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Escherichia coli SEROTYPE-39 CAPSULAR POLYSACCHARIDE: PRIMARY STRUCTURE AND DEPOLYMERISATION BY A BACTERIOPHAGE-ASSOCIATED GLYCANASE

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ABSTRACT

The acidic capsular polysaccharide isolated from *Escherichia coli* O9:K39:H9 was investigated, using n.m.r. spectroscopy, methylation analysis, uronic acid degradation of the native and methylated polysaccharides, and bacteriophage-associated enzyme degradation. The structure of the repeating unit, which is shown below, is identical to that reported for *Klebsiella* serotype-61 capsular polysaccharide.

$$\begin{array}{l}\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 3 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \uparrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 1 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \alpha\text{-D-Gal}p\end{array}$$

INTRODUCTION

The capsular antigens of *E. coli* have been divided into two groups on the basis of their physical, chemical, and microbiological characteristics[1]. Group I capsules are high-molecular-weight acidic polysaccharides, are heat stable at pH 5–6, and may contain amino sugars. The capsular polysaccharide of *E. coli* O9:K39:H9, whose structure we now report, belongs to a subgroup of I whose capsules are devoid of amino sugars and closely resemble those of *Klebsiella*.

RESULTS AND DISCUSSION

Composition and n.m.r. spectra. — *E. coli* K39 bacteria were grown on an agar medium, and the acidic polysaccharide was isolated and purified by precipitation with cetyltrimethylammonium bromide. G.l.c. analysis of the acetylated aldononitriles derived from the products in an acid hydrolysate of the polysaccharide, with and without prior reduction of the acidic function, indicated that it is composed of glucose, glucuronic acid, galactose, and mannose in the molar

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TABLE I

SUGAR ANALYSIS OF *E. coli* K39 POLYSACCHARIDE AND DERIVED PRODUCTS

Sugar	*Molar ratio*				
	Glc	*GlcA*	*Gal*	*Man*	*Glc-ol*
Polysaccharide	2.0	1.0	1.0	1.1	
P1-alditol	1.0	0.9	1.0	1.0	0.9
P2-alditol	3.0	1.9	2.1	2.0	1.0
Li product B	1.0		1.1	1.1	0.9

TABLE II

^{1}H-N.M.R. DATA FOR *E. coli* K39 POLYSACCHARIDE AND DERIVED PRODUCTS

Compound	*δ[a] (p.p.m.)*	*^{3}J Hz*	*Integral (No. of H)*	*Assignment[c]*
K39 Polysaccharide[d]	5.40	3.6	1	6-α-Glc
	5.32	n.o.[b]	1	2,3-α-Man
	5.27	2.7	1	α-Gal
	4.55	8.0	1	3-β-Glc
	4.51	8.0	1	4-β-GlcA
P1	5.42	4.0	1	α-Glc
	5.30	n.o.	0.4	2,3-α-Man-(1→3)-β-Glc
	5.31	n.o.	0.6	2,3-α-Man-(1→3)-α-Glc
	5.26	3.5	1	α-Gal
	5.23	3.8	0.6	3-α-Glc
	4.67	8.2	0.4	3-β-Glc
	4.54	8.0	0.4	4-β-GlcA···→3)-β-Glc
	4.53	8.0	0.6	4-β-GlcA···→3)-α-Glc
P1-alditol	5.44	3.4	1	α-Glc
	5.24	3.0	1	α-Gal
	5.13	n.o.	1	2,3-α-Man
	4.56	7.8	1	4-β-GlcA
Li-product B	5.28	3.8	1	α-Gal
	5.24	n.o.	1	3-α-Man
	4.53	8.0	1	3-β-Glc
P2-alditol	5.43	3.8	2	α-Glc, 6-α-Glc
	5.30	n.o.	1	2,3-α-Man-(1→3)-β-Glc
	5.27	3.0	1	α-Gal
	5.25	3.0	1	α-Gal
	5.13	n.o.	1	2,3-α-Man-(1→3)-glucitol
	4.57	8.3	1	3-β-Glc, 4-β-GlcA (2)
	4.54	7.8	1	
	4.50	7.6	1	

[a]Chemical shift relative to internal acetone at δ 2.23. [b]Not observed. [c]6-α-Glc connotes the anomeric proton of a 6-linked β-D-glucopyranosyl residue, *etc.* The absence of a numerical prefix indicates a terminal non-reducing group. [d]Recorded at 95°.

TABLE III

^{13}C-N.M.R. DATA FOR *E. coli* K39 POLYSACCHARIDE AND DERIVED PRODUCTS

Compound	*P.p.m.*[a]	*Assignment*[b]
K39 Polysaccharide	103.57	3-β-Glc
	103.37	4-β-GlcA
	101.75	α-Gal
	100.01	6-α-Glc
	99.73	2,3-α-Man
	83.35	C-3 3-β-Glc
	68.73	C-6 6-α-Glc
	62.54	C-6 α-Gal
	61.57, 61.19	C-6 2,3-α-Man and 3-β-Glc
P1	103.29	4-β-GlcA
	101.69	α-Gal
	100.05	α-Glc
	99.77	2,3-α-Man-(1→3)-α-Glc
	99.68	2,3-α-Man-(1→3)-β-Glc
	96.73	3-β-Glc
	93.03	3-α-Glc
	83.09	C-3 3-β-Glc
	80.51	C-3 3-α-Glc
	62.50	C-6 α-Gal
	61.34, 61.17, 60.84, 60.71	C-6 2,3-α-Man and 3-β-Glc
	60.62	C-6 α-Glc
P1-alditol	103.50	4-β-GlcA
	102.03, 101.49	2,3-α-Man and α-Gal
	100.01	α-Glc
	80.77	C-3 glucitol
	63.60, 63.39	C-1 and C-6 3-glucitol
	62.55	C-6 α-Gal
	60.89	C-6 2,3-α-Man
	60.61	C-6 α-Glc
P2 (salt)	103.48	3-β-Glc
	103.29	4-β-GlcA (2)
	101.68	α-Gal (2)
	100.07	α-Glc
	99.96, 99.72	2,3-α-Man (2) and α-Glc
	96.71	3-β-Glc
	93.02	3-α-Glc
	68.47	C-6 6-α-Glc

[a]Chemical shift relative to internal acetone at 31.07 p.p.m. [b]Unless otherwise indicated, 3-β-Glc connotes the anomeric carbon of a 3-linked β-D-glucopyranosyl residue, *etc.* The absence of a numerical prefix indicates a terminal non-reducing group.

ratios shown in Table I. All the sugars were shown to have the D configuration by g.l.c. of their acetylated (−)-2-octyl glycosides[2].

The ^{1}H-n.m.r. spectrum (Table II) of a partially autohydrolysed sample of the polysaccharide showed, *inter alia*, the presence of five anomeric protons corresponding to three α linkages (δ 5.27, 5.32, and 5.40) and two β linkages (δ 4.55 and 4.51). The unresolved singlet at δ 5.32 was assigned to H-1 of an α-D-mannopyranosyl residue. The ^{13}C-n.m.r. data (Table III) were in agreement with these results, showing resonances for five anomeric carbons at 103.57, 103.37, 101.75, 100.01, and 99.73 p.p.m. The spectrum also contained signals at 61.19, 61.57, and 62.54 p.p.m. These indicate that three of the four neutral hexose residues are not linked through O-6. The above results suggest that K39 polysaccharide consists of a pentasaccharide repeating-unit.

Methylation analysis. — The native polysaccharide was methylated and the alditol acetates derived from an acid hydrolysate of the methylated polysaccharide, with and without reduction of the methoxycarbonyl function, were analysed by g.l.c.–m.s. (Table IV, columns I and II). The results confirm that the polysaccharide is composed of a pentasaccharide repeating-unit; galactose occurs as a terminal group, mannose is linked through O-2 and O-3, one glucose is linked through O-3, the other through O-6, and glucuronic acid is linked through O-4. The higher proportion of 4,6-di-*O*-methylmannose released on hydrolysis after reduction of the uronic ester indicates that the glucuronic acid is linked to the mannose residue.

Uronic acid degradation[3]. — The methylated polysaccharide was subjected to a β-elimination reaction and the alditol acetates derived from an acid hydrolysate

TABLE IV

METHYLATION ANALYSES OF *E. coli* K39 POLYSACCHARIDE AND DERIVED PRODUCTS

Methylated sugar[a] *(as alditol acetate)*	*Molar ratio*[b,c]					
	I	*II*	*III*	*IV*	*V*	*VI*
1,2,3,4,5-Glc						0.42
1,2,4,5,6-Glc				0.85	0.76	
2,3,4,6-Gal	0.85	0.84	0.91	1.00	1.98	0.80
2,3,4,6-Glc				0.95	0.85	
2,4,6-Glc	1.00	1.16	1.00		1.00	1.00
2,3,4-Glc	1.06	1.00	0.21		1.02	
2,4,6-Man			0.91			0.92
4,6-Man	0.40	0.93	0.10	0.96	1.90	
2,3-Glc		0.76		0.86	1.79	

[a]1,2,3,4,5-Glc = 6-*O*-acetyl-1,2,3,4,5-penta-*O*-methylglucitol, *etc.*; all substitution patterns were confirmed by g.l.c.–m.s. [b]Determined on a DB-225 capillary column at 205°. [c]I, methylated K39 polysaccharide; II, methylated, carboxyl-reduced K39 polysaccharide; III, base-degraded methylated polysaccharide; IV, methylated, carboxyl-reduced P1-alditol; V, methylated, carboxyl-reduced P2-alditol; VI, methylated product from lithium degradation of polysaccharide. Carboxyl-reduction was performed after methylation in all cases.

of the product were analysed by g.l.c.–m.s. (Table IV, column III). The concomitant decrease in the yield of 4,6-di-*O*-methylmannose and 2,3,4-tri-*O*-methylglucose and the production of 2,4,6-tri-*O*-methylmannose demonstrate that the glucuronic acid is linked to O-2 of the mannose residue and that the 6-linked glucose residue is linked to the uronic acid. The following sequence is thus present in the repeating unit.

→6)-Glc*p*-(1→4)-Glc*p*A-(1→2)-Man*p*-(1-

Bacteriophage-mediated degradation of K39 polysaccharide. — Treatment of K39 polysaccharide with a bacteriophage which infects *E. coli* K39, followed by gel filtration of a dialysate of the digest, afforded two oligosaccharides P1 and P2. Sugar analyses (Table I) and ^{1}H- and ^{13}C-n.m.r. spectroscopy (Tables II and III) of P1, P1-alditol, P2, and P2-alditol indicated that P1 and P2 were penta- and decasaccharides, respectively. Each oligosaccharide had a glucose reducing terminus and was composed of glucose, galactose, mannose, and glucuronic acid in the same ratios as in the native polysaccharide.

P1 and P2 were reduced to their respective alditols with sodium borodeuteride and analysed by methylation. The results for P1-alditol (Table IV, column IV) show the presence of terminal galactose, terminal glucose, glucuronic acid linked through O-4, mannose linked through O-2 and O-3, and glucitol linked through O-3. These results, when compared with those for P2-alditol (Table IV, column V) and the polysaccharide (Table IV, column II), demonstrate that the bacteriophage enzyme cleaved →3)-Glc*p*-(1→6)-Glc*p*-(1- linkages in the native polysaccharide and is, therefore, a glucosidase. These results permit the following sequence to be written for the repeating unit of K39 polysaccharide.

→6)-Glc*p*-(1→4)-Glc*p*A-(1→2)-Man*p*-(1→3)-Glc*p*-(1-
3
↑
1
Gal*p*

Comparison of the ^{1}H-n.m.r. spectra of P1 and the polysaccharide (Table II) revealed that one of the β signals in the spectrum of the polysaccharide had been replaced in the spectrum of P1 by partial signals at δ 5.23 and δ 4.67. This demonstrates that the 3-linked glucose residue cleaved by the phage enzyme has the β configuration. Two further sets of partial signals were observed in the proton spectrum of P1. These are due to the α and β anomers of P1 present in solution, caused by mutarotation of the reducing glucose residue. The set of partial signals at δ 5.31 and δ 5.30 (unresolved doublets) were assigned to H-1 of the α-D-mannopyranosyl residue linked to O-3 of the reducing glucose residue. The partial signals at δ 4.54 and δ 4.53 (^{3}J 8.0 Hz) arise from a β-linked residue and are assigned to

H-1 of the glucopyranosyluronic acid since this residue is closer to the mutarotating centre than either the galactopyranosyl side-chain or the 6-linked glucopyranosyl residue which, therefore, both have the α configuration.

The ^{13}C-n.m.r. spectrum of P1 (Table III) also exhibited a number of differences from that of the polysaccharide. Apart from the expected disappearance of the anomeric carbon signal at 103.57 p.p.m. for the β-D-glucopyranosyl residue, and the twinning of the signal for the α-D-mannopyranosyl anomeric carbon, the spectrum also showed an additional resonance for an unlinked C-6 of a glycose, thus confirming that the β-D-glucopyranosyl residue was linked to O-6 of the α-D-glucopyranosyl residue in the polysaccharide.

Partial hydrolysis of K39 polysaccharide. — Paper chromatographic examination of the sugars released on heating the polysaccharide with dilute trifluoroacetic acid showed that galactose was the most acid-labile sugar in the polymer. ^{1}H-N.m.r. spectroscopy of the polymeric product recovered by gel filtration, after hydrolysis of the polysaccharide under conditions which favoured mainly the release of the galactose residues, indicated a considerable reduction (>50%) in the signal at δ 5.27, which was thus assigned to H-1 of an α-D-galactopyranosyl residue.

Lithium–ethylenediamine degradation of K39 polysaccharide[4]. — Treatment of a solution of the polysaccharide in ethylenediamine with lithium, followed by separation of the products by gel filtration on Bio-Gel P-4, afforded an oligosaccharide B. G.l.c. examination of the hydrolysis products of B (as peracetylated aldononitriles) showed it to be a tetrasaccharide-alditol composed of galactose, glucose, mannose, and glucitol (Table I). Methylation analysis of B indicated (Table IV, column VI) the presence of terminal galactose, glucose linked through O-3, mannose linked at O-3, and a 6-linked glucitol. These data and the sequence established for the polysaccharide permit the following sequence to be written for B.

Gal*p*-(1→3)-Man*p*-(1→3)-Glc*p*-(1→6)-Glc-ol

The ^{1}H-n.m.r. spectrum of B contained (Table II) H-1 resonances for one β- and two α-linked residues. The resonance at δ 5.24 (unresolved doublet) may be assigned to H-1 of the α-D-mannopyranosyl residue, while the signal at δ 4.53 (^3J 8.0 Hz) must be due to H-1 of the 3-linked D-glucopyranosyl residue which was shown to be β in the bacteriophage degradation experiment. The anomeric proton signal at δ 5.28 (^3J 3.8 Hz) may now be assigned to the D-galactopyranosyl residue, which confirms its α configuration. The structure of the repeating unit of the capsular polysaccharide of *E. coli* K39 is thus as shown in the Abstract. This is the same structure as that reported[5] for the repeating unit of *Klebsiella* K61.

EXPERIMENTAL

General methods. — These have been previously described[6,7]. In addition,

g.l.c.–m.s. was performed with a Hewlett–Packard 5988A g.l.c.–mass spectrometer, with a DB-225 bonded-phase capillary column (30 m × 0.25 mm) having a film thickness of 0.25 μm, and helium as carrier gas. The absolute configuration of hexoses was determined by the method of Leontein *et al.*[2]. Unless stated otherwise, ^{1}H- and ^{13}C-n.m.r. spectra were recorded for samples in the acid form at 30°.

Preparation of K39 polysaccharide. — A culture of *E. coli* O9:K39:H9 (A121a), obtained from Dr. I. Ørskov (Copenhagen), was grown on Mueller–Hinton agar for 18 h at 37°. The acidic capsular polysaccharide was isolated and purified by precipitation with cetyltrimethylammonium bromide as described previously[8]. An aqueous solution of the acid form of the polysaccharide was heated (0.5 h) at 90°, dialysed, and freeze-dried, and the product was examined by ^{1}H- and ^{13}C-n.m.r. spectroscopy (Tables II and III).

Sugar composition. — The polysaccharide was hydrolysed with 2M trifluoroacetic acid overnight at 100°, and the products of hydrolysis were converted into acetylated aldononitriles[9] and examined by g.l.c. A further sample of polysaccharide was treated with boiling methanolic 3% hydrogen chloride for 16 h at 80° and, after neutralisation, the products were reduced with $NaBH_4$ in anhydrous methanol and then hydrolysed with 2M trifluoroacetic acid (16 h, 100°). The products were converted into acetylated aldononitriles and analysed by g.l.c. (Table I, column I).

Methylation analysis. — K39 polysaccharide (60 mg), in the acid form, was methylated once by the Hakomori method[10] (potassium methylsulphinylmethanide) and then by the Kuhn method[11]. A portion of the methylated polysaccharide was hydrolysed with 4M trifluoroacetic acid for 1 h at 125°, and the products were reduced ($NaBH_4$), acetylated, and analysed by g.l.c.–m.s. (Table IV, column I). A further portion of the methylated product was methanolysed, reduced, hydrolysed (4M trifluoroacetic acid, 1 h, 125°), reduced, acetylated, and analysed by g.l.c.–m.s. (Table IV, column II).

Uronic acid degradation of methylated K39 polysaccharide[3]. — Methylated polysaccharide (40 mg) in Me_2SO, containing 2,2-dimethoxypropane and *p*-toluenesulphonic acid, was treated with sodium methylsulphinylmethanide under nitrogen for 16 h after which the reaction mixture was treated with CD_3I. The product was hydrolysed (4M trifluoroacetic acid, 1 h, 125°), reduced, acetylated, and examined by g.l.c.–m.s. (Table IV, column III).

Bacteriophage depolymerisation of K39 polysaccharide. — A bacteriophage which forms plaques with large haloes on *E. coli* K39 bacteria was isolated from Port Elizabeth sewage and was used to depolymerise K39 capsular polysaccharide. Separation of the oligomeric products on Bio-Gel P-4 in aqueous pyridinium acetate (pH 5.3) gave three fractions P1, P2, and P3. Solutions of P1 and P2 in D_2O were examined by ^{1}H- and ^{13}C-n.m.r. spectroscopy (Tables II and III). P1- and P2-alditol were methanolysed, reduced, and hydrolysed, and the products were converted into the acetylated aldononitriles and examined by g.l.c. (Table I). P1- and P2-alditol were methylated, methanolysed, reduced, hydrolysed, reduced, and

acetylated, and the products were examined by g.l.c.–m.s. (Table IV, columns IV and V). Solutions of P1- and P2-alditol in D_2O were examined by ^1H- and ^{13}C-n.m.r. spectroscopy (Tables II and III).

Partial hydrolysis of K39 polysaccharide. — Polysaccharide was heated with 0.5M trifluoroacetic acid at 90° until most of the galactose residues were liberated (p.c.), after which the solution was evaporated and the residue was subjected to gel filtration to afford a polymeric product which was examined by ^1H-n.m.r. spectroscopy.

Treatment of K39 polysaccharide with lithium in ethylenediamine. — Dry K39 polysaccharide (83 mg) in dry ethylenediamine (20 mL) was treated with lithium wire (1 cm of 3-mm diameter wire, Fluka) with stirring. After 30 min, a further piece of lithium wire (0.5 cm) was added in order to maintain the deep blue colour of the reaction mixture. The excess of lithium was quenched with cold water (10 mL) and the solvents were evaporated as an azeotrope with toluene. The residue was adjusted to pH 4.5 with glacial acetic acid and the resulting solution was passed down a column of Amberlite IR-120 (H^+) resin and freeze-dried. Purification of the product by gel-permeation chromatography on Bio-Gel P-4 with pyridinium acetate (pH 5.3) afforded a product (25 mg) having the elution volume of a tetrasaccharide. ^1H-N.m.r. spectroscopy of the product revealed the presence of reducing hexose. The product was therefore reduced with sodium borohydride and re-examined by ^1H-n.m.r. spectroscopy (Table II). The product was hydrolysed, and the products were converted into the acetylated aldononitriles and examined by g.l.c. (Table I). Methylation analysis of the product gave the results shown in Table IV (column VI).

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