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Structure of the amino acid-containing capsular polysaccharide from *Escherichia coli* O8:K49:H21*

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ABSTRACT

The structure of the capsular antigen of *E. coli* K49 and the oligosaccharides derived from it by partial acid hydrolysis were studied by 1D- and 2D-n.m.r. spectroscopy, g.l.c.–c.i.-mass spectrometry, and methylation analysis. The K49 polysaccharide consists of the repeating unit →4)-β-D-Glc*p*A-(1→6)-β-D-Gal*p*-(1→6)-β-D-Glc*p*-(1→3)-β-D-Gal*p*NAc-(1→. The glucuronic acid residues are substituted, in the apparent molar ratio of 4:1, with L-threonine and L-serine linked amidically to the carboxyl group.

INTRODUCTION

The acidic capsular polysaccharides of *E. coli* have been sub-divided[1] into two groups, namely, those with high molecular weight and low charge density (Group I) and those with low molecular weight and high charge density (Group II). The negative charge in Group II polysaccharides may result from the presence of phosphate, *N*-acetylneuraminic acid, or 3-deoxy-D-*manno*-2-octulosonic acid (Kdo), whereas Group I polysaccharides contain hexuronic acids and sometimes a pyruvic acid substituent. In a few microbial polysaccharides, hexuronamides that involve amino acids comprise the negatively charged components[2–7]. Only two *E. coli* capsular polysaccharides have been shown to contain such a substituent, namely, the K54 polysaccharide that contains L-threonine and L-serine[8], and the K40 polysaccharide that contains L-serine[9]. The K40 and K54 polysaccharides belong to Groups I and II, respectively.

The structure of the capsular K49 polysaccharide of *E. coli* O8:K49:H21, which belongs to Group I, is now reported

* Dedicated to Professor Leslie Hough in the year of his 65th birthday.
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RESULTS AND DISCUSSION

Isolation and characterization. — *E. coli* K49 bacteria were grown on solid medium at room temperature. The acidic polysaccharide was isolated by a sequence of precipitation with cetyltrimethylammonium bromide[10], extraction with aqueous sodium chloride, and precipitation with ethanol. The product was monodisperse by gel-permeation chromatography (M_w 420 000) and had $[\alpha]_D$ −14° (*c* 1.2, water).

Hydrolysis of the polysaccharide, using anhydrous hydrogen fluoride, and g.l.c. (program *A*) of the resulting derived alditol acetates revealed D-glucose, D-galactose, and 2-amino-2-deoxy-D-galactose in the molar ratios 1.0:1.0:0.9. Amino acid analysis revealed serine and L-threonine in the molar proportions 1:4, and the presence of a uronic acid was determined using the method of Blumenkrantz and Asboe-Hansen[11]. The absolute configurations of D-glucose and D-galactose were determined[12] by g.l.c. of their acetylated (−)-2-octyl glycosides, and those of 2-amino-2-deoxy-D-galactose and D-glucuronic acid by g.l.c. of the acetylated (+)-2-butyl glycosides[13]. The absolute configuration of L-threonine was determined by g.l.c. of its *N*-acetylated (+)-2-butyl ester[14].

Identity of the amino acids. — The amino acids could not be removed from the polysaccharide by treatment with 0.1 M NaOH (2 h, 20°), and more drastic conditions (M NaOH, 6 h, 50°, under nitrogen) caused degradation. Treatment of the polysaccharide with carbodi-imide–sodium borohydride[15] did not increase the relative amount of glucose on hydrolysis of the reduced polysaccharide, although methanolysis and reduction of the polymer did show a small but significant increase[16]. These results indicate an amide linkage between a glucuronic acid residue and the amino acids.

Methylation analysis. — The K49 polysaccharide was methylated according to the Hakomori method[17], then hydrolysed. The partially methylated sugars were converted into their alditol acetates and analysed by g.l.c.–m.s. (program *B*). The results, shown in Table I, indicated the polysaccharide to contain 6-linked glucopyranosyl, 6-linked galactopyranosyl, and 3-linked 2-acetamido-2-deoxygalactopyranosyl resi-

TABLE I

Methylation analysis data for the K49 polysaccharide and derived products

Methylated sugar[a] *(as alditol acetate)*	*Molar ratios*[b]			
	I	*II*	*III*	*IV*
1,2,3,4,5-Gal				0.2
2,3,4,6-Glc			1.0	1.0
2,3,4-Glc	1.3	1.2		
2,3,4-Gal	1.0	1.0		
2,4,6-GalNAc	0.5			0.6

[a] 2,3,4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, *etc.* [b] I, native polysaccharide; II, uronic ester-reduced aldobiouronic acid **1**; III, disaccharide **2**; IV, reduced tetrasaccharide **3**.

dues. Attempted reduction, with lithium aluminium hydride, of the ester functions in the methylated polysaccharide gave a negative result that was a further indication of an amide linkage between glucuronic acid and the amino acids.

Methanolysis. — The presence of a glucuronic acid residue was confirmed, and the position of its linkage was determined[18], by e.i.-mass spectrometry of the partially methylated methyl glycosides obtained after methanolysis. The methylated K49 polysaccharide was methanolysed (methanolic 3% HCl, 24 h, 65°), and the products were acetylated and analysed by e.i.- and c.i.-g.l.c.–m.s. (program *B*) (Table II). The characterisation of methyl (methyl 4-*O*-acetyl-2,3-di-*O*-methylglucopyranosid)uronate provided evidence for the presence of 4-linked glucuronic acid in the K49 capsular polysaccharide.

TABLE II

M.s. data of methyl glycosides obtained from the K49 polysaccharide

Partially methylated methyl glycoside[a]	*Fragment ions leading to characterisation* (m/z)	
	c.i.	*e.i.*
2,3,4-Glc	296	A_1 247
	$(M + NH_4)^+$	J_1 75 > K_2 71
2,3,4-Gal		B_1 176
		D_1 177
2,3-GlcA	310	J_1 75 > K_2 71
	$(M + NH_4)^+$	B_1 204
		K_2 71 < 10
2,4,6-GalNAc	(320)	F_1 142
	$(M + H)^+$	H_1 157 → 115
		H_1 116 (absent)

[a] 2,3,4-Glc = methyl 6-*O*-acetyl-2,3,4-tri-*O*-methylglucoside, *etc.*

The combined analytical results indicated a linear tetrasaccharide repeating-unit for the K49 polysaccharide, the sugar sequence of which was determined as follows.

Partial acid hydrolysis. — Treatment of the K49 polysaccharide with M HCl (1 h, 95°) and preparative p.c. of the products gave an acidic disaccharide **1** (R_{Gal} 0.30) as the major product. G.l.c.–c.i.-m.s. of methylated **1** gave prominent peaks at *m/z* 233, 219, and 201 (Fig. 1, Table III). After carboxyl-reduction of methylated **1**, the fragment ion at *m/z* 233 disappeared and a strong fragment ion appeared at *m/z* 205. An $(M + NH_4)^+$

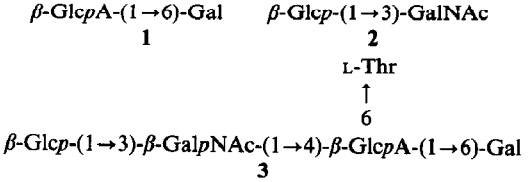

TABLE III

G.l.c.–c.i.-m.s. data from methylated disaccharides obtained from *E. coli* K49 polysaccharide

Disaccharide	*Retention time*[a]	$(M + NH_4)^+$	*Fragment ions* (m/z) *(Relative abundance)*						
			$(M+H)^+$	$(M+H-MeOH)^+$	$R^bOH_2^+$	G^{+b}	R^+	$(G-MeOH)^+$	$(R-MeOH)^+$
β-D-Glc*p*A-(1→6)-D-Gal (**1**)	1.76					233	219	201	187
	1.90					(11)	(32)	(50)	(10)
	1.98								
	2.07								
β-D-Glc*p*-(1→6)-D-Gal[c]		458			237	205	219	173	187
		(7)			(15)	(56)	(19)	(15)	(5)
β-D-Glc*p*-(1→3)-D-GalNAc (**2**)	2.57								
	2.65		496	464	278	219	260	187	228
	3.03		(6)	(20)	(10)	(3)	(15)	(27)	(90)

[a] Retention time relative to that of methylated sucrose (5.98 min), using DB17 (program *B*). [b] G and R represent the non-reducing and reducing sugars, respectively.
[c] Carboxyl-reduced methylated **1**.

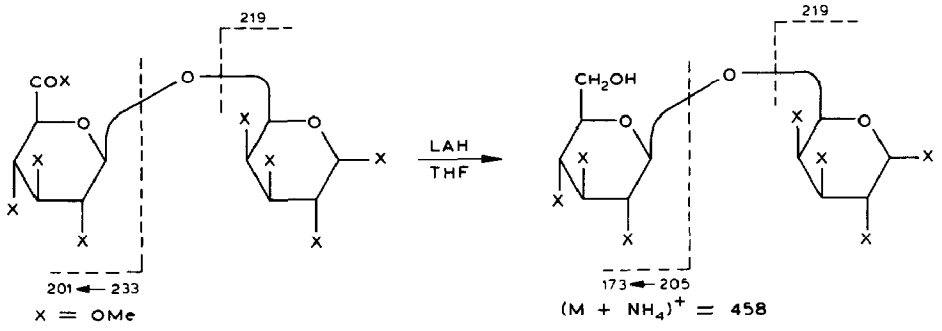

Fig. 1. Fragment ions of methylated **1** and methylated, reduced **1**.

ion at *m/z* 458 was also observed (Fig. 1, Table III). These data, together with a comparison of the relative retention times of methylated **1** with those of a standard, indicated **1** to be 6-*O*-(*β*-D-glucopyranosyluronic acid)-D-galactose. The results of methylation analysis (Table I) were also in agreement with the structure proposed for **1**.

Solvolysis with hydrogen fluoride. — Treatment of the K49 polysaccharide with anhydrous hydrogen fluoride (1 h, 0°) gave one main product (**2**) that was isolated using Bio-Gel P-2. P.c. (solvent *B*) showed **2** to be a neutral oligosaccharide with R_{Gal} 0.39. G.l.c.–c.i.-m.s. of methylated **2** gave an $(M + H)^+$ ion at *m/z* 496, along with fragment ions at *m/z* 260 and 219 (Fig. 2, Table III). Although methylation analysis of **2** gave only 2,3,4,6-tetra-*O*-methylglucose (Table I), this result, together with the g.l.c.–c.i.-m.s. data for methylated **2**, indicated D-glucopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactose to be a part of the structure of K49 polysaccharide.

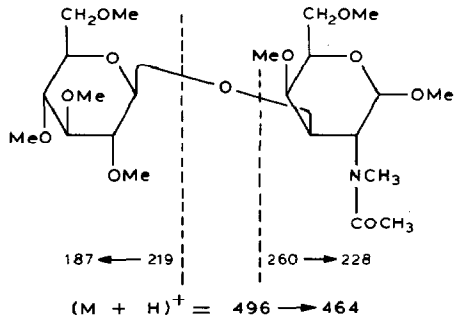

Fig. 2. Fragment ions of methylated **2**.

A milder hydrolysis of the K49 polysaccharide with hydrogen fluoride (15 min, 0°) gave a single product **3** (12%), isolated by fractionation on Bio-Gel P-2, which was shown by n.m.r. analysis to be a tetrasaccharide (see below). Methylation analysis of reduced **3** gave 1,2,3,4,5-penta-*O*-methylgalactose, 2,3,4,6-tetra-*O*-methylglucose, and 2-deoxy-4,6-di-*O*-methyl-2-methylaminogalactose (Table I) from which it can be inferred that D-galactopyranosyl-(1→6)-D-glucose is a part of the K49 polysaccharide.

Evidence for the position of substitution and type of linkage of the amino acids came from g.l.c.–m.s. of a methylated methyl glycoside of **3**, obtained by hydrolysis (15 min, 0°) of K49 polysaccharide with hydrogen fluoride in the presence of methanol and fractionation of the products on Bio-Gel P-2. Although the methylated tetrasaccharide could not be observed directly using either a DB-1 or DB-5 capillary column, fortuitously some aldobiouronic acid, minor amounts of which had co-eluted with the tetrasaccharide, was present in the sample. The aldobiouronic acid gave three components on g.l.c.–c.i.-m.s. with weak $(M + H)^+$ ions at *m/z* 598 (*A*), 584 (*B*), and 566 (*C*). Component *A* gave a strong fragment ion at *m/z* 362, showing that threonine was linked amidically to the glucuronic acid residue (Fig. 3). Component *B* was the serine analogue of *A*, and *C* was an artifact, derived from *A* by the loss of methanol (Fig. 3). A similar artifact was observed[8] during analysis of methylated 3-*O*-β-D-glucopyranosyluronic acid)-L-rhamnose, which retained its amidically-linked serine and threonine substituents.

N.m.r. spectroscopy. — The ^1H- and ^{13}C-n.m.r. data for the K49 polysaccharide (pH 4.5) are listed in Tables IV and V, respectively. The high-field region of the ^1H-n.m.r. spectrum contained signals for the methyl protons of both threonine (1.23

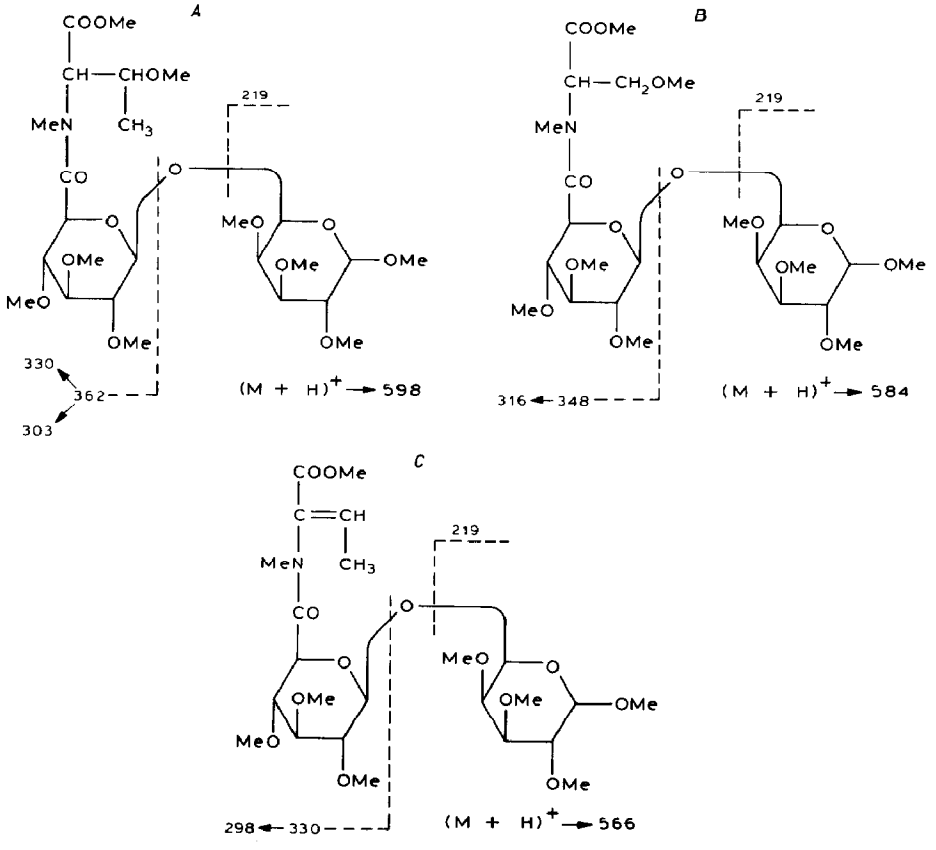

Fig. 3. Fragmentation patterns of **3***A*, **3***B*, and **3***C*.

p.p.m., d, 3 H, $J_{3,4}$ 7.6 Hz) and the *N*-acetyl group of 2-acetamido-2-deoxygalactose (2.02 p.p.m., s, 3 H). This result is consistent with the occurrence of threonine as the major substituent and suggests that the substitution is stoichiometric even though amino acid analysis showed that serine and threonine were present in the molar ratios 0.1:0.4, relative to 2-amino-2-deoxygalactose. This inconsistency could be attributed to differential degradation of the amino acids during the hydrolysis prior to amino acid analysis[19]. The region for anomeric protons contained signals at 4.59 (d, 1 H, $J_{1,2}$ 8.0 Hz), 4.55 (d, 1 H, $J_{1,2}$ 8.0 Hz), 4.46 (d, 1 H, $J_{1,2}$ 8.3 Hz), and 4.39 p.p.m. (d, 1 H, $J_{1,2}$ 7.4 Hz). The chemical shifts and coupling constants are consistent with all β residues. The corresponding ^{13}C resonances were at 103.9, 105.1, 100.2, and 104.5 p.p.m., and the $^{1}J_{C,H}$

TABLE IV

Proton chemical shifts[a] and coupling constants (Hz)[b] for the *E. coli* K49 capsular polysaccharide

Residue	*H-1* ($J_{1,2}$)	*H-2* ($J_{2,3}$)	*H-3* ($J_{3,4}$)	*H-4* ($J_{4,5}$)	*H-5*	*H-6* ($J_{5,6}$)	*H-6'* ($J_{5,6'}$, $J_{6,6'}$)
→4)-β-D-Glc*p*A-(1→	4.59[a]	3.40	3.65	3.98	4.10		
(A)	(8.0)[b]	(9.6)	(9.6)	(9.6)			
→6)-β-D-Glc*p*-(1→	4.55	3.26	3.48	3.46	3.62	4.21	3.79
(B)	(8.0)	(9.0)	(8.0)	(8.0)		(~1.0)	(ur, −11.2)
→3)-β-D-Gal*p*NAc-(1→	4.46	3.99	3.84	4.18	3.67	3.82	3.75
(C)	(8.3)	(9.6)		(~1.0)		(3.2)	(4.8, ur)
→6)-β-D-Gal*p*-(1→	4.39	3.53	3.62	3.93	3.86	4.03	3.91
(D)	(7.4)	(9.3)	(3.2)	(≤1)		(ur)	(3.2, ur)
L-Thr		4.26	4.33	1.23			
		(3.8)	(7.6)				

[a] Measured at 27° (internal acetone at 2.23 p.p.m.). [b] Observed first-order coupling constants.

TABLE V

^{13}C-N.m.r. chemical shifts for the capsular polysaccharide from *E. coli* K49

Carbon atom	*Chemical shift*[a]				
	Unit A →4)-β-D-Glc*p*A-(1→	*Unit B* →6)-β-D-Glc*p*-(1→	*Unit C* →3)-β-D-Gal*p*-NAc-(1→	*Unit D* →6)-β-D-Gal*p*-(1→	L-*Thr*
C-1	103.9	105.1	100.2	104.5	176.7
C-2	73.2	73.7	52.0	71.5	61.7
C-3	74.6	76.4	81.3	75.4	68.5
C-4	77.3	70.2	68.7	69.4	20.2
C-5	74.9	73.4	75.8	74.6	
C-6	170.2	69.6	62.0	70.5	
CH_3 } *N*-acetyl			23.3		
C=O } *N*-acetyl			175.9		

[a] In p.p.m. from internal acetone (31.07 p.p.m.); measured at pH 4.5.

values were in the range 161–164 Hz, thus confirming the presence of four β residues. Characteristic signals at 52.0, 23.3, and 175.9 p.p.m. confirmed the presence of a 2-acetamido-2-deoxyglycose residue. With reference to data on model compounds[20], the signals at 176.7 and 170.2 p.p.m. were assigned to the carboxyl groups of L-threonine and D-glucuronic acid, respectively. The resonance assigned to threonine moved from 174.7 to 177.0 p.p.m., on adjusting the pH of the solution of the polysaccharide from 2 to 9, which is in agreement with an amide linkage between the glucuronic acid and threonine.

The ^{1}H- and ^{13}C-n.m.r. data for the regions for H-1 and C-1 signals for the oligosaccharides isolated from the K49 polysaccharide are listed in Table VI. The H-1 resonances of **2** at 5.26 ($J_{1,2}$ 1–2 Hz) and 4.75 p.p.m. ($J_{1,2}$ 8.0), with integrated intensities in the ratio 0.6:0.2, can be attributed to the α and β forms of the terminal, reducing glycopyranose residue. In addition, the ^{13}C-n.m.r. spectrum of **2** contained twinned signals at 53.8/49.8 and 23.11/22.88 p.p.m. thereby confirming the presence of a terminal reducing 2-acetamido-2-deoxyglycose residue. The presence of ^{1}H and ^{13}C signals at 1.23 and 20.1 p.p.m., respectively, indicated that **3** had retained its threonine substituent.

TABLE VI

N.m.r. data for the anomeric region of oligosaccharides derived from *E. coli* K49 polysaccharide

Compound	*^{1}H.N.m.r. data*				*^{13}C-N.m.r. data*	
	δ[a] (p.p.m.)	*$J_{1,2}$ (Hz)*	*Integral (no. of H)*	*Assignment[b]*	*δ[c] (p.p.m.)*	*Assignment[d]*
β-Glc*p*A-(1→6)-Gal (**1**)	5.28	s	0.3	6-α-Gal		
	4.59	7–8	1.0	β-GlcA		
	4.50	7–8	0.7	6-β-Gal		
β-Glc-(1→3)-GalNAc (**2**)	5.26	1–2	0.6	3-α-GalNAc	175.6	C=O (*N*-acetamido)
	4.75	8	0.2	3-β-GalNAc	175.3	
	4.61	8	1.0	β-Glc	105.1	β-Glc
	4.57				104.9	
					95.9	3-β-GalNAc
					92.0	3-α-GalNAc
β-Glc*p*-(1→3)-β-Gal*p*NAc-(1→4)-β-Glc*p*A-(1→6)-β-Gal (**3**) 6 ↑ L-Thr	5.27	s	0.3	6-α-Gal	175.6	C=O (*N*-acetamido)
	4.59			4-β-GlcA	170.1	C=O (GlcA)
	4.58	7.8	3.8	β-Glc	105.0	β-Glc
	4.56			6-β-Gal	103.5	4-β-GlcA
	4.54			3-β-GalNAc	100.1	3-β-GalNAc
					97.1	6-α-Gal
					93.1	6-β-Gal

[a] Measured with reference to internal acetone, δ 2.23. [b] 4-β-GlcA refers to H-1 of a 4-linked β-glucuronosyl residue. [c] As in *a* with acetone, 31.07 p.p.m. [d] As for *b*, but for ^{13}C nuclei.

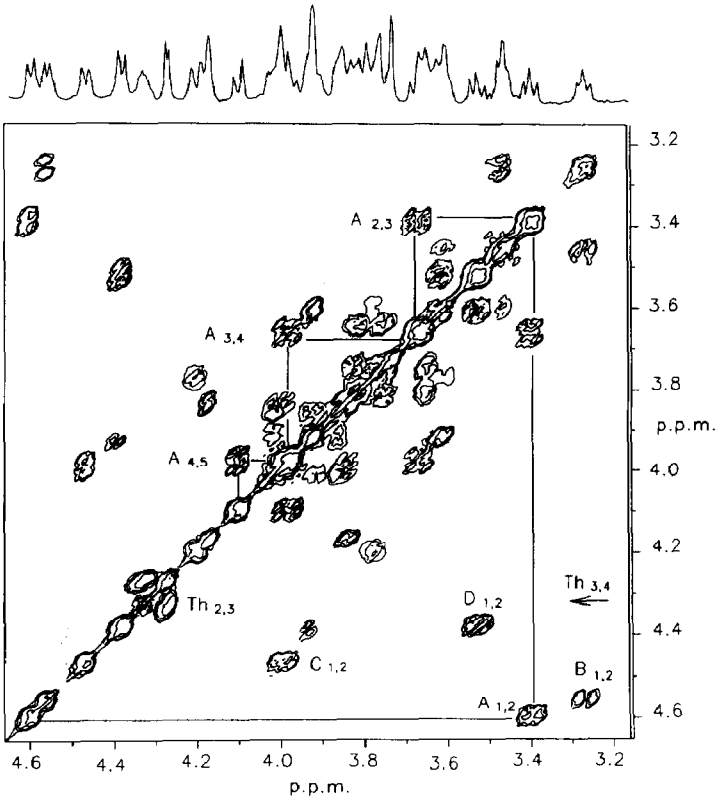

Fig. 4. Expanded COSY contour plot of the ring-proton region (4.70–3.15 p.p.m.) of *E. coli* K49 capsular polysaccharide, recorded at 27°. Cross-peaks are labelled A–D (Th = Threonine).

The COSY (Fig. 4), relayed COSY, and heteronuclear ^{13}C–^{1}H chemical-shift-correlated (Fig. 5) 2D-n.m.r. spectra of the K49 polysaccharide, recorded at 27°, permitted unambiguous assignment of all the ^{1}H and ^{13}C resonances. The resonances for anomeric protons were well resolved and were labelled arbitrarily A-1 to D-1 according to the order of decreasing chemical shift. Residues A and B were identified as *gluco* compounds from the relatively large values (~ 10 Hz) of $J_{3,4}$ and $J_{4,5}$, whereas residues C and D were identified as *galacto* compounds from the small values (~ 3.2 and ~ 1 Hz, respectively) of $J_{3,4}$ and $J_{4,5}$. On the basis of the ^{3}J values for the ring protons, it is evident that each residue was β-pyranose and had the $^{4}C_{1}$ conformation[21]. From the connectivities defined by the appropriate cross-peaks, H-2,3,4,5 of residue A were readily discernible (Fig. 4). The absence of cross-peaks relating H-5 to protons at C-6 suggested A to be the glucuronic acid residue. The significant deshielding of C-4 of this residue confirmed the position of the linkage in the polysaccharide[9,22]. Since residue B exhibited cross-peaks relating H-5 (3.62 p.p.m.), H-6 (4.21 p.p.m.), and H-6′ (3.79 p.p.m.) ($B_{5,6}$ and $B_{5,6'}$), it could be identified as the glucose residue. The assignments of the resonances of the methylene carbon atoms for residues B–D could be obtained easily from a

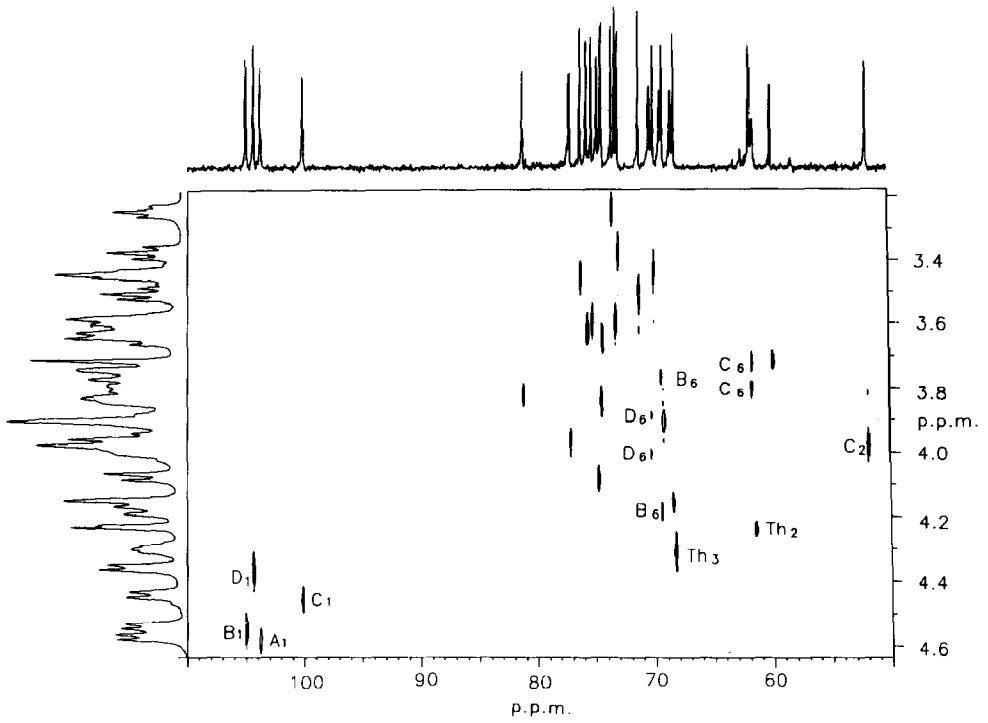

Fig. 5. Heteronuclear ^{1}H–^{13}C shift correlation map for the spectral region F_2 (110–50 p.p.m.) and F_1 (3.20–4.65 p.p.m.). The respective ^{13}C and ^{1}H 1-D spectra are displayed along the F_2 and F_1 axes, respectively, and the correlated resonances are labelled A–D (Th = Threonine).

^{13}C-DEPT experiment and, thus, the assignments of the corresponding H-6 resonances for residues B–D were confirmed from the ^{13}C–^{1}H chemical shift correlation experiment (Fig. 5). Residues C and D were identified as 2-amino-2-deoxygalactose and galactose, respectively. The correlation of the ^{13}C resonance at 52.02 p.p.m., indicative of a carbon atom linked to nitrogen, with the ^{1}H resonance at 3.99 p.p.m. assigned to H-2 of residue C (Fig. 5), confirmed the assignment of this residue as the amino sugar.

The signals corresponding to H-3 (4.33 p.p.m.) and H-2 (4.26 p.p.m.) of threonine were identified from connectivities in the COSY spectrum to the high-field H-4 resonance (1.23 p.p.m.) (Fig. 4).

The foregoing evidence unequivocally established the repeating unit of K49 capsular polysaccharide as a tetrasaccharide with the structure →4)-β-D-Glc*p*A-(1→6)-β-D-Gal*p*-(1→6)-β-D-Glc*p*-(1→3)-β-D-Gal*p*NAc-(1→. The glucuronic acid was substituted amidically with either serine or threonine. Amino acids, linked amidically to uronic acid residues, are components of other bacterial polysaccharides[2–9]; in at least one instance, the amino acid appeared to be immunodominant[9].

EXPERIMENTAL

Bacteria and cultivation. — *E. coli* O8:K49:H21 was obtained from Dr. I. Ørskov (Copenhagen) and grown on Mueller–Hinton agar (containing 0.5% of NaCl) for 3 days at 37°. The acidic capsular polysaccharide was obtained by fractional precipitation[10] with cetyltrimethylammonium bromide. The purified polysaccharide was homogeneous on Sepharose 4B and its molecular mass was estimated as 420 000 Da.

Analytical methods. — Analytical and preparative descending p.c. were performed using Whatman No.1 paper with *A*, 18:3:1:4 ethyl acetate–acetic acid–formic acid–water; and *B*, 8:2:1 ethyl acetate–pyridine–water. Alkaline silver nitrate was used for detection of sugars, or ninhydrin (0.05% in 1-butanol) for amino sugars and amino acids. Gel-permeation chromatography was performed on a column (95 × 3 cm) of Bio-gel P-2 by elution with acidified distilled water (1 drop of HCOOH per L).

Analytical g.l.c. was performed using a Hewlett–Packard 5890A gas chromatograph fitted with a dual flame -ionization detector, a 3392A recording integrator, and a DB-17 fused-silica capillary column (J & W Scientific). The following programs were used: *A* (for alditol acetates), 180° for 2 min then 5°/min to 220°; *B* (for partially methylated alditol acetates), 180° for 1 min then 2°/min to 250°; *C* (for methylated disaccharides), 210° for 1 min then 4°/min to 240°; *D* (for acetylated (+)-2-butyl derivatives of amino acids), 160° for 2 min then 2°/min to 220°.

For analysis of constituent sugars, samples of native polysaccharide (2–5 mg) were hydrolysed with 4M trifluoroacetic acid (1 mL) for 1 h at 125°, or with anhydrous hydrogen fluoride for 3 h at 24°. Mono- and oligo-saccharides were reduced with aqueous sodium borohydride at room temperature for 3 h. Alditols were acetylated with acetic anhydride–pyridine (1:1) for 30 min at 100°.

Threonine, serine, and 2-amino-2-deoxygalactose were determined, after hydrolysis (6M HCl, 24 h, 110°), with an amino acid analyser. Following hydrolysis of the K49 polysaccharide and p.c. of the hydrolysate, the configurations of 2-amino-2-deoxy-D-galactose, D-glucuronic acid, and L-threonine were established by capillary g.l.c. of their acetylated (+)-2-butyl derivatives[13,14] (program *D*). The configurations of D-glucose and D-galactose were established by formation of their acetylated (−)-2-octyl glycosides[12].

Methylation analysis. — A sample (7 mg) of the K49 polysaccharide (H^+ form) was methylated according to the Hakomori method[17], and the product was isolated by partition between $CHCl_3$ and water. A portion (2 mg) was hydrolysed (2M HCl, 6 h, 100°), and the products were reduced with aqueous sodium borohydride for 1 h at 24°, then acetylated, and analysed by g.l.c–m.s. (program *B*). The molar ratios were corrected by use of the effective carbon-response factors[23].

Methanolysis. — The methylated K49 polysaccharide (3 mg) was treated with refluxing methanolic 3% HCl (1 mL, 24 h, 65°). The solution was neutralised with silver carbonate, filtered, and concentrated to dryness in a stream of N_2, and the residue was acetylated.

Partial acid hydrolysis. — A solution of the polysaccharide (356 mg) in M HCl (100

mL) was boiled under reflux for 1 h, neutralised with lead carbonate, and centrifuged, and the supernatant solution was dialysed against distilled water. The diffusate was concentrated, deionised, and subjected to p.c. The main product, aldobiouronic acid **1**, was methylated, carboxyl-reduced, and analysed by g.l.c.–c.i.-m.s. (Table III).

Solvolysis with hydrogen fluoride. — The K49 polysaccharide (100 mg) was treated with anhydrous HF (3–5 mL) for 1 h at 0°. The reaction was quenched by pouring the mixture into a slurry of solid CO_2–calcium carbonate–dichloromethane[24], and the solvent was removed in a stream of N_2. After the addition of H_2O (5 mL), the precipitate was removed by centrifugation and the products were fractionated by gel-permeation chromatography. The neutral disaccharide **2** (R_{Gal} 0.39; yield, 5 mg) obtained was methylated, and analysed by g.l.c.–c.i.-m.s. (Table III).

The K49 polysaccharide (100 mg) was reacted with anhydrous HF for 15 min at 0° and the mixture was worked-up as described above. The main product **3** (12 mg) was reduced with aqueous sodium borohydride, and the solution was neutralised with 10% acetic acid in methanol and co-concentrated with methanol in a stream of N_2. A portion (5 mg) of the oligosaccharide-alditol was methylated and hydrolysed (2M HCl, 6 h, 100°), and the products were reduced with aqueous sodium borohydride and acetylated. The resulting partially methylated alditol acetates were analysed by g.l.c. and g.l.c.–c.i.-m.s. (Table I).

N.m.r. spectroscopy. — ^{13}C-N.m.r. spectra were recorded at 27° for solutions in D_2O, using either a Bruker AM500 or Varian XL-300 spectrometer operating at 125 or 75 MHz, respectively, in the pulsed F.t.-mode with complete proton decoupling. DEPT spectra[25] were obtained with broad-band proton decoupling, a 135° proton pulse, and a 3.3-ms delay [$0.5/(^1J_{C,H})$] between pulses to distinguish between methine and methylene carbon resonances. The $^1J_{C,H}$ values for the anomeric carbons were measured using gated decoupling[26]. Chemical shifts are reported relative to that of internal acetone (31.07 p.p.m.). ^{1}H-N.m.r. spectra were recorded at 27° or 95° with a Bruker AM500 or Bruker WH-400 instrument for solutions of oligosaccharides (5 mg) or polysaccharides (15–40 mg) in D_2O (0.5 mL, 99.8 atom %), and chemical shifts are recorded relative to that of internal acetone (2.23 p.p.m.).

2D Homonuclear chemical-shift-correlated (COSY and Relay COSY) experiments were carried out using the conventional pulse sequences[27–29]. Spectra were obtained using matrices ($t_1 \times t_2$) of 256 × 2048 data points which were zero-filled to give 1024 × 2048 data points. The spectral width was 2326 Hz and a recycle delay of 1.2 s was employed. Resolution enhancement in both dimensions was done by the unshifted sine-bell-window function prior to Fourier transformation. Magnitude spectra symmetrized about the diagonal were used to represent the data.

The ^{13}C–^{1}H chemical-shift-correlated spectrum was recorded on a concentrated solution of the polysaccharide (90 mg/mL) with proton decoupling in the F_2 domain[30]. The initial data matrix ($t_1 \times t_2$) of 128 × 4096 points was zero-filled and Fourier-transformed to give a final matrix of 512 × 4096 points. Spectral widths of 12 500 and 1047 Hz for the respective ^{13}C (F_2) and ^{1}H (F_1) chemical shift domains and a recycle delay of 1.4 s were employed. Fixed delays, r_1 and r_2, were set at 3.30 and 1.70 ms,

respectively, to select for all multiplicities. Resolution enhancement was applied in both dimensions by means of a Lorentz-to-Gauss transformation and the data were processed to give a power spectrum.

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