Structural determination of the capsular antigen of *Escherichia coli* O8:K25:H9

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Abstract

The primary structure of the acidic capsular antigen of *Escherichia coli* O8:K25:H9 was shown by monosaccharide analysis, methylation analysis, and by 1D and 2D ^{1}H and ^{13}C NMR spectroscopy to be composed of linear tetrasaccharide repeating units having the structure:

$\rightarrow$2)-β-D-Glc$p$A-(1$\rightarrow$3)-α-D-Gal$p$NAc-(1$\rightarrow$3)-α-D-Gal$p$NAc-(1$\rightarrow$2)-α-D-Fuc$p$-(1$\rightarrow$
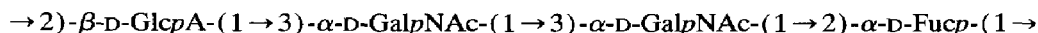
Keywords: Escherichia coli; Capsular polysaccharide; K25 antigen; NMR spectroscopy

1. Introduction

Fuc has been reported as a component of the repeating units of only four of the more than seventy known capsular antigens of *Escherichia coli* [1]. The structures of three of these have been reported viz. those of serotypes K27 [2], K28 [3], and K42 [4]. The capsular antigen of *E. coli* K25, the structure of which we now report, is the only one of the four where Fuc is found in association with aminodeoxy sugars.

2. Results and discussion

Isolation, composition, and methylation analysis.—E. coli O8:K25:H9 bacteria were grown on Mueller–Hinton agar (see Experimental section), the capsular polysaccharide was extracted from the cells with aqueous 1% phenol, and was purified by precipitation

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Abbreviations: COSY, ^{1}H–^{1}H correlation spectroscopy; HETCOR, ^{1}H–^{13}C correlation spectroscopy; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; HMBC, ^{1}H–^{13}C heteronuclear multiple bond correlation
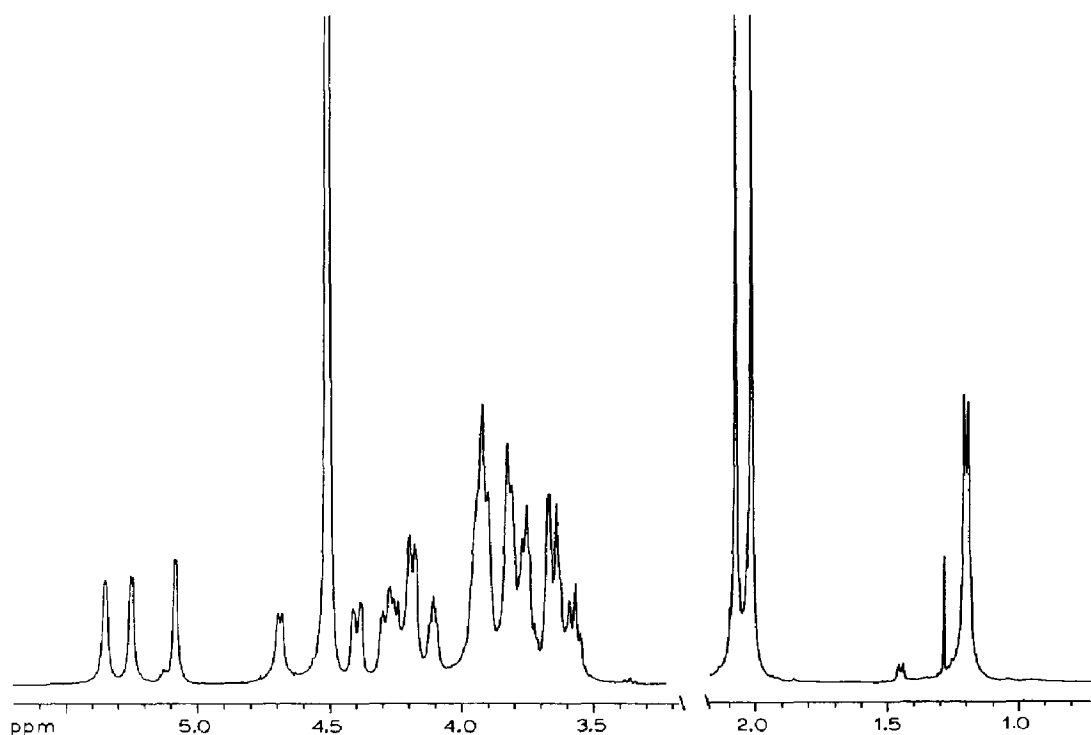 * Corresponding author.

Fig. 1. ^{1}H NMR spectrum of *E. coli* K25 capsular polysaccharide.

with cetyltrimethylammonium bromide. GLC examination of the alditol acetates derived from an acid hydrolysate of the capsular polysaccharide (**PS**) showed the presence of Fuc and GalN. Prior methanolysis of the **PS** followed by reduction of the methoxycarbonyl groups and GLC examination of the derived alditol acetates showed the presence of Glc in addition to Fuc and GalN thereby establishing the presence of GlcA in the **PS**. Fuc was shown to have the L configuration and the GlcA and GalN the D configuration by GLC examination of the derived acetylated (−)-2-octyl glycosides [5]. The ^{1}H NMR spectrum of the polysaccharide (Fig. 1) contained H-1 signals at δ 5.348 ($^3J_{H,H}$ 3.7 Hz), 5.252 ($^3J_{H,H}$ 4.0 Hz), 5.083 ($^3J_{H,H}$ 3.5 Hz), and 4.690 ($^3J_{H,H}$ 6.8 Hz), signals for the methyl protons of two NAc groups at δ 2.015 and 2.076, and a signal for the methyl protons of a deoxy sugar at δ 1.201. The ^{13}C NMR data (Fig. 2) complemented the ^{1}H NMR results with signals for C-1 at 102.60, 99.49, 99.30, and 93.38 ppm, signals at 22.97 and 23.02 ppm for the methyl carbons of the NAc groups, and a signal at 16.03 ppm for the methyl carbon of the deoxy sugar. In addition, signals for carbonyl carbons occurred at 174.81, 174.58, and 174.00 ppm. These results indicated a tetrasaccharide repeating unit for the **PS** consisting of one residue each of Fuc and GlcA and two residues of GalNAc.

Methylation analysis of the **PS** gave 3,4-di-*O*-methylfucose, 3,4-di-*O*-methylglucose (after carboxyl reduction) and 2-deoxy-4,6-di-*O*-methyl-2-methylacetamidogalactose in the molar ratios 0.9:1.0:1.6. These results indicate that the tetrasaccharide repeating unit in the **PS** is linear.

2D NMR studies of the **PS**.—The sequence of the residues in the repeating unit was established by 2D NMR experiments, which also confirmed the glycosylation sites in the **PS**. The ^{13}C and ^{1}H resonances of the residues are presented in Table 1. The residues were
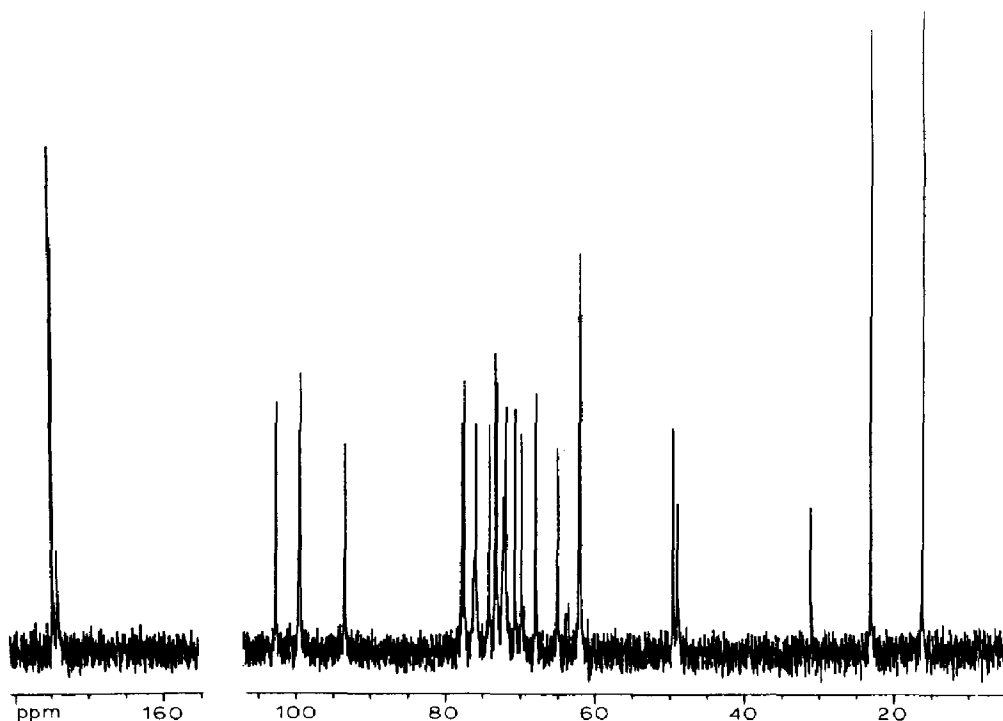
Fig. 2. ^{13}C NMR spectrum of *E. coli* K25 capsular polysaccharide.

labelled a–d in order of decreasing chemical shift of their H-1 protons. The chemical shifts of all the ^{1}H resonances of residue a and d together with H-1 to H-4 of residues b and c were easily assigned from a COSY [6] contour map of the PS. The chemical shift for H-5 of residue b was established from both the H-3/H-5 and H-4/H-5 cross-peak in a NOESY experiment (Fig. 3) [7]. H-6a and H-6b for this residue could then be assigned from the H-5/H-6a,H-6b cross-peak in the COSY spectrum. It was not possible to assign the chemical shift for H-5 of residue c in the same way because the resonances for H-3 and H-5 are very close making it impossible to verify the presence of a H-4/H-5 cross-peak. However, it was possible to assign the chemical shift for the H-6a,H-6b resonance of residue c from the H-4/H-6 NOE (Fig. 3) which was shown by all three residues with the *galacto* configuration. Returning to the COSY spectrum the chemical shift for H-5 of c could now be assigned from the H-6a,6b/H-5 cross peak.

The ^{1}H chemical shifts for each residue in the polysaccharide were then compared with data obtained from an HETCOR [8] experiment. In this way the ^{13}C resonances for C-1 to C-6 of residues a–c and C-1 to C-5 of d could be assigned. The chemical shift for the carboxyl carbon of residue d was assigned from the two (H-5/C-6) and three bond correlations (H-4/C-6) present in an HMBC experiment (Table 2) [9]. The ^{13}C and ^{1}H resonances for the NAc groups present in residues b and c were assigned from the H-2/C=O and H(CH$_3$)/C=O correlations in the HMBC experiment (Table 2).

Comparison of the ^{1}H and ^{13}C NMR data for residues a–d with literature values [10–12] for methyl glycosides permitted the residues in the repeating unit to be identified, as indicated in Table 1. The occurrence of C-1 of the GalNAc c at 93.39 ppm and of C-4 of the GalNAc b at 64.94 ppm, both unusual chemical shifts for these carbons, can be attributed

Table 1
¹H and ¹³C NMR data [a] for *E. coli* K25 polysaccharide at 50°C

Atom	Residue			
	a →2)-α-Fucp	**b** →3)-α-GalpNAc	**c** →3)-α-GalpNAc	**d** →2)-β-GlcpA
H-1	5.348	5.252	5.083	4.690
C-1	99.30	99.49	93.38	102.60
H-2	3.950	4.398	4.292	3.623
C-2	74.06	48.83	49.41	77.66
H-3	3.821	3.939	3.911	3.648
C-3	70.57	72.22	75.76	77.36
H-4	3.673	4.179	4.201	3.569
C-4	73.19	64.94	69.70	72.90
H-5	4.252	4.106	3.913	3.913
C-5	67.76	71.93	71.74	75.76
H-6a	1.201	3.756	3.813	
H-6b		3.756	3.813	
C-6	16.03	62.03	61.74	174.00
C=O		174.81	174.58	
CH₃ (NAc)		2.015	2.076	
CH₃ (NAc)		22.97	23.02	

[a] Chemical shifts in ppm with acetone as internal reference, δ 2.23 and 31.07 ppm for ¹H and ¹³C respectively.

to the ''γ-gauche effect'' [13]. This describes the interaction between H-1 of **c** and H-4 of **b** and establishes that **c** is α-linked, that **b** is substituted at O-3 and that -α-D-GalpNAc-(1→3)-D-GalpNAc is present in the repeating unit of the **PS**. The linkage positions of **a**, **c**, and **d** were established from the significant deshielding observed for the linkage carbon atoms. Thus O-2 of **a** and **d** and O-3 of **b** and **c** were identified as the linkage positions of the residues in the repeating unit. These findings accord with the results of the methylation analysis.

The sequence of the residues in the repeating unit was established by NOESY and HMBC experiments. The intraresidue NOEs and pertinent interresidue NOEs are shown in the NOESY contour plot (Fig. 3). The α-linked pyranoside residues **a**–**c** showed characteristic intramolecular NOEs between H-1 and H-2, while the β-linked residue **d** showed an NOE to H-3 and possibly an NOE to H-5. Intense interresidue NOEs between the anomeric protons and the protons across the glycosidic linkages were observed for residues **a** and **b**. Although an intense NOE was observed between H-1 of **d** and a proton at where H-3 of **c** resonates, this is also the position of an expected intraresidue NOE between H-1 and H-5 of residue **d**. It was thus necessary to unambiguosly establish this linkage from the HMBC experiment. H-1 of residue **c** showed an intense NOE to H-4 of **b** and a less intense NOE to H-3 of the same residue. In this case the linkage position was clearly identified as O-3 both from the β-effect experienced by C-3 of residue **c** and the results of the methylation analysis of the polysaccharide. It should be noted, however, that whereas a stronger NOE
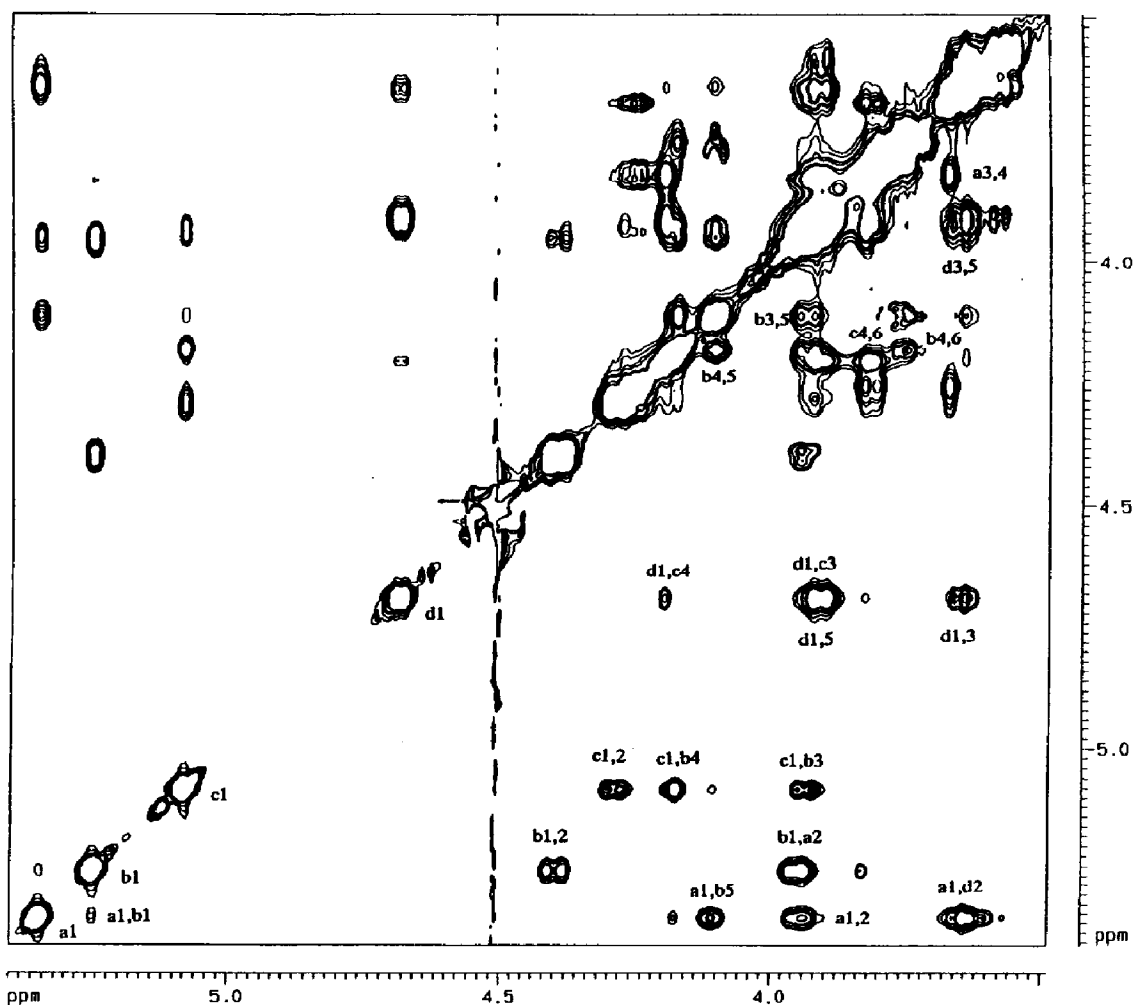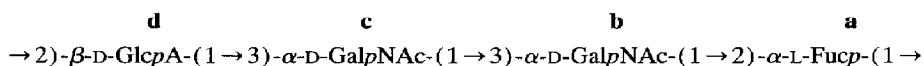
Fig. 3. NOESY contour plot of the region δ 5.40–3.49: a1 connotes H-1, a1,2 connotes the cross-peak between H-1 and H-2 of residue **a**, and a1,b5 connotes the cross-peak between H-1 of residue **a** and H-5 of residue **b**, etc.

to H-4 of a 3-linked Gal has been frequently observed [14–16], a 4-linked Gal will only show the expected NOE to H-1 of the glycosylating sugar. H-1 of residue **a**, in addition to the intraresidue NOE to its H-2 and the interglycosidic NOE to H-2 of **d**, showed an interresidue NOE to H-5 of **b**. This latter NOE not only indicates the proximity between H-1 of **a** and H-5 of **b** but also establishes the relationship for the partial structure **b-a** as D-L or L-D.

In order to unambiguously establish the linkage between **d** and **c** and to confirm the sequence of the residues in the repeating unit an HMBC experiment [9] was performed. The inter- and intra-residue correlations observed are listed in Table 2. Strong interresidue correlations were observed between H-1 of **a** and C-2 of **d**, H-1 of **b** and C-2 of **a**, H-1 of **c** and C-3 of **b**, and H-1 of **d** and C-3 of **c**. These data establish the structure of the repeating unit of the capsular polysaccharide of *E. coli* K25 as:

Table 2
HMBC data for *E. coli* K25 polysaccharide

Residue	Proton	Correlation to
a		
→ 2)-α-Fuc*p*	H-1	70.57 (**a**, C-3), 67.76 (**a**, C-5), 77.66 (**d**, C-2)
	H-4	16.03 (**a**, C-6)
b		
→ 3)-α-Gal*p*NAc	H-1	72.22 (**b**, C-3), 74.06 (**a**, C-2)
	H-4	62.03 (**b**, C-6)
	H-2	174.81 (C=O of NAc)
	CH$_3$ (δ 2.015)	174.81 (C=O of NAc)
c		
→ 3)-α-Gal*p*NAc	H-1	75.76 (**c**, C-3), 72.22 (**b**, C-3)
	H-2	174.56 (C=O of NAc)
	CH$_3$ (δ 2.076)	174.56 (C=O of NAc)
d		
→ 2)-β-Glc*p*A	H-1	75.76 (**c**, C-3)
	H-4, H-5	174.00 (**d**, C-6)

 d   **c**   **b**   **a**

→ 2)-β-D-Glc*p*A-(1 → 3)-α-D-Gal*p*NAc-(1 → 3)-α-D-Gal*p*NAc-(1 → 2)-α-L-Fuc*p*-(1 →

The above structure closely resembles the repeating unit of the *O*-deacetylated O-antigen polysaccharide of *E. coli* O127 [17]. In the latter polysaccharide a 2-linked Gal replaces the 2-linked GlcA in the repeating unit of the **PS**.

3. Experimental

General methods.—Analytical GLC was performed with a Hewlett–Packard 5890A gas chromatograph, fitted with flame-ionisation detectors and a 3392A recording integrator, with He as the carrier gas. Either a J&W Scientific fused-silica DB-225 or DB-17 bonded-phase capillary column (30m × 0.25 mm, 0.25 μm film) was used for separating alditol acetates, partially methylated alditol acetates, and acetylated (−)-2-octyl glycosides. The operating conditions have been previously described [18,19]. Hydrolysis of samples with CF$_3$CO$_2$H, carboxyl reduction of methyl esters resulting from methanolyses, determination of absolute configuration of the sugars, preparation of alditol acetates and partially methylated alditol acetates, and methylation analysis of the polysaccharide were carried out as previously described [18].

Preparation of polysaccharide.—An authentic culture of *E. coli* O8:K25:H9 bacteria (Culture no. Bi 7575-41) was grown on Mueller–Hinton agar (meat infusion 5 g, casein hydrolysate 17.5 g, soluble starch 1.5 g, agar 14 g, distilled water to 1 L) at 37°C and the capsular polysaccharide was isolated and purified as previously described [20].

NMR Spectroscopy.—Samples were deuterium-exchanged by freeze-drying several times from D$_2$O and were then dissolved in 99.99% D$_2$O (0.5 mL) containing a trace of acetone as internal reference (δ 2.23 for ^{1}H and 31.07 ppm for ^{13}C). Spectra were recorded on a

Bruker AMX-400 NMR spectrometer equipped with an X32 computer using UXNMR software. Experiments on **PS** were recorded at 323 K and the parameters used for the 2D experiments were as follows: COSY and NOESY: 256×2048 data matrix, zero filled to 1024 data points in t_1; 64 scans per t_1 value; spectral width 1960 Hz; recycle delay, 1.0 s; unshifted sine-bell window function in t_1 and t_2 (COSY) and shifted sine-squared window function in t_1 and t_2 (NOESY). The mixing delay in the NOESY was 0.2 s. HETCOR: 256×2048 data matrix, zero filled to 512 in t_1; spectral width, 10 000 Hz in t_2 and 1960 Hz in t_1; 1600 scans per t_1 value; recycle delay, 1.2 s; Gaussian window functions. HMBC: 256×4096 data matrix, zero filled to 1024 in t_1; spectral width, 20826 Hz in t_1 and 1960 Hz in t_2; 80 scans per t_1 value; recycle delay, 1.0 s; delay for evolution of long range coupling, 60 ms; shifted sine-squared window function.

Acknowledgements

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