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STRUCTURE OF *Escherichia coli* CAPSULAR ANTIGEN K34*

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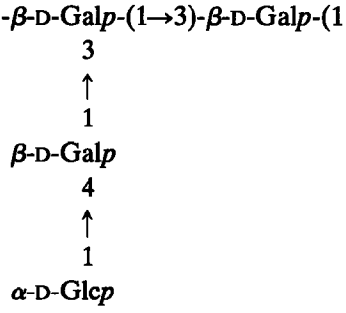
ABSTRACT

The structure of the heat-stable K antigen of *Escherichia coli* serotype K34 has been determined by n.m.r. spectroscopy, methylation analysis, and Smith degradation to be



The assignment of the single α-linkage to the D-glucosyl residue was confirmed by the positive reaction of an α-D-glucosidase on the oligosaccharide obtained by bacteriophage depolymerization. It is noteworthy that the same enzyme was without action on the native polysaccharide.

INTRODUCTION

The capsular antigens of *Escherichia coli* have been designated[1] heat-labile or heat-stable, and some of the latter (type A) are said to resemble those of *Klebsiella*. As part of a continuing programme we now report the structure of the capsular antigen of *E. coli* K34.

RESULTS AND DISCUSSION

Composition. — Analysis of the native polysaccharide before and after reduction[2] of the uronic acid gave glucose and galactose in the ratios of 1.0:2.6 and

*Dedicated to Dr. R. Stuart Tipson.

TABLE I

SUGAR ANALYSIS OF K34 POLYSACCHARIDE AND DERIVED PRODUCTS

Sugar (as alditol acetate)	*Mole ratio*[a]				
	I[b]	II	III	IV	V
Galactose	2.6	2.7	2.0	2.0	0.03
Glucose	1.0	2.0		0.6	1.0
Glyceraldehyde			1.2		

[a]Using DB-17 column programmed for 180° for 2 min, 5°/min to 220°. [b]I, original acid polysaccharide; II, carboxyl-reduced polysaccharide; III, oligosaccharide obtained from Smith degradation of carboxyl-reduced polysaccharide (**N1**); IV, oligosaccharide obtained from selective Smith degradation of acidic polysaccharide; V, product obtained from chromium trioxide oxidation of acidic polysaccharide.

2.0:2.7, respectively (Table I), suggesting a composition of glucuronic acid, glucose, and galactose in the ratios of 1:1:3. The n.m.r. data were consistent with a pentasaccharide repeating-unit having one α and four β linkages and demonstrated the absence of deoxy sugar and ester or acetal substituents (Table II).

The n.m.r. spectra of the native polysaccharide showed little detail on account of the viscosity of the solution. An improved proton spectrum was obtained by examination of the polyol produced by periodate oxidation and borohydride reduction, but without acidic hydrolysis. The polyol gave a solution of noticeably lower viscosity and despite the cleavage of certain sugar rings, chemical shifts were substantially unchanged (Table II). Oxidation[3] of the acetylated polysaccharide by chromium trioxide gave glucose as the only identifiable product, which was therefore assigned the α-linkage. The individual sugars were each determined to be of the D configuration by comparison of the circular-dichroism spectra[4] of their methylated derivatives with standards.

Methylation analysis[5]. — Methylation analyses, with and without reduction of the uronic acid, gave the results shown in Table III, columns I and II, from which it may be deduced that the glucose residue occupies a terminal position and a galactose residue constitutes the branch point. The uronic acid, which is 2-substituted, was shown by a β-elimination experiment[6] (Table III, column III) to be linked to the branch point galactose at C-4. These results do not indicate whether the side-chain consists of one or more sugar residues.

Smith degradation[7]. — A sample of the native polysaccharide was reduced by the carbodiimide procedure[8] and oxidized with periodate. Slightly more than four moles of periodate per repeating unit were consumed and, following borohydride reduction, dialysis yielded a polymeric product whose ^{1}H-n.m.r. spectrum was recorded (Table II). Smith hydrolysis of the polyol, followed by paper chromatography, yielded an oligosaccharide (**N1**), analysis of which gave a ratio of galactose:glyceraldehyde of 2.0:1.2 (Table I) and the ^{1}H-n.m.r. spectrum showed

TABLE II

N.M.R. DATA FOR *E. coli* K34 CAPSULAR POLYSACCHARIDE AND DERIVED PRODUCTS

Compound	*^{1}H-n.m.r. data*				*^{13}C-n.m.r. data*	
	δ[a]	$J_{1,2}$[b] *(Hz)*	*Integral (H)*	*Assignment*[c]	*p.p.m.*[d]	*Assignment*[c]
K34 capsular polysaccharide	5.17	n.o.[b]	1	α-Glc	98.71	α-Glc
	4.71	b	1	β-GlcA	102.90	β-GlcA
	4.57	b	3	β-Gal	103.99	β-Gal
					104.44	β-Gal (×2)
					179.45	CO of α-GlcA
Polyol of K34 polysaccharide	5.18	n.o.	1	α-Glc[f]		
	4.71	b	1	β-GlcA		
	4.59	b	2.1	β-Gal		
	4.57	6	1	β-Gal		
Gal(1→3)Gal—O—CH(CHO[g])CH$_2$OH	4.53	8		β-Gal		
2-GlcA(1→4)Gal(1→3)Gal—[h]	4.71	8	1	β-GlcA		
	4.57	8	2	β-Gal		
Phage oligosaccharide **P1**	5.17	3	1.0	α-Glc		
	4.97	2	0.8	α-Gal		
	4.82	7	1.2	β-GlcA		
	4.65	8	0.4	β-Gal		
	4.53	7	2.2	β-Gal		

[a]Chemical shift relative to internal acetone; δ 2.23 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate. [b]Key: b = broad, unable to assign accurate coupling constant; s = singlet; n.o. = not observed. [c]For example, β-Gal = proton on C-1 of β-linked D-Gal residue. [d]Chemical shift in p.p.m. relative to internal acetone; 31.07 p.p.m. downfield from D.S.S. [e]As for [c], but for anomeric ^{13}C nuclei. [f]Assignments for the polyol signals correspond to the intact sugar residues in the native polysaccharide. [g]Oligosaccharide (**N1**) obtained from Smith degradation of carbodiimide-reduced polysaccharide. [h]Polysaccharide obtained from selective Smith degradation of acidic polysaccharide.

both sugars to be β-linked (Table II). Furthermore, methylation analysis of **N1** proved it to be a (1→3) galactobiose derivative (Table III, column IV).

Selective Smith degradation. — Kinetic data published by Painter and colleagues[9] have demonstrated significant differences in the rate of periodate oxidation of various sugars including glycosyluronic residues. Accordingly, a sample of the native polysaccharide was oxidized with dilute periodate for a limited time. When the polyol, obtained by reduction and purified by gel chromatography, was subjected to a Smith hydrolysis a polymeric product was obtained in which (following reduction of the uronic acid) galactose and glucose were shown to be present in the ratio of 2.0:0.6 (Table I, column IV). The formation of a polymeric product with the loss of two sugars and the fact that selective removal of only the

TABLE III

METHYLATION ANALYSIS OF K34 POLYSACCHARIDE AND DERIVED PRODUCTS

Methylated sugars[a] *(as alditol acetate)*	*Mole %*[b]				
	I[c]	II	III	IV	V
2,3,4,6-Glc	28.4	18.7	23.3		
2,3,4,6-Gal				41.7	
2,4,6-Gal	19.8	21.9	50.6	58.3	47.5
2,3,6-Gal	22.2	21.9	26.1		40.9
2,6-Gal	29.6	19.4			12.6
3,4-Glc		18.1			

[a]2,3,4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, etc. [b]Values are corrected by use of the effective, carbon-response factors given by Albersheim *et al.*[15]. Determined on a DB-17 column programmed for 180° for 1 min, 2°/min to 250°. [c]I, original acid polysaccharide; II, reduction of uronic ester; III, product from β-elimination and remethylation; IV, product **N1** from Smith degradation; V, product from selective Smith degradation.

glucosyl residue proved impossible, demonstrate that the 4-substituted galactosyl unit forms part of the side chain. Methylation of the degraded polyol, without reduction of the uronic acid, indicated one galactose residue to be 4-substituted and the other to be linked through C-3 (Table III, column V). These results also indicated that the hydrolysis of the side chain was incomplete.

Comparison of the ^{1}H-n.m.r. spectrum (Table II) of the degraded polymer with that of the native polysaccharide demonstrated the absence of a signal at δ 5.17, for the terminal α-D-glucopyranosyl residue, and that the signal at δ 4.57 now integrated for two protons, instead of three as in the native material. These results suggest that the signal at δ 4.71 may be assigned to the β-D-glucosyluronic residue.

Bacteriophage depolymerization[10]. — A phage was isolated from Vancouver sewage and propagated on *E. coli* K34 to give a final concentration of 9.0×10^{11} p.f.u.mL^{-1}. Depolymerization of K34 polysaccharide yielded an oligosaccharide (**P1**), analysis of which showed the phage enzyme to have a β-D-galactosidase activity[11]. The ^{1}H-n.m.r. spectra was recorded for **P1** (Table II) and for **P1** following borohydride reduction (no significant changes in chemical shifts).

Incubation of **P1** with an α-D-glucosidase liberated glucose (paper chromatography) confirming the assignment of the single α-linkage to this residue. It is of interest that the same enzyme was without detectable action on the native polysaccharide, thus providing a further illustration of the utility of phage-generated oligosaccharides in structural studies.

CONCLUSION

The partial structure —GlcA$\overset{4}{\underset{|3}{-}}$Gal— has been established by methylation

and β-elimination. The side chain, consisting of the terminal glucopyranosyl residue and one galactopyranosyl unit, is eliminated by the selective Smith degradation. Since the residual polymer contains both 3- and 4-substituted galactose units, one may write —GlcA—$\overset{4}{\underset{|3}{}}$Gal—$\overset{3}{}$Gal—, whence the structure for the *E. coli* K34 antigen is as shown in the abstract.

EXPERIMENTAL

General methods. — These, and the instrumentation used, have been described previously[12]. Analytical paper chromatography was carried out on Whatman No. 1 paper using either solvent *1*, 18:3:1:4 EtOAc–AcOH–HCO_2H–H_2O, or solvent *2*, 4:1:5 1-butanol–EtOH–H_2O (upper phase). Preparative chromatography employed Whatman 3MM and solvent *1*.

Analytical g.l.c. was carried out in fused silica capillary columns, (*A*) DB-17-15N or (*B*) DB-225-15N, using flame-ionization detectors. Preparative g.l.c. used a F and M model 720 instrument with a stainless-steel column (1.8 m × 6.3 mm) packed with 3% SP-2340 on Supelcoport (100–200 mesh) programmed from 175° to 240° at 1°/min.

Isolation and purification of K34 polysaccharide. — An actively growing culture was used to inoculate 100 mL of Mueller–Hinton broth, which was incubated at 37° for 4 h when the turbid suspension was poured into a metal tray (60 × 40 cm) containing Mueller–Hinton agar medium. After 4 days at 37°, the bacterial slime was scraped off, treated with 1% phenol, and the polysaccharide was isolated and purified by Cetavlon precipitation[13]. Further purification was achieved by use of a column of Biogel P2.

Sugar analysis and composition. — Hydrolysis of a sample (20 mg) of K34 polysaccharide with 2M CF_3CO_2H for 20 h at 95°, removal of excess acid by co-evaporation with water, followed by paper chromatography (solvent *1*) showed glucose, galactose, glucuronic acid, and an aldobiouronic acid. The sugars released were analyzed as alditol acetates by g.l.c. (column *A*, programmed from 180° to 220° at 5°/min) with the results shown in Table I, column I.

A sample of K34 polysaccharide (13 mg), dried *in vacuo* and under an i.r. lamp, was treated with methanolic HCl (3%) and refluxed overnight on a steam-bath under anhydrous conditions. The excess of acid in the mixture was neutralized with $PbCO_3$. The resultant mixture was centrifuged, the supernatant was evaporated to dryness, and the product obtained was reduced[2] with $NaBH_4$ in anhydrous MeOH. The residue, after removal of borate, was hydrolyzed with 2M CF_3CO_2H on a steam-bath (20 h) after which the acid was removed by codistillation with water. The sugars in the hydrolyzate were determined as their alditol acetates (see Table I, column II).

N.m.r. spectroscopy (^1H and ^{13}C) was performed on the original poly-

saccharide. The principal signals for both ^{1}H-n.m.r. and ^{13}C-n.m.r. are recorded in Table II.

Chromium trioxide oxidation. — A sample (10 mg) of the polysaccharide was dissolved in formamide (5 mL) and treated with Ac_2O (1 mL) and C_5H_5N (1 mL) overnight at room temperature. The acetylated material (12 mg) was recovered by dialysis and freeze-drying. The acetylated polysaccharide dissolved in AcOH was treated with CrO_3 (100 mg) for 2 h at 50°. The material was recovered by partition between $CHCl_3$ and water. Sugar analysis was then performed on the recovered material with the results shown in Table I, column V.

Methylation analysis. — The capsular polysaccharide (30 mg) in the free-acid form, obtained by passing the sodium salt through a column of Amberlite IR-120 (H^+) resin, was dissolved in anhydrous Me_2SO (5 mL) and methylated[5] by treatment with 5 mL dimethylsulfinyl anion for 4 h and then with 10 mL MeI for 1 h. The methylated polysaccharide was recovered by dialysis (mol.wt. cut off 13,500) against distilled water overnight. The methylated polysaccharide was purified by partition between CH_2Cl_2 and water, as well as by gel-permeation chromatography (Sephadex LH 20). Drying *in vacuo* and under an i.r. lamp, followed by i.r. spectroscopic analysis, indicated complete methylation (no absorptions at 3625 and 3200–3500 cm^{-1}).

A portion (15 mg) of this product was hydrolyzed with 2M CF_3CO_2H for 20 h at 95°. The excess of acid was removed by codistillation with water and the hydrolyzate was analyzed by paper chromatography (solvent *2*), developed with *p*-anisidine hydrochloride) and as alditol acetates using columns *A* and *B*, Table III, column I.

A portion of the methylated polysaccharide was reduced with $LiAlH_4$ in refluxing oxolane overnight. The methylated and carboxyl-reduced polysaccharide was hydrolyzed with 2M CF_3CO_2H on a steam bath for 20 h. Reduction of the hydrolyzate with $NaBH_4$, followed by acetylation (with Ac_2O–C_5H_5N), and g.l.c. and g.l.c.-m.s. analyses (column *A* programmed from 180° to 250° at 2°/min) gave the data in Table III, column II.

Uronic acid degradation[6]. — A sample (20 mg) of methylated K34 polysaccharide was dried and then, with a trace of *p*-toluenesulfonic acid, was dissolved in 19:1 Me_2SO–2,2-dimethoxypropane (12 mL) and the flask was sealed under nitrogen. Dimethylsulfinyl anion (5 mL) was added and allowed to react for 18 h at room temperature. Methyl iodide (3 mL) was added to the cooled mixture and stirring was continued for 1 h. The methylated, degraded product was isolated by partition between $CHCl_3$ and water. The product was then purified by gel-permeation chromatography (Sephadex LH-20). The degraded product was hydrolyzed with 2M CF_3CO_2H for 8 h at 95° and the partially methylated alditol acetates were prepared as described earlier. G.l.c. analysis and g.l.c.-m.s. were conducted using column *A* programmed from 180° to 250° for 2°/min (Table III, column III).

Carbodiimide reduction of K34 polysaccharide[8]. — A portion (120 mg) of K34 polysaccharide (H^+ form) was dissolved in water (30 mL). 1-Cyclohexyl-3-(2-

morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (423 mg) was added to the polysaccharide solution. As the reaction proceeded (with consumption of hydrogen ions), the pH was maintained at 4.75 by adding 0.1M HCl dropwise. When consumption of hydrogen ions ceased, approximately 2 h later, an aqueous 3M solution of $NaBH_4$ was added dropwise. Foaming was controlled by constant stirring. Approximately 100 mL of $NaBH_4$ solution was added over a period of 2 h. Throughout the base addition, the solution was maintained at about pH 7 by titrating with aq. HCl. After concentrating, the polysaccharide was dialyzed against distilled water for 2 days when lyophilization gave 124 mg of product.

Smith degradation. — Carboxyl-reduced K34 polysaccharide (120 mg) was dissolved in 30 mL water and 20 mL 0.06M $NaIO_4$ was added. The reaction was conducted at room temperature and in the dark. Aliquots (0.1 mL) of the solution were withdrawn periodically, diluted 250 times, and analyzed spectrophotometrically. The periodate consumption reached a plateau after about 122 h when ~4 mol IO_4^- had been consumed per repeating unit. The excess of periodate was decomposed by adding ethylene glycol (1 mL) and the product was dialyzed overnight against distilled water. Sodium borohydride (0.5 g) was added and the solution was left overnight. The solution was deionized with Amberlite IR-120 [H^+] resin and several portions of MeOH were distilled from the product. The derived polyalcohol was dissolved in 10 mL 0.5M CF_3CO_2H and stirred for 48 h at room temperature. The product was dialyzed against 2 L of distilled water. The dialyzate was concentrated and freeze-dried. Two compounds were isolated by preparative paper chromatography of the freeze-dried product, using solvent *1*. The slower-moving compound (**N1**) was subjected to ^{1}H-n.m.r. spectroscopy, sugar (Table I, column III), and methylation analyses (Table III, column IV).

Selective Smith degradation. — A solution of K34 acidic polysaccharide (20 mg) in water was mixed with 0.02M $NaIO_4$ (20 mL) and kept in the dark for 3 h at room temperature. Ethylene glycol (0.2 mL) was added, the mixture was stirred for 1 h and the polyaldehyde formed was dialyzed overnight. This polyaldehyde was reduced to the polyol by $NaBH_4$ and Smith hydrolysis was effected by reaction with 0.5M CF_3CO_2H for 24 h at room temperature. Following dialysis against 1 L of distilled water, the nondialyzable material was freeze dried, examined by ^{1}H-n.m.r. spectroscopy (Table II), and analyzed, with reduction[2] of the uronic acid (Table I, column IV).

This degraded product was methylated according to Hakomori's procedure[5] and the analytical data are given in Table III, column V.

Determination of the configuration (D *or* L) *of the sugars.* — The hydrolyzate from the methylation analysis was separated into individual partially methylated alditol acetates using preparative g.l.c. (column SP 2340 programmed from 175° to 240° at 1°/min). Each of these alditol acetates was dissolved separately in MeCN and their c.d. spectra were recorded.

Bacteriophage ø34. — The phage was isolated from Vancouver sewage and propagated by tube and flask lysis to a concentration of 0.9×10^{11} p.f.u.mL^{-1}. The

solution was dialyzed against a buffer of pH 7 [NH_4OAc and $(NH_4)_2CO_3$] for 3 days. The solution was concentrated and the total of 7.2×10^{12} p.f.u. was added to an aqueous solution of 250 mg of K34 polysaccharide. Depolymerization was carried out for 48 h at 37°, after which time the solution was dialyzed against distilled water (3 × 1 L) and the product was recovered by lyophilization. The crude product was deionized by passage through a column of Amberlite IR 120 (H^+) and the eluate was concentrated and added to a column of Bio-Gel P2 (400 mesh) which was eluted with water at 6.6 $mL.h^{-1}$. Oligosaccharide **P1** was collected between 40–80 mL to give 86 mg, 34% yield.

The nature of the reducing end was established by the method of Morrison[14] (column *A*) and the ^1H-n.m.r. spectra of **P1** and reduced **P1** were recorded.

A portion of **P1** (3 mg) was dissolved in 1 mL of NaOAc buffer (pH 7.0) and a solution of α-D-glucosidase (Sigma, 0.5 mg in 1 mL of buffer) was added. The mixture was incubated at 37° for 2 days when the reaction was terminated by addition of one drop of 50% AcOH. The product, isolated by lyophilization, was examined by paper chromatography (solvent *1*).

Native K34 polysaccharide (5 mg) was similarly examined.

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