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Structure of the capsular polysaccharide (K98 antigen) of *E. coli* O7:K98:H6

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ABSTRACT

The capsular polysaccharide (K98 antigen) of *E. coli* O7:K98:H6 contains rhamnose, glucuronic acid, and acetate in the molar ratios 3:1:0.6. Methylation analysis, oligosaccharide analysis, and 1D- and 2D-n.m.r. spectroscopy revealed the polysaccharide to be a glucuronic acid-substituted rhamnan with the structure

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→3)-α-L-Rha-(1→3)-α-L-Rha-(1→2)-α-L-Rha-(1→
                          2
                          ↑
                          1
                    β-D-GlcA
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Of the 3-linked rhamnose residues, ~60% are *O*-acetylated at position 2.

INTRODUCTION

The capsular antigens (K antigens) of *Escherichia coli* are acidic polysaccharides which are classified into two groups[1,2] on the basis of chemical, biochemical, and genetic parameters. These parameters include the temperature-regulation of the expression of the capsular antigens and their occurrence with certain O-antigens. There are several *E. coli* strains having capsular polysaccharides that do not fit this classification and constitute a third group of K antigens, related to group II[3] to which the K98 polysaccharide belongs. Like K antigens of group I, the K98 antigen is also expressed at low temperatures of growth and, like K antigens of group II, it occurs together with several O-antigens. The structure of the K98 antigen is now reported.

RESULTS AND DISCUSSION

Isolation and characterisation of the K98 capsular polysaccharide. — The polysaccharide, isolated[4] in a yield of 100 mg/L of culture medium, consisted of rhamnose, glucuronic acid, and acetate in the molar ratios 3:1:0.6. G.l.c. of the derived (+)-2-octyl rhamnoside[5] indicated the rhamnose to be L.

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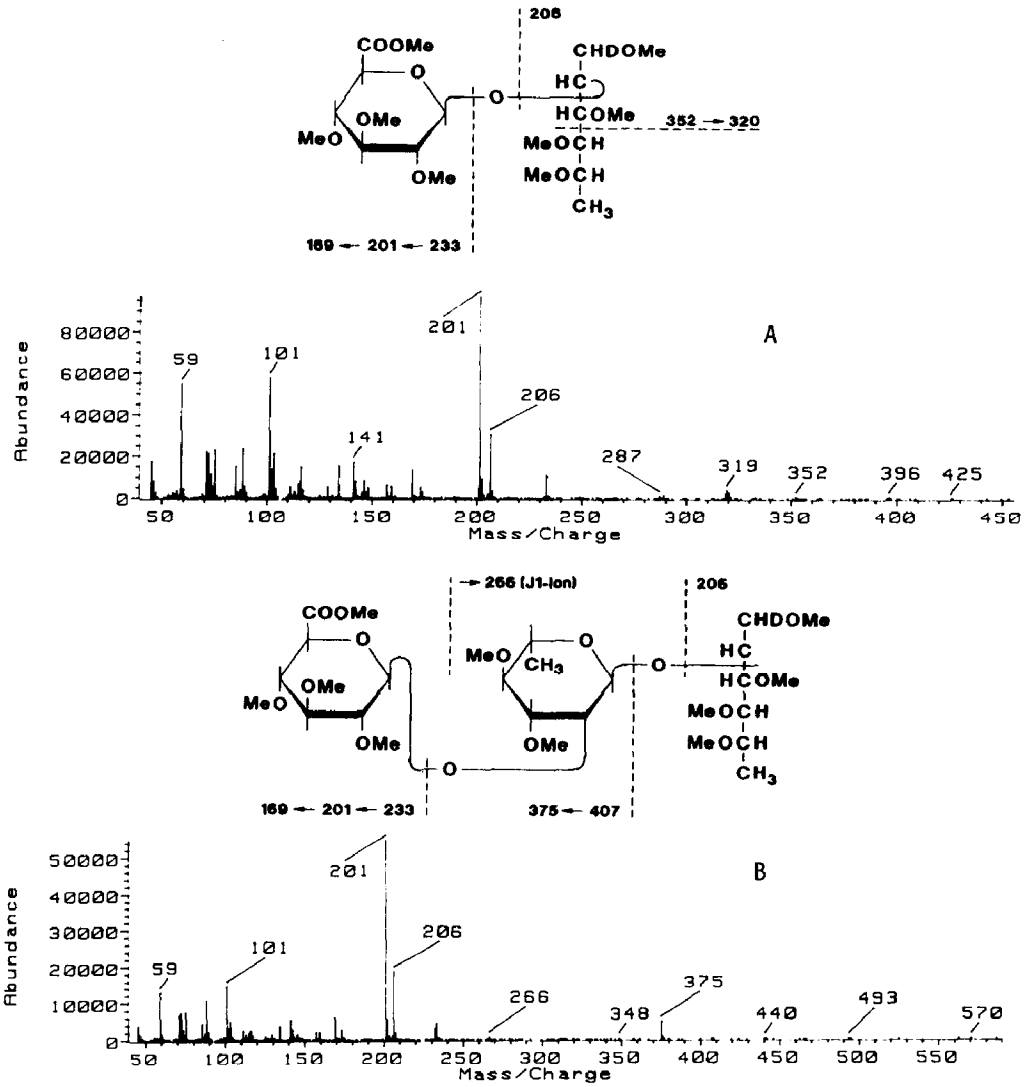
TABLE I

Composition[a] of the K98 polysaccharide (PS) and its carboxyl-reduced (PS_{red}) and periodate-oxidised (PS_{ox}) forms

Preparation	*Constituents*							
	GlcA		*Rha*		*OAc*		*Glc*	
	%	*M.r.*	%	*M.r.*	%	*M.r.*	%	*M.r.*
PS	25.0	0.9	70.2	3.0	5.1	0.6	0	0
PS_{red}	0	0	69.1	3.0	0	0	22	0.85
PS_{ox}	0	0	67.3		0	0	0	0

[a] M.r., molar ratio; % by weight.



Carboxyl reduction[6] of the polysaccharide converted the GlcA into D-Glc which was determined subsequently with D-glucose oxidase. Periodate oxidation destroyed the GlcA and one Rha residue. The sugar compositions of the native, carboxyl-reduced, and periodate-oxidised polysaccharides are shown in Table I.

In immunoelectrophoresis, passive haemagglutination, and ELISA[7], the polysaccharide reacted with the K98-specific serum of the non O-cross-reactive *E. coli* O107:K98:H6, which indicated K98 specificity.

Methylation of the carboxyl-reduced K98 polysaccharide. — Methylation was effected with a modification[8] of the Hakomori procedure[9], the product was hydrolysed, and the sugar derivatives released were treated with sodium borohydride, then acetylated, and analysed by g.l.c.–m.s. The results (Table II) show that the K98 polysaccharide, after loss of the *O*-acetyl group, contained one 2-linked and one 3-linked Rha, one 2,3-disubstituted Rha, and a terminal GlcA.

Oligosaccharide analysis. — The K98 polysaccharide was hydrolysed (M trifluoroacetic acid, 1 h, 100°) and the neutralised hydrolysate was subjected to high-voltage paper electrophoresis (40 V/cm, pH 5.4, 90 min). Three oligosaccharides were detected, namely, OS-1 (M_{GlcA} 0.65, GlcA:Rha 1:1), OS-2 (M_{GlcA} 0.49, GlcA:Rha 1:2), and OS-3 (M_{GlcA} 0.37, GlcA:Rha 1:3), which were isolated by preparative electrophoresis, purified by chromatography on Fraktogel TSK HW 40, and reduced with sodium borodeuteride.

Each reduced oligosaccharide was methylated, and the methylated derivatives (OS-1m/3m) were purified on Sepak-18 and subjected to g.l.c.–m.s. The c.i. (ammonia)-mass spectra contained peaks for $(M + NH_4)^+$ at *m/z* 473 (OS-1m), 647 (OS-m2), and 821 (OS-3m), indicative of di-, tri-, and tetra-saccharides, respectively. The e.i.-mass spectra of OS-1m/3m are shown in Fig. 1. The fragments with *m/z* 233, 201, and 169

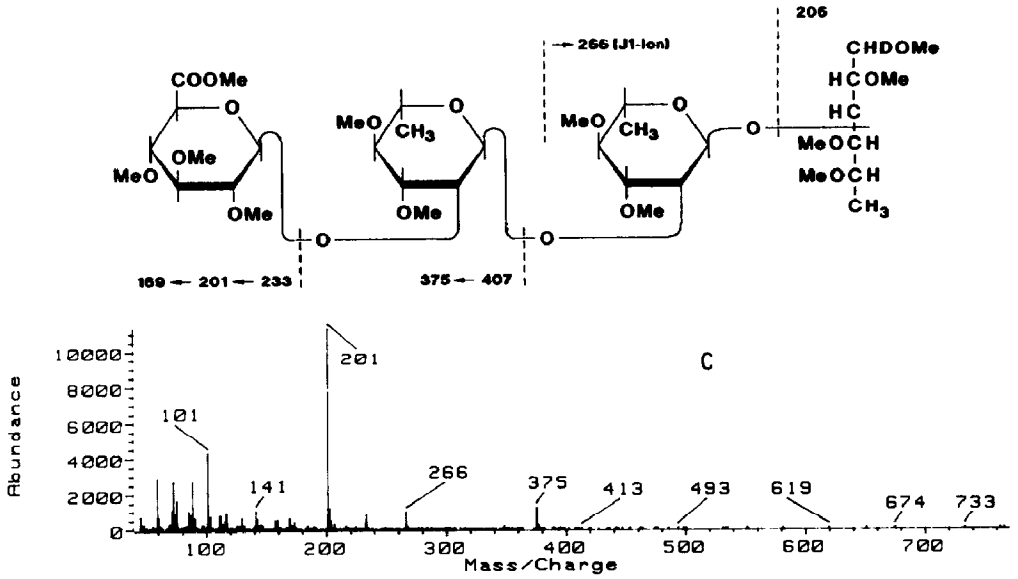


Fig. 1. E.i.-mass spectra of A, OS-1m; B, OS-2m; C, OS-3m.

TABLE II

Products of methylation analysis of the K98 polysaccharide before (I) and after carboxyl reduction (II)

Molar ratio		*Interpretation of the mass spectra*
I	*II*	
0.8	0.9	1,2,5-Tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol
0.9	0.8	1,3,5-Tri-*O*-acetyl-2,4-di-*O*-methylrhamnitol
1.0	1.0	1,2,3,5-Tetra-*O*-acetyl-4-*O*-methylrhamnitol
0	1.1	1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol

were formed by cleavage of the terminal GlcA acid, and the fragments with *m/z* 206 and 59 were derived from Rha-ol-*l-d*. The (1→2) linkage in OS-1m was indicated[10] by the fragments with *m/z* 319 and 287 (319−32). The fragment with *m/z* 375 (407−32) (ref. 11) and the J_1 ion with *m/z* 266 (ref. 12) indicated the central Rha in OS-2m to be 2-linked. Thus, OS-1/3 had the structures **1–3**, respectively.

β-D-GlcA-(1→2)-L-Rha-ol

1

β-D-GlcA-(1→2)-α-L-Rha-(1→2)-L-Rha-ol

2

β-D-GlcA-(1→2)-α-L-Rha-(1→2)-α-L-Rha-(1→3)-L-Rha-ol

3

N.m.r. spectroscopy. — The ^{13}C-n.m.r. spectra of the native and deacetylated K98 polysaccharides are shown in Fig. 2. The signal at δ 104.8 was assigned to C-1 of GlcA (ref. 13), that at δ 102.8 to C-1 of the 3-linked Rha, and that at δ 101.5 or 101.7 to C-1 of the 2-linked Rha (refs. 13 and 14). Therefore, the signal at δ 101.7 or 101.5 must be due to C-1 of the 2,3-linked rhamnose. The signal at δ 176.1 was due to COOH of GlcA, and the three signals in the region δ 17.4–17.6 to Me-5 of Rha. The signals at δ 173.4 and 21.0, present in the spectrum of the native polysaccharide, were assigned to OAc.

For complete assignment of the signals, 2D measurements[15] were used. In a ^{1}H,^{1}H-correlated (COSY) spectrum of the *O*-deacetylated K98 polysaccharide, the ^{1}H resonances were assigned starting from those for H-1 and their cross-peaks. The C–H signal correlations were obtained in a ^{1}H,^{13}C-correlated (COSY) spectrum. The assignments of the C-1/4 resonances are shown in Table III. Although the C-5 and C-6 signals could not be assigned in these spectra, their chemical shifts were apparent from the ^{13}C-n.m.r. spectrum. Thus, the signals at δ 70.1 and 70.3 were due to C-5 of Rha and those at δ 17.4, 17.5, and 17.6 to the C-6 of Rha. A definitive allocation to RhaI, RhaII,

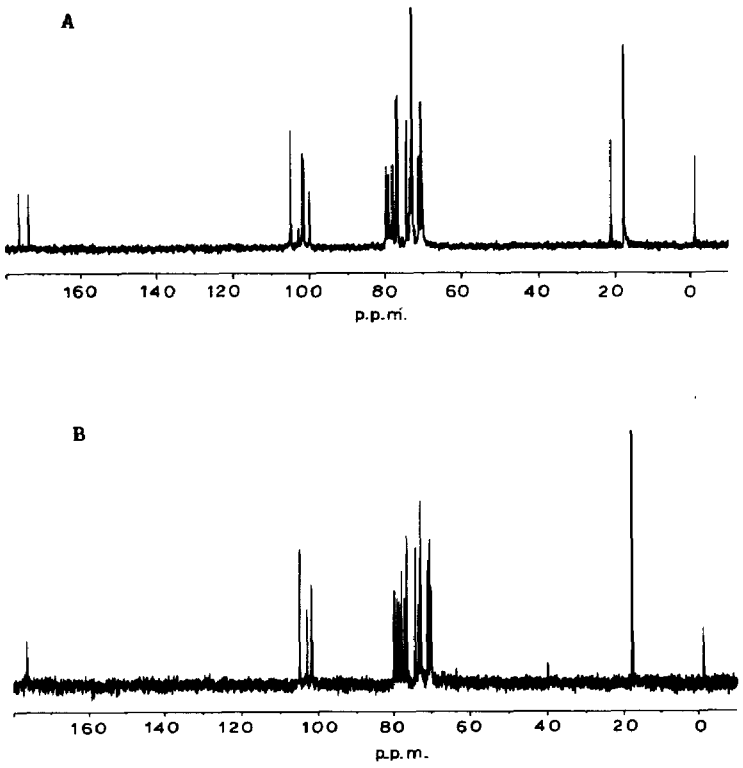


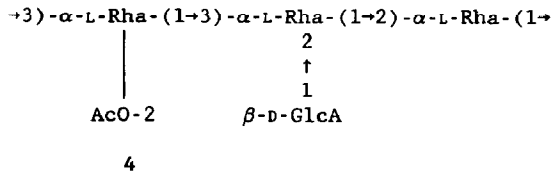
Fig. 2. ^{13}C-N.m.r. spectra of solutions of the native (A) and the *O*-deacetylated (B) K98 capsular polysaccharides in D_2O at 70°.

or RhaIII (Table III) was not possible. The signals at δ 76.4 and 176.1 were assigned to C-5 and C-6 of GlcA, respectively.

The anomeric configurations of the sugar constituents were determined using gated decoupling[16]. The $J_{C\text{-}1,H\text{-}1}$ value of 165 Hz indicated GlcA to be β and those of 171, 177, and 178 Hz indicated Rha to be α.

The ^{13}C-n.m.r. spectrum of the K98 polysaccharide contained pairs of signals at δ 99.8 and 102.8, 73.1 and 70.8, and 76.6 and 78.4. A spectrum in the inverse-gated mode[17] showed that these pairs had relative intensities of ~3:2. In the spectrum of the *O*-deacetylated K98 polysaccharide, only the signals at 102.8, 70.8, and 78.4 (C-1,2,3 of RhaI) were apparent. The upfield shift of the C-2 signal and the downfield shifts of those for C-1,3 indicated that C-2 of RhaI was the site of acetylation. The above ratio of intensities indicated the extent of acetylation to be ~60%. Thus, the substitutions of the Rha backbone with OAc and with β-GlcA occur on the rhamnoses I and II, respectively, and the structure of the K98 polysaccharide can be formulated as **4**.

The sequence of the sugar units was confirmed with a 2D-NOESY experiment[18]. Thus, for the *O*-deacetylated polysaccharide, there were cross-peaks assignable to inter-ring interactions of the anomeric protons. The n.O.e. cross-peaks corresponded to



a (1→2) linkage between RhaII and RhaIII (at 4.0 p.p.m.), a (1→3) linkage between RhaIII and RhaI (at 3.7 p.p.m.), and a (1→2) linkage between GlcA and RhaII (at 4.1 p.p.m.) (see Table III).

Serological analysis. — In an enzyme-linked immunoabsorbent assay (ELISA)[7], using a K98-specific antiserum (rabbit), the K98 polysaccharide had a reciprocal titre of 64,000. The *O*-deacetylated polysaccharide and the carboxyl-reduced polysaccharide had reciprocal titres of 16 and 0, respectively. These results were supported by inhibition of the ELISA with the native, *O*-deacetylated, and carboxyl-reduced polysaccharides. A 50% inhibition was obtained with 20μM K98 polysaccharide and with 1mM *O*-deacetylated or carboxyl-reduced polysaccharide. These results indicated the OAc group to be a

TABLE III

^{13}C- and ^{1}H-n.m.r. data for the *O*-deacetylated K98 polysaccharide

Residue[a]	*Proton or carbon*	^{1}H (δ, *p.p.m.*)	^{13}C (δ, *p.p.m.*)
RhaI	1	4.98	102.8
	2	4.10	70.8
	3	3.76	78.4
	4	3.54	72.6
RhaII	1	5.20	101.5
	2	4.16	79.6
	3	3.88	77.6
	4	3.60	72.6
RhaIII	1	5.12	101.7
	2	4.01	78.9
	3	3.84	70.8
	4	3.43	73.3
GlcA	1	4.52	104.8
	2	3.30	74.2
	3	3.45	76.4
	4	3.58	72.8

[a] →3)-α-L-RhaI-(1→3)-α-L-RhaII-(1→2)-α-L-RhaIII-(1→
2
↑
1
β-D-GlcA

serological determinant. In order to evaluate the contribution of the rhamnose backbone to the serological specificity, the K3 polysaccharide, which has the same rhamnose backbone as the K98 polysaccharide but which is substituted with a 4-deoxy-2-hexulosonic acid[19], was analysed in an ELISA with the anti-K98 antiserum. The K3 polysaccharide had a reciprocal titre of 4000, indicative of a distinct contribution of the rhamnose backbone to the serological K98 specificity.

EXPERIMENTAL

Bacteria and cultivation. — *E. coli* 21511 (O7:K98:H6) was obtained from Drs. I. and F. Ørskov (Copenhagen), and grown to the late logarithmic phase (5–7 h) in a fermenter at 37° in 10-L batches containing (per L) $K_2PO_4 \cdot 3H_2O$ (9.2 g), KH_2PO_4 (2 g), sodium citrate·$5H_2O$ (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.1 g), $(NH_4)_2SO_4$ (1 g), casamino acids (20 g), D-glucose (2 g), and the dialysable part of yeast (100 mL, from 500 g, in 5 L of deionised water).

Isolation and purification of the K98 polysaccharide. — The polysaccharide and the bacterial cells were precipitated from the liquid culture by the addition of 1 vol. of aqueous 2% cetyltrimethylammonium bromide (Cetavlon). Each of the following operations was carried out 4°. The polysaccharide was extracted from the precipitate with M calcium chloride and purified by three cycles of precipitation from aqueous solution with ethanol (80%, final concentration), followed by repeated extraction with cold aqueous 80% phenol (buffered to pH 6.8 with sodium acetate) to remove contaminating proteins[4]. The combined aqueous phases were centrifuged for 4 h at 100 000*g* and the supernatant solution was lyophilised. The residue was purified by elution from Sephadex G-50 with water.

Analytical methods. — Glucuronic acid and rhamnose were determined in the polysaccharide with the carbazole and cysteine reagents, respectively[20,21]. Acetic acid was characterised as its hydroxamate[22], protein determinations were done according to Bradford[23], and nucleic acid was determined by u.v. spectroscopy of samples in 10mM sodium hydroxide at 258 nm.

N.m.r. spectra (related to Me_4Si) were obtained with a Bruker WM 300 spectrometer in the F.t. mode. G.l.c.–m.s. was performed with a Hewlett–Packard HP 5988 A mass spectrometer (70 eV) combined with an HP-5980 gas chromatograph equipped with an SP-2330 capillary column.

O-*Deacetylation.* — A solution of the K98 polysaccharide (100 mg) in 0.25M sodium hydroxide (5 mL) was kept for 1 h at 37°, neutralised with hydrochloric acid, dialysed, and lyophilised, to give *O*-deacetylated polysaccharide (85 mg).

Carboxyl reduction. — The K98 polysaccharide (60 mg) was treated with 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide at pH 4.7 and the resulting esters were reduced with sodium borohydride[6]. After dialysis and lyophilisation, carboxyl-reduced polysaccharide (47 mg) was obtained. The almost quantitative conversion of GlcA into Glc was confirmed by the ^{13}C-n.m.r. spectrum of the product, which contained a signal at δ 61.2 for CH_2OH of Glc, but no signal for COOH of GlcA.

Methylation analysis. — Polysaccharide preparations (5 mg) were each methylated with methyl sulfoxide–potassium hydride–methyl iodide in a modification[8] of the procedure described by Hakomori[9]. After hydrolysis of the products, followed by reduction with sodium borodeuteride and acetylation, the partially methylated alditol acetates were analysed by g.l.c.–m.s.

Isolation of the oligosaccharides. — Oligosaccharides OS-1/3 were obtained after hydrolysis of the K98 polysaccharide (100 mg) in M trifluoroacetic acid (10 mL) at 100° for 1 h. The neutralised hydrolysate was concentrated and subjected to high-voltage paper electrophoresis (40 V/cm, pH 5.4, 90 min). The separated oligosaccharides were eluted from the paper with water and purified by chromatography on Fraktogel TSK HW40.

Serological studies. — An antiserum against *E. coli* O107:K98:H6 was obtained from rabbits as described[13]. The enzyme-linked immunosorbent assay[7] (ELISA) was used with the following modifications. Microtitre plates were pretreated at 37° for 1 h with a 0.5% solution of polylysine (mol. wt. 7 000–15 000) in phosphate-buffered saline (PBS, 50 μL/well). After washing with 0.05% Tween-20 in PBS, each well was coated with 50 μL of a solution of polysaccharide (0.1 mg/mL water) at 37° overnight. After blocking with 4% bovine serum albumin in PBS (250 μL/well) for 4 h and washing with PBS, serial dilutions of the antiserum (specific for K98, not cross-reactive with O7) were set up in the coated wells. After washing with 0.05% Tween-20 in PBS, the wells were incubated with peroxidase-conjugated goat (anti-rabbit) antibody. After washing, the antigen–antibody interaction was detected by incubation with hydrogen peroxide (0.002% final concentration) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid).

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