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Escherichia coli serotype K45 capsular antigen: a glycan containing 3-acetamido-3,6-dideoxygalactopyranose

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ABSTRACT

The primary structure of the acidic capsular antigen of *Escherichia coli* O8:K45:H9 was shown by glycose analysis, methylation analysis, and one- and two-dimensional ^{1}H and ^{13}C NMR spectroscopy to be composed of repeating branched tetrasaccharide units having the structure:

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→ 6)-α-D-GlcpNAc-(1 → 4)-β-D-GalpA-(1 → 3)-β-D-GlcpNAc-(1 →
               3
               ↑
               1
     β-D-Fucp3NAc
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The polysaccharide contains the uncommon amino sugar 3-acetamido-3,6-dideoxy-D-galactopyranose, which has not been previously identified in a capsular antigen.

INTRODUCTION

The capsular (K) antigen of *Escherichia coli* O8 : K45 : H9 belongs to the group I polysaccharides of this genus[1] and as such is a high molecular weight, acidic polysaccharide which contains amino sugars. The polysaccharide is unusual in that it contains the amino sugar 3-acetamido-3,6-dideoxy-D-galactopyranose (D-Fuc3NAc) which has previously been shown to be present in lipopolysaccharides and cell-wall antigens of *E. coli*[2], *Pseudomonas maltophilla*[3], *Eubacterium saburreum*[4], *Proteus penneri*[5], and *Xanthomonas campestris*[6]. This is the first reported occurrence of this amino sugar in a capsular polysaccharide.

RESULTS AND DISCUSSION

Isolation, composition, and linkage analysis of the capsular antigen.—*E. coli* K45 bacteria were grown on Mueller–Hinton agar, and the acidic capsular polysaccha-

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ride (**PS**) was isolated and purified by precipitation with cetyltrimethylammonium bromide, followed by delipidation with aqueous 1% acetic acid at 100°C for 1 h. The average molecular weight of **PS** was found to be M_r 1.5×10^6 on a dextran-calibrated column of Sephacryl S500. Hydrolysis of **PS** followed by GLC–MS examination of the derived alditol acetates showed the presence of GlcN and a 3-amino-3,6-dideoxyhexose in the ratio 2:1. Methanolysis of **PS** followed by reduction of the methoxycarbonyl groups, hydrolysis, and GLC analysis of the derived alditol acetates revealed the presence of Gal. These results indicate that **PS** comprises a tetrasaccharide repeating unit consisting of GlcNAc, GalA, and 3-amino-3,6-dideoxyhexose in the approximate molar ratios 2:1:1.

GlcNAc was shown to have the D configuration by GLC analysis of the derived acetylated (−)-2-octyl glycoside[7]. A sample of **PS** was partially hydrolysed with 0.5 M trifluoroacetic acid and, after *N*-reacetylation of the sample, both GalA and the 3-acetamido-3,6-dideoxyhexose were isolated from the hydrolysate by paper chromatography. The 3-acetamido-3,6-dideoxyhexose was further purified by semi-preparative HPLC and identified as 3-acetamido-3,6-dideoxygalactose on the basis of the ^{1}H NMR spectrum. The $[\alpha]_D$ was determined to be +89° (H_2O), indicating that the sugar has the D configuration[8]. The separated GalA fraction was also shown to have the D configuration by GLC analysis of the acetylated (−)-2-octyl glycoside[7] of the derived Gal.

The ^{1}H NMR spectrum of **PS** in D_2O (Table I) contained signals for H-1 of an α-linked sugar at δ 4.94 ($J_{1,2}$ 3.6 Hz) and for H-1 of β-linked sugars at 4.62 ($J_{1,2}$ 7.4 Hz), 4.55 ($J_{1,2}$ 7.9 Hz), and 4.48 ($J_{1,2}$ 7.8 Hz), and two signals for the methyl protons of NAc groups at δ 2.02 (3 H) and 2.06 (6 H), respectively. A doublet centred at δ 1.26 ($J_{5,6}$ 6.3 Hz; 3 H) indicated the presence of a 6-deoxy sugar. The ^{1}H-decoupled ^{13}C NMR spectrum confirmed a tetrasaccharide repeating unit with signals for C-1 at 104.55, 104.34, 102.28, and 99.40 ppm, and signals for carbonyl carbons at 175.15, 175.06, and 171.23 ppm. Three signals, at 53.07, 55.43, and 55.63 ppm, indicated the presence of three C–N bonds, and confirmed the presence of

TABLE I

NMR data [a] for *E. coli* K45 polysaccharide

Residue		Proton or carbon						
		1	2	3	4	5	6	6′
→ 3,6)-α-D-GlcNAc (**a**)	H	4.94	4.02	3.85	3.57	4.32	3.82	4.12
	C	99.40	53.07	81.32	69.14	71.43	69.23	
→ 3)-β-D-GlcNAc (**b**)	H	4.62	3.88	3.85	3.62	3.52	3.80	3.96
	C	102.28	55.43	84.68	69.88	76.32	61.77	
→ 4)-β-D-GalA (**c**)	H	4.55	3.62	3.83	4.38	4.44		
	C	104.34	70.87	72.39	78.07	73.69	171.23	
β-D-Fuc3NAc (**d**)	H	4.48	3.52	3.93	3.74	3.91	1.25	
	C	104.55	69.06	55.63	70.63	72.85	16.22	

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

three NAc groups. A signal at 16.22 ppm confirmed the presence of a 6-deoxy sugar.

Methylation of the polysaccharide followed by GLC and GLC–MS analysis of the permethylated alditol acetates derived from the products of an acid hydrolysate gave 3,6-dideoxy-2,4-di-*O*-methyl-3-methylacetamidogalactose, 2-deoxy-4-*O*-methyl-2-methylacetamidoglucose, 2-deoxy-4,6-di-*O*-methyl-2-methylacetamidoglucose, and 2,3-di-*O*-methylgalactose (after carboxyl reduction). These results indicate the presence of 3,6-linked GlcNAc, 4-linked GalA, 3-linked GlcNAc, and terminal Fuc3NAc in a branched tetrasaccharide repeating unit with GlcNAc as the branch point.

2D NMR studies of the E. coli *K45 capsular polysaccharide.*—The identities and sequence of the residues in the repeating unit were established by 2D NMR experiments, which also confirmed the glycosylation sites in the polysaccharide. The residues in the repeating unit were labelled **a**–**d** in order of decreasing chemical shift of their anomeric protons (Fig. 1). The chemical shifts of the proton resonances of residue **a**–**d** were established from COSY[9] and 2D homonuclear Hartmann–Hahn (HOHAHA)[10] (Fig. 1) experiments.

Residue **a** *[→ 3,6)-α-*D*-GlcNAc].*—The ^{1}H resonances for residue **a** were readily traced via their cross-peaks in the COSY spectrum as far as H-3. The H-3/4 cross-peak overlapped with several other signals, but its position could easily be identified with the help of the HOHAHA spectrum. The chemical shifts of the remaining ^{1}H resonances were then assigned using both the HOHAHA and COSY spectra. As expected for a *gluco*-type residue, magnetism relayed well through the spin system. The ^{13}C resonances for residue **a** were assigned by comparing the ^{1}H assignments with the ^{1}H–^{13}C correlation data from an HMQC[11] experiment.

Residue **b** *[→ 3)-β-*D*-GlcNAc].*—The ^{1}H resonances for residue **b** were traced as far as H-3 via the cross-peaks in the COSY spectrum. The H-3/4 cross-peak overlapped with that for residue **a** and the H-4 resonance had, therefore, to be assigned from the HOHAHA spectrum. Magnetism also relayed well through this spin system, and the remaining ^{1}H resonances for this residue were readily assigned from the HOHAHA spectrum and confirmed from the COSY spectrum. The ^{13}C resonances were then assigned from the HMQC spectrum as before.

Residue **c** *[→ 4)-β-*D*-GalA].*—In the COSY spectrum, the H-2/3 cross-peak for residue **c** overlapped with the H-3/4 cross-peaks for residues **a** and **b**; however, the H-3 resonance could be assigned from the HOHAHA spectrum. The magnetism in this spin system did not relay beyond H-4 and the chemical shift of the H-5 resonance was assigned from the small H-4/5 cross-peak in the COSY spectrum.

Residue **d** *[β-*D*-Fuc3NAc].*—Beginning with the H-1/2 cross-peak in the COSY spectrum, the ^{1}H resonances for residue **d** were traced as far as H-4. The magnetism in this spin system did not appear to relay beyond H-4 so that no further information could be obtained from the HOHAHA spectrum. The remaining ^{1}H resonances were assigned by working back from the H-6 signal at δ 1.26 in

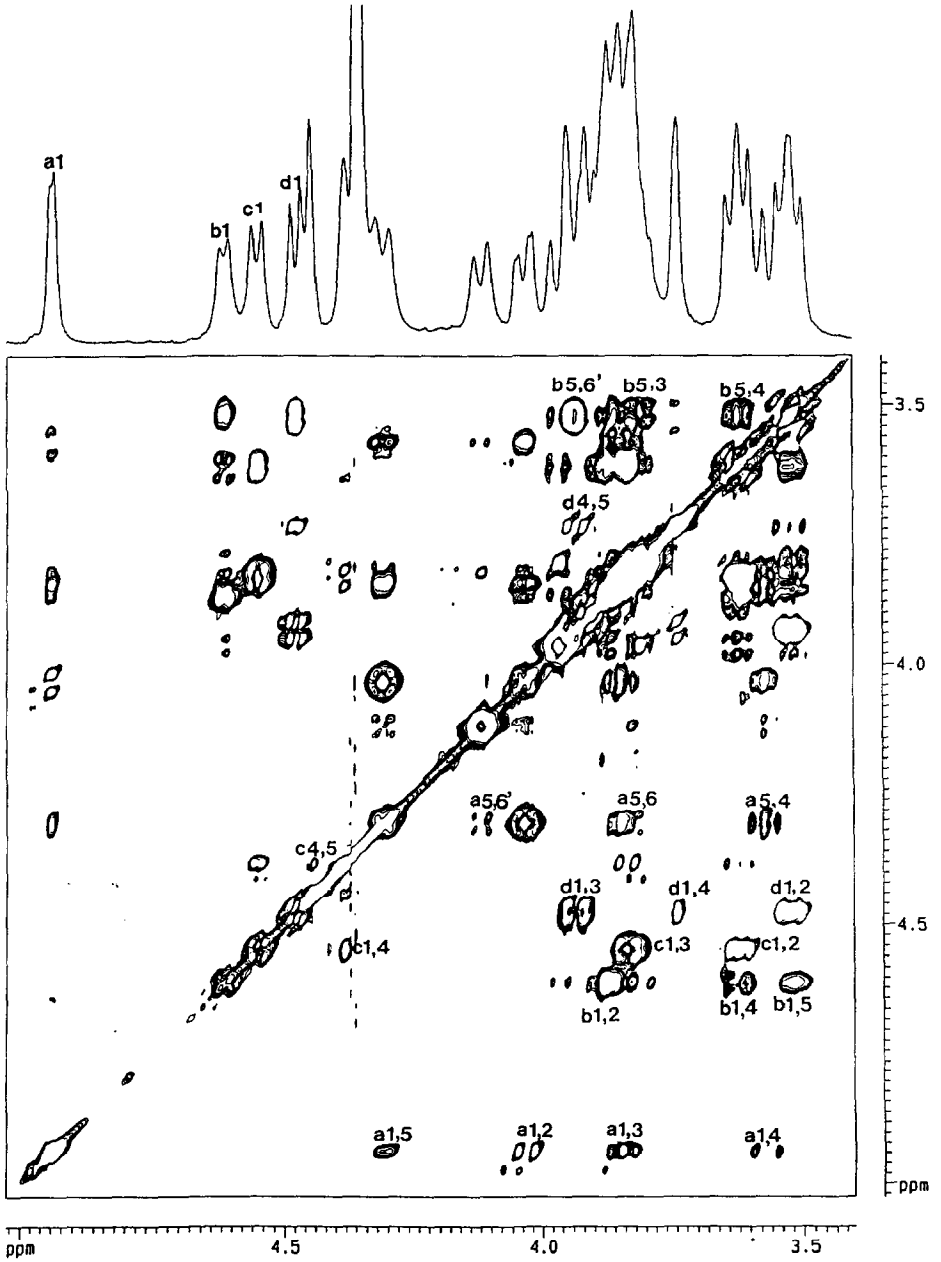


Fig. 1. HOHAHA contour plot of **PS**: **a1** connotes H-1 of residue **a**, **a1,2** connotes the cross-peak between H-1 and H-2 of residue **a**, etc. (See Table I for identification of **a–d**).

the COSY spectrum, which allowed the resonance for H-5 to be assigned and showed that the H-3 and H-5 resonances in fact overlap, resulting in an overlap of the H-3/4 and H-4/5 cross-peaks. The ^{13}C resonances were assigned by compar-

TABLE II

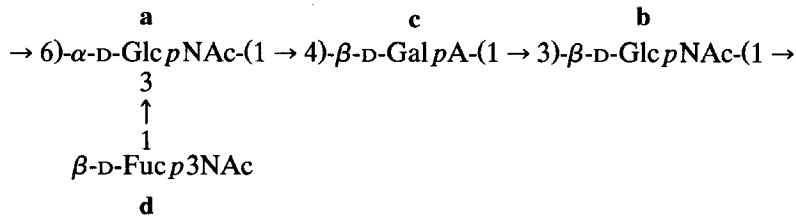
Two- and three-bond $^{1}H-^{13}C$ correlations for **PS**

Residue	Proton	Correlation to
a	H-1	81.32(**a**,C-3), 71.43(**a**,C-5), 78.07(**c**,C-4)
	H-3	104.55(**d**,C-1)
	H-6	102.28(**b**,C-1),
b	H-1	69.23(**a**,C-6)
	H-2	102.28(**b**,C-1), 55.43(**b**,C-2)
	H-3	55.43(**b**,C-2), 104.34(**c**,C-1)
	H-4	84.68(**b**,C-3), 61.77(**b**,C-6)
c	H-1	84.68(**b**,C-3)
	H-2	104.34(**c**,C-1)
	H-3	104.34(**c**,C-1), 78.07(**c**,C-4)
	H-4	70.87(**c**,C-2), 73.69(**c**,C-5), 99.40(**a**,C-1)
d	H-1	81.32(**a**,C-3)
	H-2	104.55(**d**,C-1)
	H-3	69.06(**d**,C-2)
	H-5	55.63(**d**,C-3), 70.63(**d**,C-4)

ing the ^{1}H assignments with the $^{1}H-^{13}C$ correlation data. The carbon resonance at 55.63 ppm could have been assigned to C-3 or C-5, due to the overlap of these two proton signals. However, GLC–MS analysis of the hydrolysed polysaccharide and identification of this sugar component, as described earlier, pinpointed the acetamido function at C-3, allowing chemical shifts to be assigned for C-3 and C-5, respectively.

Comparison of the chemical shift data for residues **a–d** with those reported for methyl glycosides[12–14] permitted identification of residue **a** as 3,6-linked α-GlcNAc, residue **b** as 3-linked β-GlcNAc, residue **c** as 4-linked β-GalA, and residue **d** as terminal β-Fuc3NAc.

The sequence of the residues **a–d** in the repeating unit was established by a heteronuclear multiple bond correlation (HMBC) experiment[16], which measures through-bond connectivity between C and H atoms two and three bonds distant. Correlations between H-1 of α-GlcNAc and H-4 of β-GalA, between H-1 of β-GalA and H-3 of β-GlcNAc, between H-1 of β-GlcNAc and H-6 of α-GlcNAc, and between H-1 of β-Fuc3NAc and H-3 of α-GlcNAc were clearly observed (Table II). The combined chemical and NMR data permit the structure of the tetrasaccharide repeating unit of the E. coli K45 capsular polysaccharide to be written as:



CONCLUSION

Although Fuc3NAc has been isolated from lipopolysaccharides and cell-wall antigens of *E. coli*[2] and is therefore not new to the genus, this is the first occurrence of this uncommon sugar in a capsular antigen. It is also worth noting that, in previously reported occurrences of this sugar, it has been α-linked in all cases but one[1–6], and that the β-linked Fuc3N reported in *Proteus penneri* contained a butyramido group rather than an NAc group[5]. This is therefore, to the best of our knowledge, the first β-linked Fuc3NAc to be reported.

EXPERIMENTAL

General methods.—Analytical GLC was performed on a Hewlett–Packard 5890A gas chromatograph, fitted with flame-ionization detectors and a 3392A recording integrator, with He as carrier gas. A J&W Scientific fused-silica DB-17 bonded-phase capillary column (30 m × 0.25 mm; film thickness, 0.25 μm) was used for separating partially methylated alditol acetates (programme I), alditol acetates (programme II), and acetylated octyl glycosides (240°C isothermal, pressure 140 kPa). The temperature programmes used were: I, 180°C for 2 min, then 3°C min^{-1} to 240°C, 100 kPa; II, 180°C for 2 min, then 2°C min^{-1} to 240°C, 100 kPa. The identities of all derivatives except Fuc3NAc were determined by comparison with authentic standards and confirmed by GLC–MS on a Hewlett–Packard 5988A instrument. Spectra were recorded at 70 eV and an ion-source temperature of 200°C, using the appropriate column. GPC of the K45 polysaccharide was performed on a dextran-calibrated column (1.6 × 65 cm) of Sephacryl S500, using 0.1 M sodium acetate buffer (pH 5.00) as eluent. HPLC was carried out on a Supelcogel C-611 column (30 cm × 7.8 mm), using 10^{-4} M NaOH as eluent.

Polysaccharide samples were hydrolysed with anhyd liquid HF for 3 h at room temperature. Alditol acetates were prepared by reduction of the products in aqueous solutions of hydrolysates with $NaBH_4$ for 1 h followed by acetylation with 2:1 Ac_2O–pyridine for 1 h at 100°C. Samples were methanolysed by refluxing with methanolic 3% HCl for 16 h. Native and methylated polysaccharides were carboxyl-reduced with $NaBH_4$ in dry MeOH after methanolysis. Methylations were carried out on the acid form of the polysaccharide, using tetramethylurea[16], potassium dimsyl[17], and MeI in Me_2SO.

Preparation of the K45 polysaccharide.—An authentic culture of *E. coli* O8:K45:H9 was obtained from Dr. I. Ørskov (Copenhagen) and propagated on Mueller–Hinton agar. The capsular polysaccharide was extracted with aq 1% phenol, separated from the cells by ultracentrifugation, and purified by precipitation with cetyltrimethylammonium bromide. This was followed by delipidation of **PS** with 1% acetic acid at 100°C for 1 h, followed by centrifugation, dialysis, and freeze-drying.

Partial hydrolysis.—K45 polysaccharide (200 mg) was hydrolysed with 0.5 M CF_3CO_2H at 100°C for 1 h and the hydrolysate in water (5 mL) containing MeOH (0.5 mL) was *N*-reacetylated by treatment with Ac_2O (2 mL) for 2 h at room temperature[18]. After concentration of the solution, the residue in water (2 mL) was treated with NH_3 (0.5 mL) before being subjected to paper chromatography on Whatman No. 1 paper, using EtOAc–AcOH–pyridine–H_2O (5:1:5:3) as eluent. The components were visualised with basic silver nitrate[19] and ninhydrin[20], and extracted from the paper with distilled water. The fraction containing Fuc3NAc was further purified by semi-preparative HPLC. The identity of the compound was confirmed by NMR data and had $[\alpha]_D$ +89° (*c* 3.20, H_2O).

NMR spectroscopy.—Samples were deuterium-exchanged by freeze-drying several times from D_2O and then examined as solutions in 99.99% D_2O containing a trace of acetone as internal standard (δ 2.23 for ^1H and 31.07 for ^{13}C). Spectra were recorded at 65°C on a Bruker AMX-400 spectrometer equipped with an X32 computer. The parameters used for 2D experiments were as follows: COSY, HOHAHA [512 × 2048 data matrix, zero-filled to 1024 data points in t_1; 64 or 72 scans per t_1 value; spectral width, 1805 Hz; recycle delay, 1.0 s; unshifted sine-bell filtering in t_1 and t_2 (COSY) and shifted sine-squared filtering in t_1 and t_2 (HOHAHA)]. HMQC [512 × 4096 data matrix, zero-filled to 1024 data points in t_1; spectral width, 11 067 Hz in t_1 and 1805 Hz in t_2; 64 scans per t_1 value; recycle delay, 1.0 s; fixed delay, 3.46 ms; shifted sine-squared filter). HMBC [256 × 4096 data matrix, zero-filled to 1024 in t_1; spectral width, 20 826 Hz in t_1 and 1805 Hz in t_2; 72 scans per t_1 value; recycle delay, 1.0 s; fixed delay, 3.46 ms; shifted sine-squared filter].

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REFERENCES

1 K. Jann and B. Jann, *Rev. Infect. Dis.*, 9 (1987) s517–s526.
2 P-E. Jansson, H. Lenholm, B. Lindberg, U. Lindquist, and S.B. Svenson, *Carbohydr. Res.*, 161 (1987) 273–279.
3 J.L. Di Fabio, M.B. Perry, and D.R. Bundle, *Biochem. Cell. Biol.*, 65 (1987) 968–977.
4 P-E. Jansson, B. Lindberg, M. Spellman, T. Hofstad, and N. Skaug, *Carbohydr. Res.*, 137 (1985) 197–203.
5 E.V. Vinogradov, Z. Sidorczyk, A. Swierzko, A. Rożalski, E.D. Daeva, A.S. Shashkov, Y.A. Knirel, and N.K. Kochetkov, *Eur. J. Biochem.*, 197 (1991) 93–103.
6 J. Hickman and G. Ashwell, *J. Biol. Chem.*, 241 (1966) 1424–1428.
7 K. Leontein, B. Lindberg, and J. Lönngren, *Carbohydr. Res.*, 62 (1978) 359–362.
8 A.C. Richardson, *Carbohydr. Res.*, 4 (1967) 415–421.
9 A. Bax and R. Freeman, *J. Magn. Reson.*, 44 (1981) 542–561.
10 A. Bax and D.G. Davis, *J. Magn. Reson.*, 65 (1985) 355–360.

11 A. Bax and S. Subramanian, *J. Magn. Reson.*, 67 (1986) 565–569.
12 K. Bock and H. Thörgersen, *Annu. Rep. NMR Spectrosc.*, 13 (1982) 1–57.
13 K. Izumi, *Carbohydr. Res.*, 170 (1987) 19–25.
14 J.H. Bradbury and G.A. Jenkins, *Carbohydr. Res.*, 126 (1984) 125–156.
15 A. Bax and M.F. Summers, *J. Am. Chem. Soc.*, 108 (1986) 2093–2094.
16 T. Narui, K. Takahashi, M. Kobayashi, and S. Shibata, *Carbohydr. Res.*, 103 (1982) 293–295.
17 L.R. Phillips and B.A. Fraser, *Carbohydr. Res.*, 90 (1981) 149–152.
18 E. Altman, M.B. Perry, and J.-R. Brisson *Carbohydr. Res.*, 191 (1989) 295–303.
19 W.E. Trevelyan, D.P. Procter, and J.S. Harrison, *Nature (London)*, 166 (1950) 444.
20 S.C. Churms, in G. Zweig and J. Sherma (Eds.), *Handbook of Chromatography*, Vol. 1, CRC Press, Boca Raton, FL, 1982, p. 193.