{glc}}^{\text{(Solvent D)}}$
		Glucose	Mannose	Glucuronic Acid	Formate	Acetate	Pyruvate		
P 1	All type 2 polysaccharides	2.34	1.29	1.30	1.27	0	0	0.45	0.36
P 2	22 only	2.92	1.43	1.50	0.02	0	1.44	0.94	0.66
P 3	243 2930 2895	2.97	1.65	1.51	1.58	1.54	0	0.41	0.45

* Figures are given for material from strains 418, 22 and 243 respectively. Other preparations showed no significant differences from these in the molar ratios of the constituents.

† Electrophoretic mobility relative to glucuronic acid. (M_{GlcA}).

enzyme were unsuccessful. Carboxyl reduction, the conversion of the glucuronic acid residues to glucose, leaves the polymer structure intact. This change was sufficient to render type 2 polysaccharide resistant to enzymatic hydrolysis and thus defines further the substrate specificity of the phage-induced enzyme. It appears to be a mannosidase hydrolysing the mannosidic bond in polymers containing the sequence –D-glucuronosyl–D-mannosyl–D-glucose – identified by Gahan *et al.* (1967). It was not active against polysaccharides of types 30 and 69 which are known to cross-react serologically with type 2 *Klebsiella* strains (Dr I. Ørskov, personal communication). Either the serological cross-reaction is not due to the particular mannosidic bond, or the remaining glycosidic bonds and chemical structure of the polymers may be sufficiently different to account for the lack of enzyme action.

Surprisingly, phage-induced enzyme preparations from *Klebsiella aerogenes* type 54 also hydrolysed type 2 polysaccharides, forming the same products as the enzyme induced by phage in type 2 bacteria. This result could be due to (i) a gratuitous impurity in the type 54 enzyme preparations or (ii) a much lower substrate specificity in the type 54 enzyme systems. The latter is more probably true, as these enzymes had previously been shown to hydrolyse *K. aerogenes* type 54 polysaccharide, de-acylated polymer and oligosaccharides derived from it (Sutherland & Wilkinson, 1968) as well as hydrolysing the capsular polysaccharide of similar but not identical structure formed by *Escherichia coli* K27 (Sutherland, Jann & Jann, 1970). Both the enzymes induced in *Klebsiella* type 2 bacteria and those isolated from phage-infected *K. aerogenes* type 54 cultures appear to be hydrolases. The hydrolysis products contained glucuronic acid and not a 4, 5 unsaturated uronic acid of the type formed by the eliminase acting on *Xanthomonas phaseoli* capsular polysaccharide (Lesley, 1961) and similar systems.

Of the nine serotype 2 *Klebsiella* strains examined, only one capsular polysaccharide was pyruvylated confirming the results of Gormus, Wheat & Porter (1971). Pyruvate is now known to be a common substituent of bacterial exopolysaccharides, having been identified in such polymers from *Xanthomonas* species (Orentas, Sloneker & Jeanes, 1963); *Escherichia coli* (Lawson *et al.* 1969) and several other bacteria. Recently, Gormus *et al.* (1971) identified pyruvic acid as a component of exopolysaccharides from various *Klebsiella* species including serotypes 1 and 3–6, but failed to find it in the polysaccharide of the one type 2 strain tested. The present study shows that not all strains of one serotype possess the same acyl substituents on their exopolysaccharides despite the probable constancy of the carbohydrate structure. Formate, previously identified as a component of *Klebsiella aerogenes* type 54 capsular material (Sutherland, 1970) is prevalent in type 2 polysaccharides as well.

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