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STRUCTURE OF THE SERINE-CONTAINING CAPSULAR POLYSACCHARIDE K40 ANTIGEN FROM *Escherichia coli* O8:K40:H9*

THOMAS DENGLER, BARBARA JANN, AND KLAUS JANN
Max-Planck-Institut für Immunbiologie, D-7800 Freiburg (F.R.G.)
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ABSTRACT

The structure of the K40 antigenic capsular polysaccharide (K40 antigen) of *E. coli* O8:K40:H9 was elucidated by determination of the composition, ^{1}H- and ^{13}C-n.m.r. spectroscopy, periodate oxidation and Smith degradation, and methylation analysis. The K40 polysaccharide consists of [(*O*-β-D-glucopyranosyluronic acid)-(1→4)-*O*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-(1→6)-*O*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-(1→4)] repeating units. All of the glucuronic acid residues are substituted amidically with L-serine.

INTRODUCTION

The capsular polysaccharides of *E. coli* can be characterised on the basis of physical and chemical properties as well as their genetic and cellular expression[1–3]. Whereas many capsular polysaccharides occur preponderantly together with the O8 or O9 cell-wall antigen (lipopolysaccharide), *i.e.*, preponderantly in O groups 8 or 9, others occur together with any of the many O-antigenic lipopolysaccharides (in many *E. coli* O groups). The latter capsular polysaccharides express their negative charge through *N*-acetylneuraminic acid, 3-deoxy-D-*manno*-2-octulosonic acid (KDO), or phosphate[2–9]. Capsular polysaccharides occurring in O groups 8 or 9 contain hexuronic acids as negatively charged components. This group consists of two types of polysaccharides, namely, those, such as the K27, K30, and K42 polysaccharides, which, like the *Klebsiella* capsular polysaccharides[10], do not contain amino sugars, and those, such as the K85 and K87 polysaccharides[2,3], which contain amino sugars. The structure of the capsular K40 polysaccharide of *E. coli* O8:K40:H9, which belongs to the second type, is now reported.

RESULTS AND DISCUSSION

Isolation and characterisation. — The capsular (K40) polysaccharide was

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isolated from dialysable cultures of *E. coli* 2175 (O8:K40:H9) by a sequence[4,11,12] of precipitation with cetyltrimethylammonium bromide (Cetavlon), extraction with aqueous calcium chloride, precipitation with ethanol, and extraction with cold phenol (pH 6.5). The yield was 100 mg/L of liquid culture. The K40 polysaccharide consisted of glucuronic acid, 2-acetamido-2-deoxyglucose, and serine in the molar ratios 1:2:1. The absolute configuration of serine was determined as L by g.l.c. of its (+)-2-butyl ester.

In immunoelectrophoresis, the precipitin reaction, and passive haemagglutination, the K40 polysaccharide reacted with an anti-K40 serum but not with an anti-O8 serum, indicating serological K40 specificity. The polysaccharide had $[\alpha]_D^{22}$ +103° (*c* 0.7, water) and an average molecular weight of ~300,000 (Yphantis[13]).

Nature of the serine substitution. — Treatment[14] of the K40 polysaccharide with 2M NaOH (4 h, 20°) did not cleave serine from the polymer. More-drastic conditions (2 h, 100° under nitrogen) resulted in partial degradation of the polysaccharide without significant liberation of serine. Likewise, treatment of the polysaccharide with carbodi-imide–sodium borohydride[15] did not reduce the glucuronic acid to glucose. These results indicated an amide linkage between glucuronic acid and serine.

Methylation analysis. — The K40 polysaccharide was methylated (Hakomori[16,17]) and then hydrolysed[18], and the products were converted into alditol acetates. G.l.c. [80 → 260° at 10°/min, on CB SE-54 (25 m × 0.25 mm)] showed four major components, the relative retention times and mass-spectral fragments of which are shown in Table I. The results showed that the K40 polysaccharide contained equimolar amounts of 4- and 6-linked 2-acetamido-2-deoxyglucopyranosyl residues.

Attempts to convert the glucuronic acid residue of the methylated polysaccharide into glucose using lithium aluminium hydride were unsuccessful, further indicating amide substitution of the glucuronic acid by serine.

Periodate oxidation and Smith degradation. — Oxidation of the K40 polysaccharide followed by borohydride reduction resulted in the complete loss of the

TABLE I

METHYLATION ANALYSIS (G.L.C./M.S.) OF THE K40 POLYSACCHARIDE

		R_{Glc}[a]	*Mass-spectral fragments* (m/z)							
			117	*144*	*145*	*158*	*188*	*189*	*202*	*233*
A[b]	4GlcN	6.6	+	+			+			+
B	6GlcN	7.7		+	+		+	+		+
C	4GlcNMe	9.5	+			+		+		+
D	6GlcNMe	10.3			+	+		+	+	+

[a] R_{Glc}, retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol. [b] Methylated *N*-acetyl and *N*-acetyl-*N*-methyl derivatives of 4- and 6-substituted 2-amino-2-deoxyglucose residues.

glucuronic acid and destruction of 50% of the 2-acetamido-2-deoxyglucose, confirming the (1→6) linkage of one of the 2-acetamido-2-deoxyglucosyl residues.

Treatment of the K40 polysaccharide, in sequence, with periodate, borohydride, and mild acid (Smith degradation[19]) gave two main products which were eluted from Biogel P-2 in the di- to tri-saccharide region (Kd 0.44 and 0.40). After methylation, the products were characterised by g.l.c.–m.s. as **1** and **2**. C.i.(ammonia)-m.s. of **1** and **2** gave peaks for $(M + NH_4)^+$ with *m/z* 397 (**1**) and 325 (**2**), indicating molecular weights of 379 and 307, respectively. The fragmentation patterns of **1** and **2** in e.i.-m.s. are shown in Fig. 1. The structure of **1**, which comprises one 2-acetamido-2-deoxyglucosyl residue (GlcNAc) and the C-4,5,6 region of the other residue (GlcNAc'), indicated, together with the results of the methylation analysis, 2-acetamido-2-deoxy-D-glucopyranosyl-(1→6)-2-acetamido-2-deoxy-D-glucopyranose to be a part of the K40 polysaccharide. The structure of **2** confirmed the presence of a 4-linked glucuronic acid residue, substituted amidically with serine.

N.m.r. spectroscopy and the structure of the K40 polysaccharide. — The signals in the ^{13}C-n.m.r. spectrum of the K40 polysaccharide and their assignments are listed in Table II. Only 23 of the 25 signals expected from the composition of the polysaccharide were seen in the spectrum, since the signals from the *N*-acetyl groups of the two 2-acetamido-2-deoxyglucosyl residues coincided. A comparison with signals given by the structurally related K5 polysaccharide of *E. coli*, which has [(*O*-β-D-glucopyranosyluronic acid)-(1→4)-*O*-(2-acetamido-2-deoxy-α-D-glucopyranosyl-(1→4)] repeating units[20], was made. One of the 2-acetamido-2-

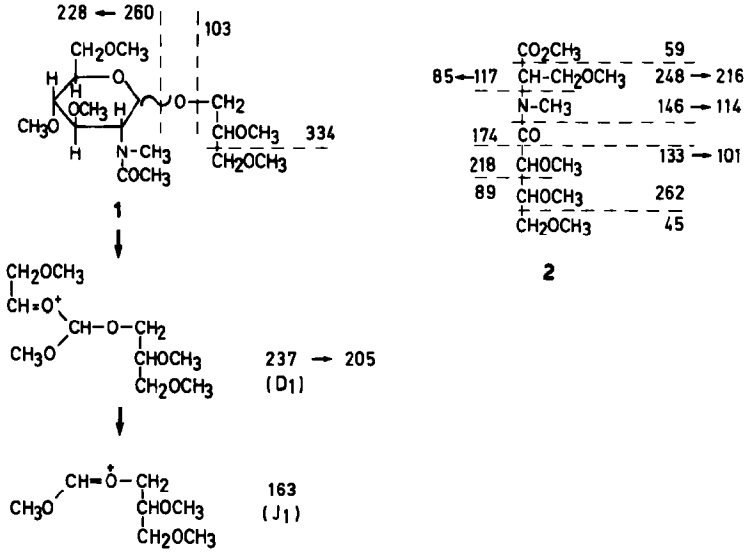


Fig. 1. Fragmentation patterns of the methylated products **1** and **2** from the Smith degradation of the capsular K40 polysaccharide.

deoxyglucosyl residues of the K40 polysaccharide (GlcNAc) gave signals with chemical shifts the same as those of the K5 polysaccharide, and the other residue (GlcNAc') exhibited an α-shift (+5 p.p.m.) at C-6 and a negative shift of −7 p.p.m. at C-4. These values, when compared with those reported[21] for methyl 2-acetamido-2-deoxy-β-D-glucopyranoside (C-4, 70 p.p.m.; C-6, 61.1 p.p.m.), indicated a (1→4) linkage of GlcNAc and a (1→6) linkage of GlcNAc'. The signals due to GlcA and serine were similar to those of methyl β-D-glucopyranosiduronyl-6-(N)-serine[14] except for an α-shift (+7.6 p.p.m.) of the C-4 signal, indicating a (1→4) linkage of the glucuronic acid residue. Substitution of C-6 was evident from an α-shift of −5.5 p.p.m., as compared to unsubstituted (1→4)-linked glucuronic acid of the K5 polysaccharide.

Assignment of signals in the ^{13}C-n.m.r. spectrum of the K40 polysaccharide was facilitated by the use of the attached proton test (APT)[22,23]. In spectra recorded with this spin echo technique, signals due to =CH- and CH_3-groups are positive and those due to ≡C- and -CH_2-groups are negative. As shown in Table II, the

TABLE II

^{13}C-N.M.R. SIGNALS OF THE K40 AND K5 POLYSACCHARIDES OF *E. coli*, METHYL β-D-GLUCOPYRANOSIDURONYL-6-(*N*)-SERINE[25], AND METHYL 2-ACETAMIDO-2-DEOXY-α-D-GLUCOSIDE[35]

		K40 δ	*APT*	*K5* δ	*Me β-GlcA–Se* δ	*α-GlcNAc-OMe* δ
GlcA	C-1	103.2	+	103.6	103.5	
	C-2	74.1	+	74.7	73.2	
	C-3	75.5	+	77.3	75.9	
	C-4	79.9	+	80.1	72.0	
	C-5	76.2	+	77.8	75.3	
	C-6	169.9	−	175.4	170.5	
GlcNAc	C-1	97.7	+	97.9		98.6
	C-2	54.3	+	54.5		54.3
	C-3	70.4	+	70.6		71.9
	C-4	77.0	+	78.0		70.4
	C-5	71.7	+	72.0		72.2
	C-6	60.8	−	61.1		61.4
GlcNAc'	C-1	98.0	+			98.6
	C-2	54.6	+			54.3
	C-3	72.1	+			71.9
	C-4	70.7	+			70.4
	C-5	71.5	+			72.2
	C-6	65.9	−			61.4
Serine	C-1	175.2	−		176.0	
	C-2	56.7	+		62.5	
	C-3	62.7	−		57.2	
NHAc	Me	22.9	+	23.0		23.1
	CO	175.2	−	175.3		175.2

signals due to all C-6 atoms and to C-1 and C-3 of serine are negative.

The chemical shifts of the C-1 signals of all sugar constituents and of C-2 signals of GlcNAc and GlcNAc′ indicated the glucuronic acid to be β and both 2-acetamido-2-deoxy-D-glucopyranosyl residues to be α. This was verified by a gated decoupling experiment[23–25] in which glucuronic acid showed a $J_{C\text{-}1,H\text{-}1}$ value of 165 Hz and each 2-acetamido-2-deoxyglucosyl residue showed a $J_{C\text{-}1,H\text{-}1}$ value of 175 Hz.

The ^{1}H-n.m.r. spectrum (Fig. 2) of the K40 polysaccharide contained signals at δ 5.35 (J 3.3 Hz), 4.84 (J 2.4 Hz), and 4.55 (J 7.8 Hz) which, together with their chemical shifts, indicated two 2-acetamido-2-deoxyglucosyl residues and one β-D-glucosyluronic acid residue. As with the ^{13}C-n.m.r. spectrum, the ^{1}H-n.m.r. spectrum was similar to that of the K5 polysaccharide[20]. The latter contained signals at δ 5.36 (J 3.3 Hz) and 4.50 (J 8.5 Hz) (not shown) and, since only one 2-acetamido-2-deoxyglucosyl residue was present in the K5 polysaccharide, lacked the signal at δ 4.84. The spectrum of the K40 polysaccharide contained a singlet at δ 1.9 due to the NAc group.

The foregoing results indicate that the K40 polysaccharide can be formulated as **3**. The K5 polysaccharide has a very similar structure, but lacks the serine substituent and has a β-GlcA-(1→4)-α-GlcNAc repeating-unit which is also part of the K40 polysaccharide chain. The K5 polysaccharide is not immunogenic which was explained[20] by the structural identity with the first polymeric precursor in

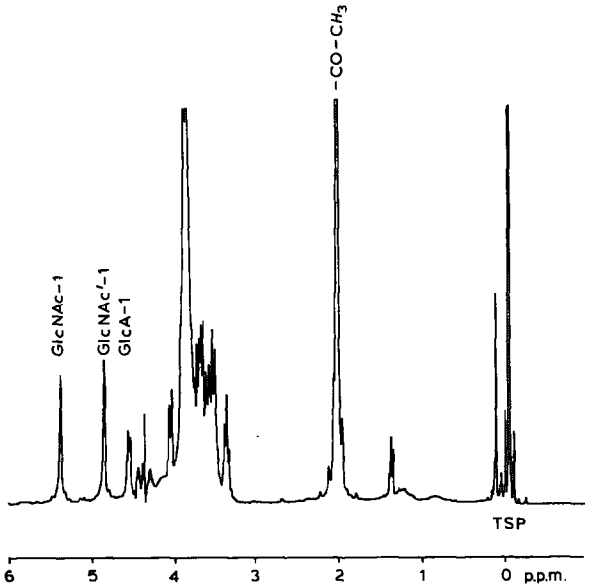
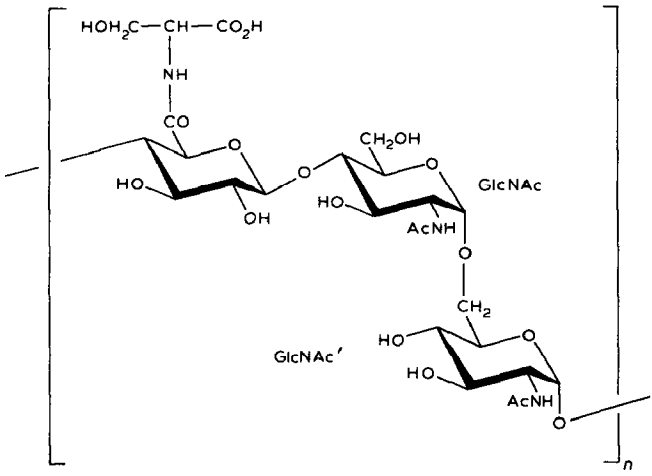


Fig. 2. ^{1}H-N.m.r. spectrum (300 MHz) of the K40 polysaccharide [D_2O, 70°, external sodium 4,4-dimethyl-4-sila-(2,2,3,3-$^2H_4$)pentanoate, Me_4Si], run with solvent suppression by gated decoupling.



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heparin biosynthesis. The immunogenicity of the K40 polysaccharide, in which the glucuronic acid is amidically linked to serine, is in keeping with this interpretation. An immunodominance of the serine substituent is indicated by the fact that the K5 polysaccharide did not react with an anti-K40 antiserum.

Whereas *O*-acetylation is common with microbial polysaccharides, substitution with amino acids is less frequently encountered. Amino acids involved in ester linkages are found in teichoic acids[26]. Amide linkages in which the carboxyl group of *N*-acetylalanine is involved are found in the cell-wall lipopolysaccharide of *E. coli* O114[27] and in the cell-wall polysaccharide of *Staphylococcus aureus*[28]. Amide linkages involving the amino group of the amino acid are encountered with serine in the cell-wall lipopolysaccharide of *Shigella boydii* type 8[29], with lysine in the lipopolysaccharide of *Proteus mirabilis*[30], with threonine in the lipopolysaccharide of *Rhodopseudomonas sphaeroides* ATCC 17023[31], with threonine and serine in the capsular K54 polysaccharide of *E. coli*[32], and with alanine, serine, and threonine in the capsular polysaccharide of *Haemophilus influenzae* type d. The capsular K40 polysaccharide of *E. coli* O8:K40:H9 also has this type of substitution.

EXPERIMENTAL

Bacteria and cultivation. — *E. coli* A51d (O8:K40:H9) was obtained from Drs. I. and F. Ørskov (Copenhagen) and grown to the late log phase (5–7 h) in a fermentor at 37° in 10-L batches, which contained per L: $K_2HPO_4 \cdot 3\ H_2O$ (9.7 g), KH_2PO_4 (2 g), sodium citrate $\cdot 5\ H_2O$ (0.5 g), $MgCl_2 \cdot 7\ H_2O$ (0.1 g), casamino acids (20 g), and the dialysable part of yeast (100 mL from 500 g in 5 L of deionised water).

Isolation and purification of the K40 polysaccharide. — The acidic capsular

polysaccharide and the bacterial cells were precipitated from the liquid culture by addition of 1 vol. of aqueous 2% cetyltrimethylammonium bromide (Cetavlon). All following operations were performed at 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 extractions with cold phenol (80%, buffered to pH 6.5 with sodium acetate)[4,11]. The combined, final aqueous phases were centrifuged for 4 h at 100,000*g* and the supernatant solution was lyophilised. The residue was further purified by chromatography on Sephadex G-50.

For isolation of the polysaccharide from agar-grown bacteria[12,33], the bacteria were suspended in 0.25M NaCl (120 mL per 100 14-cm plates). After heating the suspension (60°, 20 min), the bacteria were removed by centrifugation and Cetavlon was added to 1%. The precipitate was removed by centrifugation and the supernatant solution was diluted with 4 vol. of water. The precipitated Cetavlon salt was collected by centrifugation, dissolved in the minimum amount of M sodium chloride, precipitated with 4 vol. of ethanol, dialysed, ultracentrifuged, and lyophilised.

Analytical methods. — Glucuronic acid was determined with the carbazole reagent, and 2-acetamido-2-deoxyglucose and serine were determined (after hydrolysis with 4M HCl, 100°, 18 h) with a Durrum D-500 amino acid analyser. 2-Acetamido-2-deoxyglucose was also determined with the Morgan–Elson reagent[34].

To determine the absolute configuration of serine, 20 mg of K40 polysaccharide were hydrolysed (4M HCl, 16 h, 100°), and the hydrolysate was neutralised with Amberlite IRA-410 (Na^+) resin and lyophilised. The residue was dissolved in 1 mL of (−)-2-butanol (1 mL), trifluoroacetic acid (20 μL) was added, and the mixture was kept for 15 h at 100°. Excess of reagent was removed *in vacuo* and the residue was acetylated. The product was analysed by g.l.c. (ECNSS-M, 140°) in comparison with the products obtained from D- and L-serine.

Optical rotations were measured with a Perkin–Elmer 141 polarimeter, and g.l.c. was performed with a Varian Aerograph Series 1400 instrument equipped with an autolinear temperature programmer and a Hewlett–Packard 3380 integrator. N.m.r. spectra were recorded with a Bruker WM 300 spectrometer in the F.t. mode at 70° [external sodium 4,4-dimethyl-4-sila(2,2,3,3-$^2H_4$)pentanoate]. The ^{13}C values were corrected (−1.31 p.p.m.) using 1,4-dioxane (δ 67.4 based on Me_4Si), and the ^1H values by −007 p.p.m. (1,4-dioxane signal at δ 3.7). Mass spectra were obtained with a Finnigan 1020 mass spectrometer.

Periodate oxidation and methylation. — The procedures have been described in detail[28].

Smith degradation. — The K40 polysaccharide (50 mg) was oxidised in 0.4 M $NaIO_4$ (1 mL; 90 h, room temperature) and the product was reduced with $NaBH_4$ (100 mg). Excess reagents were removed with methanol–acetic acid *in vacuo* and by dialysis. The product was isolated by lyophilisation and treated with aqueous 80%

acetic acid (30 mg in 2 mL; 100°, 1.5 h), and an equal volume of water was then added. Removal of the acetic acid *in vacuo* was followed by chromatography of the resulting aqueous solution (~1 mL) on a column (1.5 × 90 cm) of Biogel P-2. The effluent was monitored by the absorbance at 206 nm, and the materials from the peaks with Kd 0.44 (**1**) and 0.40 (**2**) were isolated by lyophilisation.

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