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THE USE OF BACTERIOPHAGE-MEDIATED DEPOLYMERISATION IN THE STRUCTURAL INVESTIGATION OF THE CAPSULAR POLYSACCHARIDE FROM *Escherichia coli* SEROTYPE K36*

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ABSTRACT

The structure of the repeating unit of the capsular polysaccharide from *Escherichia coli* serotype K36 has been established from the results of spectroscopic and chemical analyses of (*a*) **P1**, the tetrasaccharide obtained on depolymerisation of the polysaccharide with a bacteriophage-borne endo-galactosidase, (*b*) **P1**-alditol, and (*c*) the original polysaccharide. The repeating unit, which is identical to that reported for *Klebsiella* K57, has the following structure.

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

INTRODUCTION

The capsular (K) antigens of *E. coli* are either polysaccharides or proteins[1]. The polysaccharide K antigens have been subdivided into two groups[1], namely, those associated with O groups 8, 9, 20, and 101, and those found with other O groups. The K antigens belonging to the former group are either high-molecular weight (3×10^5 to 10^6) heterogeneous acidic polymers devoid of amino sugars (and which resemble the K antigens of *Klebsiella*) or homogeneous amino sugar-containing acidic polymers, generally of lower molecular weight (1×10^3 to 3×10^5).

The capsular polysaccharide of *E. coli* K36, whose structure is now reported, is associated with O antigen 9 and contains no amino sugars. The structure of the polysaccharide was established mainly from a study of the oligosaccharide obtained from the bacteriophage-mediated depolymerisation of the original polysaccharide.

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RESULTS AND DISCUSSION

Preparation of the capsular polysaccharide. — *E. coli* K36 was grown on Mueller–Hinton agar, and the polysaccharide was isolated and purified as previously described[2]. The purified product obtained by precipitation with cetyltrimethylammonium bromide had $[\alpha]_D$ +63°, and was shown to be monodisperse (M_r = 2.7 × 10^7) by gel-permeation chromatography on Sepharose 4B CL. Analysis of hydrolysates of the polysaccharide with and without prior reduction of the uronic acid showed it to have the composition mannose, galactose, and galacturonic acid in the ratios of 2:1:1. Each sugar was shown to be D by g.l.c. analysis of the acetylated (−)-2-octyl glycosides[3].

Preparation of oligosaccharide **P1**. — Depolymerisation of *E. coli* K36 capsular polysaccharide by its homologous bacteriophage Φ36 afforded oligosaccharide **P1** in 78% yield. Analysis of **P1**, with and without reduction of the uronic acid, showed it to have the same composition as the original polysaccharide. Comparison of the optical rotation of the polysaccharide with that of **P1** suggests that the phage enzyme had cleaved a β linkage.

Methylation of **P1**-*alditol.* — Oligosaccharide **P1** was reduced with sodium borohydride and the derived oligosaccharide-alditol was methylated by the Hakomori method[4]. G.l.c. and g.l.c.-m.s. analyses of the partially methylated alditol acetates obtained after reduction of the uronic acid (Table I, column I) show **P1** to be a linear tetrasaccharide with a mannosyl unit as the non-reducing end group and a 3-linked galactose residue as the reducing terminus. Thus, the structure of **P1** is either Man-(1→2)-Man-(1→4)-GalA-(1→3)-Gal or Man-(1→4)-GalA-(1→2)-Man-(1→3)-Gal. The precise location of the 2-linked mannosyl residue and the nature of the configuration of the anomeric linkages may be obtained from a study of the n.m.r. spectra of **P1** and **P1**-alditol.

TABLE I

METHYLATION ANALYSES OF K36 POLYSACCHARIDE AND **P1**-ALDITOL

Methylated sugar [a]	T[b]	*Molar ratio*[c]		
(as alditol acetate)	*(OV-225)*	*I*	*II*	*III*
1,2,4,5,6-Gal	0.75	1.0		
2,3,4,6-Man	0.98	1.0	1.0	1.0
3,4,6-Man	1.55	1.1	1.1	1.3
2,4,6-Gal	1.70		1.3	1.2
2,3-Gal	3.21	0.8		
2-Gal	4.03			1.1

[a]1,2,4,5,6-Gal = 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methylgalactitol, *etc.* [b]Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on DB-225 (J & W fused-silica capillary column, 0.25-μm film thickness, 30 m × 0.25 mm) isothermal at 205°. [c]I, Methylated, carboxyl-reduced **P1**-alditol; II, methylated native polysaccharide; III, methylated reduced native polysaccharide.

TABLE II

^{1}H-N.M.R. DATA FOR *E. coli* K36 POLYSACCHARIDE AND DERIVED OLIGOSACCHARIDES

Compound	*Chemical shift*[a] *(p.p.m.)*	*No. of H*	*Assignment*[b]
P1[c]	5.32	~0.1	→3)-β-Gal*f*
	5.28	0.45	→3)-α-Gal*p* →3)-α-Gal*f*
	5.27	1.0	→4)-α-Gal*p*A
	5.24	0.35	→2)-α-Man*p*-(1→3)-α-Gal*p*
	5.23	0.45	→2)-α-Man*p*-(1→3)-β-Gal*p*
	5.16	~0.1	→2)-α-Man*p*-(1→3)-β-Gal*f*
	5.13	~0.1	→2)-α-Man*p*-(1→3)-α-Gal*f*
	4.93	1.0	α-Man*p*
	4.74	1.0	H-5 of →4)-α-Gal*p*A
	4.62	0.45	→3)-β-Gal*p*
	4.49	1.0	H-4 of →4)-α-Gal*p*A
P1-alditol	5.29	1.0	→4)-α-Gal*p*A
	5.22	1.0	→2)-α-Man*p*
	4.94	1.0	α-Man*p*
	4.72	1.0	H-5 of →4)-α-Gal*p*A
	4.47	1.0	H-4 of →4)-α-Gal*p*A
Polysaccharide	5.23	2	→3,4)-α-Gal*p*A →2)-α-Man*p*
	5.05	1.0	α-Man*p*
	4.68	1.0	→3)-β-Gal*p*
	4.63	1.0	H-5 of →3,4)-α-Gal*p*A
	4.43	1.0	H-4 of →3,4)-α-Gal*p*A

[a]Chemical shift relative to that of internal acetone at δ 2.23. [b]→4)-α-Gal*p*A refers to the anomeric proton of a 4-linked α-galactopyranosyluronic acid residue. [c]For the origin of the compounds, see text.

^{1}H-N.m.r. data for **P1** *and* **P1**-*alditol.* — The 500-MHz ^{1}H-n.m.r. data for **P1** and **P1**-alditol recorded at 95° are collected in Table II. The spectrum of **P1** shows three fractional resonances at δ 5.32 (0.1 H), 5.28 (0.45 H), and 4.62 (0.45 H), which represent the anomeric protons of the β-furanosyl and α- and β-pyranosyl forms of the terminal galactose residue. The fractional signal representing H-1 of the α-galactofuranosyl residue is known to occur to higher field than that for the β-furanosyl residue and is probably overlapped[5] with the α-pyranosyl signal. The fractional signals at δ 5.24, 5.23, 5.16, and 5.13 are attributed to a 2-linked mannosyl residue adjacent to the reducing end and arise from the four **P1** oligosaccharides in solution caused by the α and β configurations of both ring forms of the terminal galactose. A similar set of signals has been reported[5] for H-1 of a 2-linked mannosyl residue interior to a reducing galactose terminus in the pentasaccharide obtained by the bacteriophage-mediated degradation of *Klebsiella* K3.

The accuracy of the above interpretation is verified by the spectrum of **P1**-

alditol (Table II) where the terminal galactose residue has been reduced to galactitol, causing H-1 of the 2-linked mannose to appear as a singlet at δ 5.22. The signals at δ 5.27 ($J_{1,2}$ 3.0 Hz) and 5.29 ($J_{1,2}$ 3.0 Hz) in the spectra of **P1** and **P1**-alditol, respectively, may be assigned to H-1 of α-galactopyranosyluronic acid residues. Of the three unassigned resonances in the anomeric region of the spectra of **P1** and **P1**-alditol, one signal is due to H-1 of the terminal mannose residue while the others must arise from non-anomeric ring protons. A COSY 45 experiment performed on **P1**-alditol readily established the signals at δ 4.72 and 4.47 as those of H-5 and H-4, respectively, of the α-galactopyranosyluronic acid residue. The resonances at δ 4.94 and δ 4.93 in the spectra (Table II) of **P1**-alditol and **P1**, respectively, may now be assigned to H-1 of the pendant mannopyranosyl groups. The COSY experiment also establishes that H-2 of the pendant mannosyl group resonates at δ 3.88, whereas H-2 of the 2-linked α-mannopyranosyl residue resonates 0.17 p.p.m. to lower field at δ 4.05.

^{13}C-N.m.r. data for **P1** *and* **P1**-*alditol.* — The ^{13}C-n.m.r. spectral data (Table III) of **P1** accord with a tetrasaccharide structure. The signals at 93.1 and 97.1 p.p.m. correspond[6] to the α- and β-anomeric carbons of the terminal galactose residue in **P1**, while the fractional signals at 95.4 and 95.1 p.p.m. are attributed to the anomeric carbon of the 2-linked mannose adjacent to the galactose. The signals at 102.5 and 101.8 p.p.m. in the spectrum of **P1** and the signals at 102.5 and 101.6 p.p.m. in the spectrum of **P1**-alditol are assigned to the anomeric carbon atoms of the 4-linked α-galactopyranosyluronic acid residues and the terminal mannopyranosyl groups, respectively. These assignments were confirmed by a 2D heteronuclear shift correlation experiment on **P1**-alditol. The anomeric configuration of the pen-

TABLE III

^{13}C-N.M.R. DATA FOR **P1**, **P1**-ALDITOL, AND K36 POLYSACCHARIDE

Compound	*Chemical shift*[a] *(p.p.m.)*	$^{1}J_{C\text{-}1,H\text{-}1}$ *(Hz)*	*Assignment*[b]
P1	102.5		→4)-α-Gal*p*A
	101.8		α-Man*p*
	97.1		→3)-β-Gal
	95.4		→2)-α-Man*p*-(1→3)-β-Gal
	95.1		→2)-α-Man*p*-(1→3)-α-Gal
	93.1		→3)-α-Gal
P1-alditol	102.5	169	→4)-α-Gal*p*A
	101.6	170	α-Man*p*
	100.3	170	→2)-α-Man*p*
Polysaccharide K36	105.2		→3)-β-Gal*p*
	101.7		→3,4)-α-Gal*p*A
	100.8		α-Man*p*
	95.5		→2)-α-Man*p*

[a]Chemical shift relative to internal acetone at 31.07 p.p.m. [b]As for footnote *b* in Table II.

dant mannosyl group cannot be unambiguously assigned from the ^{13}C and ^{1}H chemical shift data for **P1** and **P1**-alditol. However, measurement of the $^{1}J_{C\text{-}1,H\text{-}1}$ values[7] for **P1**-alditol (Table III) clearly establishes the α-configuration for the terminal mannosyl groups and confirms the α-configuration for the in-chain mannose and galacturonic acid residues. Thus, the structure of **P1** is α-D-Man*p*-(1→4)-α-D-Gal*p*A-(1→2)-α-D-Man*p*-(1→3)-D-Gal.

N.m.r. data for the K36 polysaccharide. — The ^{1}H-n.m.r. spectrum of a partially autohydrolysed sample of the polysaccharide shows (Table II) five signals in the anomeric region, one of which integrated for two protons. The assignment of the ^{1}H resonances in the anomeric region of the spectrum follows from the analysis of the spectra of **P1** and **P1**-alditol. Comparison of the ^{1}H spectra of K36 polysaccharide and **P1** shows that the signal at δ 4.68 ($J_{1,2}$ 7.5 Hz) disappears on formation of the oligosaccharide and is replaced by the fractional signals assigned to the terminal galactose residue in **P1**. These data show that the galactose residue must be β-linked in the polysaccharide. A similar comparison of the ^{13}C spectra of K36 polysaccharide and **P1** (Table III) permits the assignment of the signal at 105.4 p.p.m. to the anomeric carbon atom of the β-galactopyranosyl residue. The assignments of the ^{13}C anomeric resonances for K36 polysaccharide were confirmed by a heteronuclear shift correlation experiment.

Methylation analysis of K36 polysaccharide. — The only outstanding piece of information required to specify fully the structure of K36 polysaccharide is the site of attachment of the β-galactopyranosyl residue in the polysaccharide repeating-unit. Comparison of the results of the methylation analysis (Table I) of **P1**-alditol with those of the polysaccharide before and after reduction of the uronic ester demonstrates that the β-galactopyranosyl residue is linked to O-3 of the α-galactopyranosyluronic acid in the polysaccharide.

Thus, the polysaccharide of *E. coli* serotype K36 has the structure shown in the ABSTRACT and is identical to that reported[8] for *Klebsiella* K57. The structure is also closely related to that[6] of the capsular polysaccharide of *Klebsiella* K68. In this polysaccharide, the β-galactosyl residue is linked to O-2 of the galactosyluronic acid and the pendant mannosyl group carries a 1-carboxyethylidene group. The polysaccharide of *E. coli* K36 is only the second capsular polysaccharide to be reported in the *E. coli* series which has galacturonic acid in the repeating unit.

EXPERIMENTAL

General methods. — Solutions were concentrated under reduced pressure at 40° (bath). Optical rotations were measured at 23 ±2° with a Perkin-Elmer Model 141 polarimeter for aqueous solutions in a 1-cm cell. Descending p.c. was performed on Whatman No. 1 paper with (*1*) 8:2:1 ethyl acetate–pyridine–water, (*2*) 2:1:1 1-butanol–acetic acid–water, (*3*) 5:1:5:3 ethyl acetate–acetic acid–pyridine–water, and (*4*) 40:11:9 1-butanol–ethanol–water, and detection with alkaline silver nitrate[9]. Gel-permeation chromatography was performed on a column of Sepharose 4B CL, cali-

brated with dextrans, and eluted with M NaCl. Fractions were analysed by the phenol–sulphuric acid method[10]. Analytical g.l.c. was performed at 205° and 225°, using a Hewlett–Packard 5890A gas chromatograph fitted with flame-ionisation detectors, a 3392A recording integrator, and a DB-225 bonded-phase fused-silica capillary column (30 m × 0.25 mm) having a film thickness of 0.25 μm, and with helium as carrier gas. G.l.c.-m.s. was conducted with a V.G. Micromass 16F spectrometer with an ionisation energy of 40 eV and an ion-source temperature of 170°. ^{1}H-N.m.r. spectra (internal acetone, δ 2.23) were recorded at 30° and 95° with a Bruker WM 500 Ft spectrometer. Samples in the acid form were deuterium-exchanged by freeze-drying solutions in D_2O (99.8 and 99.96%). ^{13}C-N.m.r. spectra were recorded with the same spectrometer on the same solutions (internal acetone, 31.07 p.p.m.) at 30°. 2D ^{1}H–^{1}H correlation spectroscopy (COSY 45) and 2D ^{1}H–^{13}C correlation spectroscopy were performed using standard Bruker software.

Preparation and properties of E. coli *K36 polysaccharide.* — A culture of *E. coli* O9:H19:K36, obtained from Dr. I. Ørskov (Copenhagen), was propagated on Mueller–Hinton agar. The acidic capsular polysaccharide was isolated and purified by precipitation as the cetyltrimethylammonium salt[2]. The yield of purified polysaccharide was 235 mg per tray (1.5 L of medium with a surface area of 1,650 cm^2). The polysaccharide had $[\alpha]_D$ +63° and on gel-permeation chromatography was shown to be monodisperse ($M_r = 2.7 \times 10^7$).

Bacteriophage-mediated depolymerisation of polysaccharide K36. — Polysaccharide (509 mg) was dissolved in 250 mL of a partially purified lysate (containing 2.8×10^{13} plaque forming units) of K36 bacteriophage which was isolated and propagated by methods described previously[11,12]. The mixture was stirred at 29° with added chloroform to discourage bacterial growth. After 3 days, the mixture was freeze-dried, and a solution of the residue in water (50 mL) was dialysed against distilled water (5 × 50 mL, mol.wt. cut-off 8,000). The combined dialysates were freeze-dried and the residues were subjected to paper chromatography (solvent *3*) to give **P1**, $[\alpha]_D$ +89°.

Analysis of the constituent sugars of the native polysaccharide and **P1**. — Samples of K36 polysaccharide and **P1** (5 mg each) were heated for 16 h (100°) with 2M trifluoroacetic acid, and the acid was then evaporated. The hydrolysis products were converted into the peracetylated aldononitriles[13] and analysed by g.l.c. Further samples each of K36 polysaccharide and **P1** were treated with refluxing methanolic 3% hydrogen chloride for 16 h at 80°, the mixtures were then neutralised, and 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 the peracetylated aldononitriles and examined by g.l.c. Each hydrolysate was examined by p.c. (solvents *1–4*).

P1-*alditol.* — A solution of **P1** (40 mg) in water (2 mL) was reduced with sodium borohydride and, after the usual work-up, **P1**-alditol was obtained, which was examined by ^{1}H- and ^{13}C-n.m.r. spectroscopy.

Methylation analysis of **P1**-*alditol and K36 polysaccharide.* — **P1**-alditol (20

mg) was methylated by the Hakomori procedure[4], the permethylated product was methanolysed, hydrolysed, reduced with sodium borohydride, and acetylated, and the products were examined by g.l.c. and g.l.c.-m.s. (Table I, column I).

Polysaccharide K36 (48 mg) in the acid form was methylated under Hakomori conditions[4] and then by the Kuhn procedure[14]. Successive hydrolysis of the methylated product with 2M trifluoroacetic acid, reduction with sodium borohydride, and acetylation with pyridine and acetic anhydride afforded a mixture of partially methylated alditol acetates which were examined by g.l.c. and g.l.c.-m.s. (Table I, column II).

Methylated polysaccharide (23 mg) was reduced with $LiAlH_4$ in anhydrous oxolane, the product was hydrolysed, reduced with sodium borohydride, and acetylated with pyridine–acetic anhydride, and the mixture was examined by g.l.c. and g.l.c.-m.s. (Table I, column III).

Determination of absolute configuration. — The absolute configurations of the constituent monosaccharides were determined by g.l.c. analysis of the derived (−)-2-octyl glycoside acetates[3].

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