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The structure of the capsular polysaccharide of *Escherichia coli* O9:K35:H$^-$

P. Linton Hackland and Haralambos Parolis

School of Pharmaceutical Sciences, Rhodes University, Grahamstown 6140 (South Africa)

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ABSTRACT

The annexed structure of the capsular polysaccharide of *Escherichia coli* O9:K35:H$^-$ (A104a) has been determined by glycose and methylation analysis, base-catalysed degradation, and 1D and 2D NMR spectroscopy on the polysaccharide and the products obtained by bacteriophage-mediated depolymerisation.

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

INTRODUCTION

E. coli capsular polysaccharides (K-antigens) have been arranged into 72 distinct serotypes[1], the structures of the repeating units of 55 of which have been reported[2]. The capsular antigen of *E. coli* O9:K35:H$^-$, the structure of which is now reported, has the same qualitative sugar composition as those of *E. coli* K29[3], K39[4], and K102[5].

RESULTS AND DISCUSSION

Composition and NMR spectra.—E. coli O9:K35:H$^-$ bacteria (culture No. A104a) were grown on an agar medium and the acidic polysaccharide (**PS**), isolated and purified as described[6], showed a broad distribution of molecular weights in gel-permeation chromatography on Sephacryl S500 with a maximum at

Correspondence to: Professor H. Parolis, School of Pharmaceutical Sciences, Rhodes University, Grahamstown 6140, South Africa.

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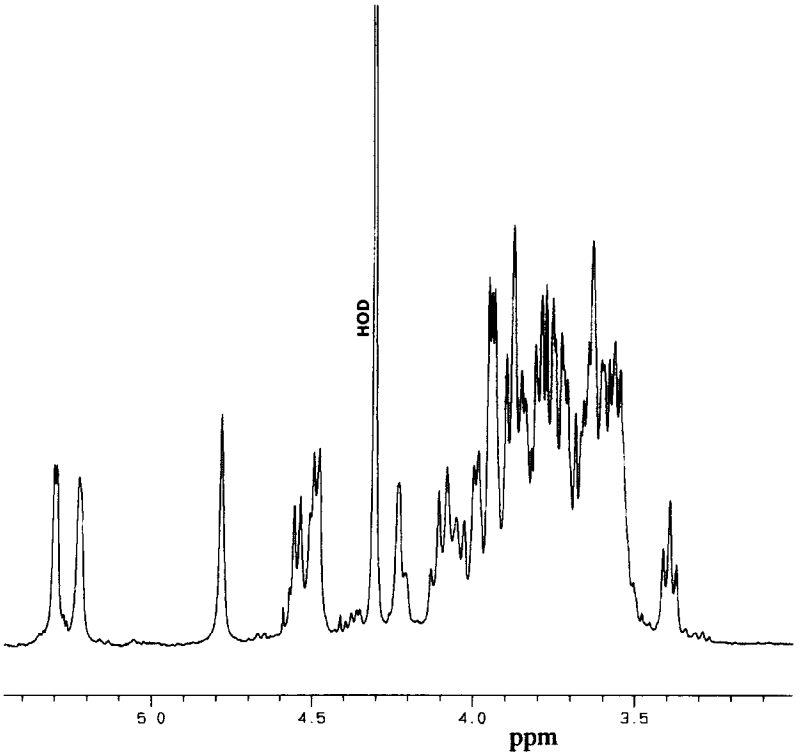


Fig. 1. 400-MHz ^{1}H NMR spectrum of **PS** at 70°.

10[7]. GLC of the acetylated aldononitriles[7], derived from the products in an acid hydrolysate of **PS** with and without prior reduction of the uronic acid group, showed it to be composed of Glc, Man, Gal, and GlcA in the molar ratios 2:1:1:1. Each of the sugars was shown to be D by GLC of the acetylated (−)-2-octyl glycosides[8].

The ^{1}H NMR spectrum of **PS** (Fig. 1) at 70° contained, inter alia, H-1 resonances for α-residues at δ 5.30 ($J_{1,2}$ 3.7 Hz) and 5.23 ($J_{1,2}$ 3.8 Hz), and β-residues at δ 4.79 (unresolved doublet), 4.55 ($J_{1,2}$ 7.9 Hz), and 4.49 ($J_{1,2}$ 7.6 Hz), and an H-5 resonance of α-GlcA at δ 4.51 ($J_{4,5}$ 9.2 Hz). The resonance at δ 4.79 was assigned to H-1 of β-D-Man*p*. The ^{13}C NMR spectrum contained C-1 signals at 103.23 (2 C), 102.09, 100.62, and 99.90 ppm and a signal for C=O at 172.80 ppm. These results suggested **PS** to have a pentasaccharide repeating unit.

Methylation analysis and uronic acid degradation.—**PS** was methylated by the Hakomori method[9,10] and the partially methylated alditol acetates, prepared from an acid hydrolysate of the polysaccharide without and with reduction of the methoxycarbonyl function, were analysed by GLC–MS (Table I, columns I and II). The results indicated terminal Gal, 4-linked GlcA, 3,4-linked Man, and 3- and 4-linked Glc.

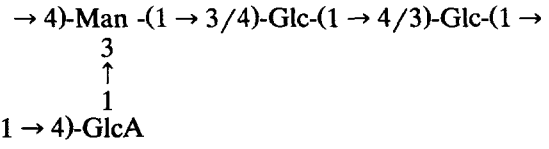
TABLE I

Methylation analysis data for the *E. coli* K35 polysaccharide and derived products

Methylated sugar [a] (as alditol acetate)	Molar ratio [b,c]							
	I	II	III	IV	V	VI	VII	VIII
1,2,4,5,6-Glc							0.47	0.62
2,3,4,6-Gal	0.40	0.34	0.14	0.14	0.14	0.13	0.33	1.44
2,4,6-Glc	1.00	0.78	0.96	0.90	0.87	0.63		1.00
2,4,6-Man							0.73	0.94
2,3,6-Man			0.38	0.47	0.57	0.68		
2,3,6-Glc	1.00	1.00	1.00	1.00	1.00	1.00	1.00	2.15
2,6-Man	0.79	0.84	0.55	0.47	0.38	0.21		0.91
2,3-Glc		0.31					0.44	1.52

[a] 1,2,4,5,6-Glc = 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methylglucitol, etc. [b] Determined on a DB-225 capillary column at 210°. [c] I, methylated K35 polysaccharide; II, methylated carboxyl-reduced K35 polysaccharide; III–VI, base-degraded methylated polysaccharide 1, 2, 4, and 6 h, respectively; VII, methylated, carboxyl-reduced **P1**-alditol; VIII, methylated, carboxyl-reduced **P2**-alditol.

Methylated **PS** was subjected to a β-elimination reaction[11] using potassium dimsyl as base. At intervals of 1, 2, 4, and 6 h, the products were trideuteriomethylated and the partially methylated alditol acetates derived from the acid hydrolysates of the products were analysed by GLC and GLC–MS (Table I, columns III–VI). The results, when compared with those for methylated **PS** (Table I, column I), showed a decrease in the proportions of 2,6-di-*O*-methylmannose and 2,3,4,6-tetra-*O*-methylgalactose and the concomitant production of 2,3,6-tri-*O*-methylmannose. These results indicated the GlcA to be present in the side chain and 3-linked to Man, the terminal Gal to be linked to the GlcA, and the partial structure **1**.



1

Bacteriophage-mediated depolymerisation of **PS**.—Depolymerisation of **PS** with a viral-borne (bacteriophage) endoglycanase[12] gave the oligosaccharides **P1** and **P2**, which were shown to be a penta- and deca-saccharide, respectively, by the method of Morrison[13]. Both **P1** and **P2** had Glc as a reducing terminus and each had Glc, Gal, Man, and GlcA in the same ratios as in **PS**.

P1 and **P2** were reduced with sodium borodeuteride and then methylated. The partially methylated alditol acetates, prepared from acid hydrolysates after carboxyl reduction of each methylated oligosaccharide-alditol, were examined by GLC and GLC–MS (Table I, columns VII and VIII) which established **P1** to be the repeating unit and **P2** to be the double repeating unit of **PS**. Comparison of the methylation results for **P1**-alditol (Table I, column VII) with those for **P2**-alditol

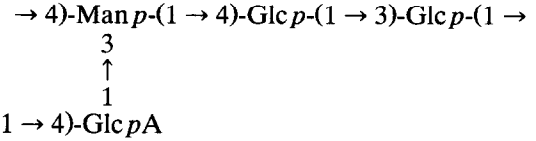
TABLE II

^{1}H NMR data for oligosaccharide products derived from *E. coli* K35 polysaccharide at 30°

Compound	Chemical shift [a] (ppm)	^3J (Hz)	No. of H	Assignment [b]
P1 [c]	5.38	3.9	0.4	4-α-Glc-(1 → 3)-α-Glc
	5.35	3.9	0.6	4-α-Glc-(1 → 3)-β-Glc
	5.29	3.9	1.0	4-α-GlcA
	5.24	3.8	0.4	3-α-Glc
	4.79	n.o. [d]	0.4	3-β-Man · · · α-Glc
	4.78	n.o.	0.6	3-β-Man · · · β-Glc
	4.67	8.0	0.6	3-β-Glc
	4.48	7.8	1.0	β-Gal
	4.46	9.7	1.0	H-5 of 4-α-GlcA
P1-alditol	5.30	3.8	1.0	4-α-GlcA
	5.14	3.9	1.0	4-α-Glc
	4.79	n.o.	1.0	3-β-Man
	4.50	9.3	1.0	H-5 of 4-α-GlcA
	4.48	7.7	1.0	β-Gal

[a] Chemical shift relative to that for internal acetone at δ 2.23. [b] 4-α-Glc connotes H-1 of a 4-linked α-D-glucopyranosyl residue, etc. [c] For the origin of the compounds, see text. [d] Not observed.

(Table I, column VIII) and for **PS** (Table I, column II) demonstrated that the bacteriophage enzyme was a glucosidase that catalysed the hydrolysis of → 3)-Glc*p*-(1 → 4)-Man*p*-(1 → linkages in **PS**. Thus, the repeating unit of **PS** has the structure **2**.



2

The H-1 resonances of the residues in **P1** and **P1**-alditol and those for H-5 of GlcA are collected in Table II. Comparison of the ^{1}H NMR data for **PS** (see above) and **P1** (Table II) shows that a signal for H-1β (δ 4.55) for **PS** was replaced by partial signals at δ 5.24 and 4.67 for **P1**. The latter signals were assigned to H-1α and H-1β of the reducing Glc terminus and established that a 3-linked β-Glc was cleaved by the bacteriophage from the 4-linked Man. These assignments were confirmed by the absence of the partial signals from the ^{1}H NMR spectrum of **P1**-alditol. The ^{1}H NMR spectrum of **P1** contained two further sets of fractional signals at δ 5.38 and 5.35 (d) and at 4.79 and 4.78 (unresolved d). These fractional signals occurred in the same ratio as the H-1 signals assigned to the reducing 3-linked Glc and reflect the mutarotational equilibrium of the reducing terminus. The sets of unresolved doublets at δ 4.79 and 4.78 were assigned to the 4-linked β-Man and those at δ 5.38 and 5.35 were assigned to the residue closest to the mutarotational centre, namely, the 4-linked Glc which was established as α. The

GlcA was established as α by the occurrence of the resonance of H-5 ($J_{4,5}$ 9–10 Hz) in the region for H-1 resonances of **PS** (see above), **P1**, and **P1**-alditol (Table III). The signal at δ 5.29 in the spectrum of **P1** was therefore assigned to the α-GlcA and the remaining signal at δ 4.48 to the terminal Gal which was established as β. The assignment of most of the resonances for **P1**-alditol follows from the assignment of the resonances for **P1**. The resonance at δ 5.14 for **P1**-alditol was assigned to H-1 of the 4-linked Glc rather than the 4-linked GlcA since the former is linked to the terminal Glc-ol and would be expected to show the largest change in chemical shift. The above results establish the structure of **P1** and **PS** as **3** and **4**, respectively.

β-D-Gal*p*-(1 → 4)-α-D-Glc*p*A-(1 → 3)-β-D-Man*p*-(1 → 4)-α-D-Glc*p*-(1 → 3)-α,β-D-Glc*p*

3

→ 4)-β-D-Man*p*-(1 → 4)-α-D-Glc*p*-(1 → 3)-β-D-Glc*p*-(1 →
3
↑
1
β-D-Gal*p*-(1 → 4)-α-D-Glc*p*A

4

2D NMR spectroscopy of **PS**.—The sequence and anomeric configuration of each sugar residue in the repeating unit **4** was confirmed by ^1H–^1H correlation experiments on **PS**. The chemical shifts of all of the ^1H resonances of residues **a**–**d** and H-1/5 of residue **e** were established from COSY[14], HOHAHA[15], and NOESY[16] experiments (Table III, Figs. 2 and 3). Commencing from H-1, the chemical shifts of the ^1H resonances of each residue were traced via their cross-peaks.

Residue **a** *[→ 4)-Glc*p*].*—The chemical shifts for the H-1/4 resonances were established from the COSY spectrum, and the connectivities up to H-5 were noted

TABLE III

^1H NMR data for *E. coli* K35 polysaccharide at 70°

Residue [b]	Chemical shifts (ppm) [a]						
	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
a							
4-α-Glc*p*	5.30	3.64	3.92	3.70	4.07	3.76	3.84
b							
4-α-Glc*p*A	5.23	3.65	3.98	3.91	4.51		
c							
3,4-β-Man*p*	4.79	4.24	3.88	4.12	3.61	3.88	4.03
d							
3-β-Glc*p*	4.55	3.39	3.67	3.58	3.58	3.77	3.99
e							
β-Gal*p*	4.49	3.58	3.67	3.97	3.74		

[a] Relative to internal acetone at δ 2.23. [b] 4-α-Glc connotes a 4-linked α-D-glucopyranosyl residue, etc.

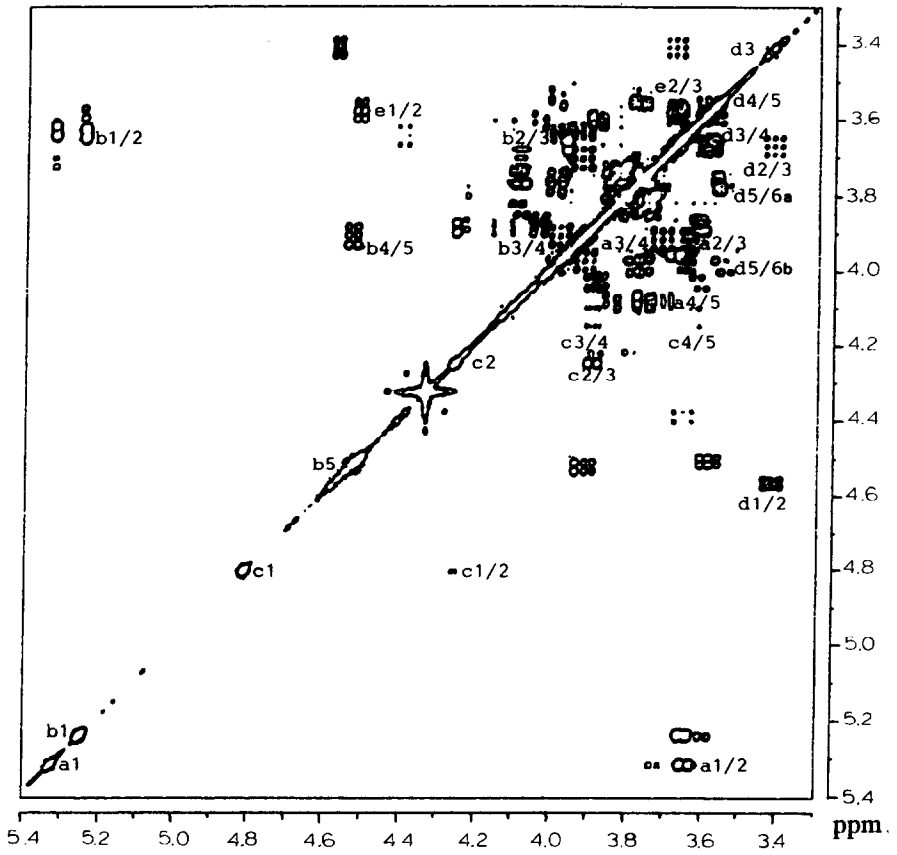


Fig. 2. COSY contour plot of **PS**: **a**1 connotes H-1 of residue **a**, **a**1,2 connotes the cross-peak between H-1 and H-2 of residue **a**, etc. (See Table III for identification of **a**–**e**).

in the H-1 track in the HOHAHA contour map. The chemical shifts for the H-6a and H-6b resonances in the COSY spectrum could then be traced from H-5.

Residue **b** *[→4)-α-Glc*p*A]*.—For this residue, the H-5 resonance provided a second window into the spin system. The connectivities from H-1 to H-5 were established easily from the COSY spectrum and confirmed in the HOHAHA contour plot.

Residue **c** *[→3,4)-β-Man*p*]*.—The chemical shifts for the H-1/4 resonances were established from the COSY spectrum, and that for H-5 from the H-2 track in the HOHAHA spectrum. The chemical shift of the latter resonance was confirmed by the NOE between H-1 and H-5 in the NOESY experiment. A weak H-4/H-5 cross-peak was observed in the COSY contour plot from which the chemical shifts for the H-5, H-6a, and H-6b resonances were determined.

Residue **d** *[→3)-β-Glc*p*]*.—The chemical shifts for the H-1/4 resonances were traced in the COSY spectrum, and that for the H-5 resonance was determined from the expected NOE between H-1 and H-5 in the NOESY experiment. The

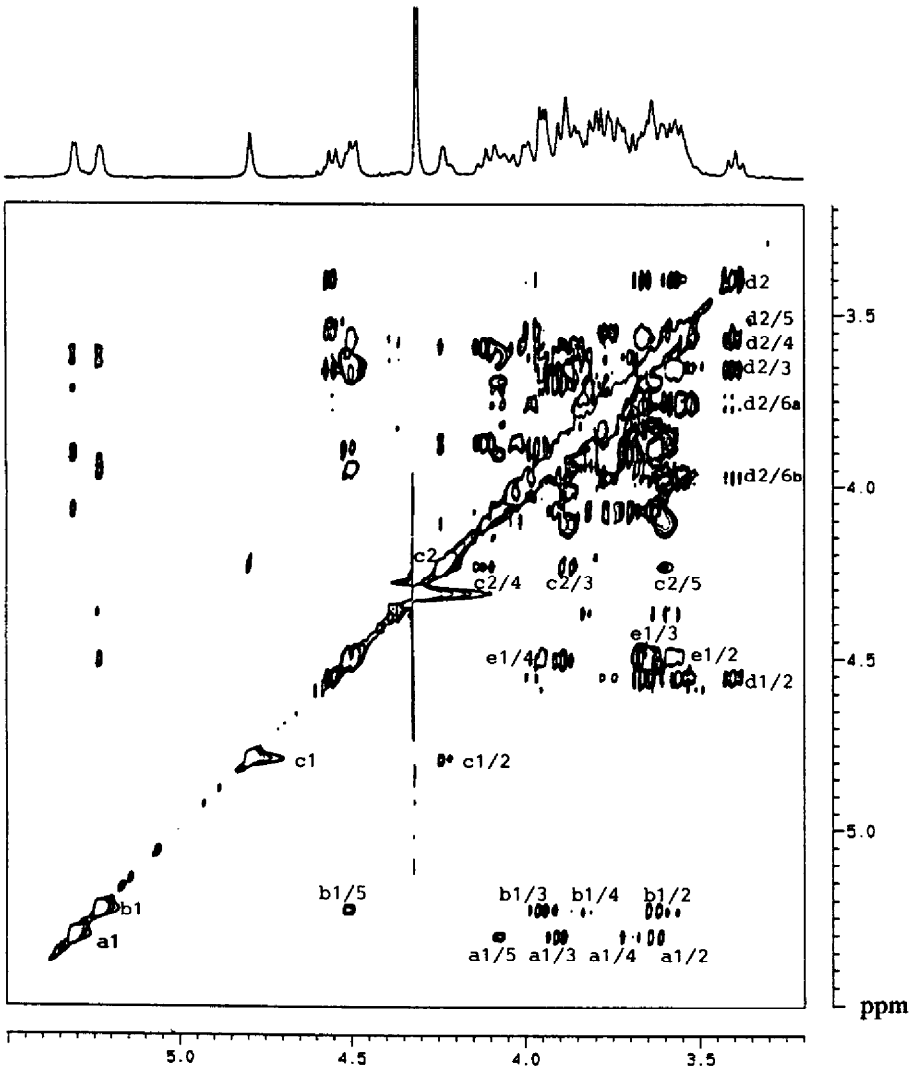


Fig. 3. HOHAHA contour plot of **PS**: **a**1, **a**1,2, etc. as for Fig. 2. The ^{1}H NMR spectrum is projected along the f_2 axis.

H-6a and H-6b resonances were then identified from the COSY spectrum. These assignments were confirmed from the H-2 track in the HOHAHA contour plot.

Residue **e** *(β-Gal*p*).*—The chemical shifts for the H-1/4 resonances were established from the COSY contour map, and that for the H-5 resonance from the NOESY spectrum.

The sequence of the residues in the repeating unit of the polysaccharide was investigated by a phase-sensitive NOESY experiment. The observed inter- and intra-residue NOE contacts are presented in Table IV. The α-pyranosyl residues **a**

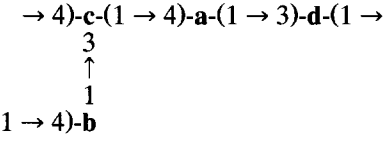
TABLE IV

NOE contacts for *E. coli* K35 polysaccharide

Proton irradiated [a]	NOE contact to
a H-1	3.67 (**d**, H-3), 3.64 (**a**, H-2)
b H-1	4.24 (**c**, H-2), 3.88 (**c**, H-3), 3.65 (**b**, H-2)
c H-1	3.70 (**a**, H-4), 4.24 (**c**, H-2), 3.88 (**c**, H-3), 3.61 (**c**, H-5)
c H-2	3.88 (**c**, H-3)
d H-1	4.12 (**c**, H-4), 3.67 (**d**, H-3), 3.58 (**d**, H-5)
e H-1	3.91 (**b**, H-4), 3.67 (**e**, H-3), 3.74 (**e**, H-5)

[a] See Table III for identification of **a**–**e**.

and **b** showed characteristic NOEs between H-1 and H-2 while the β-pyranosyl residues **c**–**e** showed the expected contacts from H-1 to H-3 and H-5. Residue **c** also showed intra-residue NOEs between H-1 and H-2 and H-3, confirming that it had the *manno* configuration. Intense inter-residue NOE contacts were also observed between the H-1 resonances of residues **a**, **c**, and **d** and H-3 of **d**, H-4 of **a**, and H-4 of **c**, respectively, whereas a less intense NOE was observed between H-1 of **e** and H-4 of **b**. Residue **b**, on the other hand, showed intense NOEs between its H-1 and H-2 and H-3 of **c**. The inter-residue NOE data, together with the results of the methylation analysis data of **PS**, established the sequence shown in **5**.



5

The identity of residues **a**–**e** and the anomeric configuration of the residues followed from the ^{1}H NMR data (Tables III and IV).

Thus, the structure of the repeating unit of **PS**, as deduced from the study of the products obtained by bacteriophage-mediated degradation, was confirmed by the 2D NMR study of **PS**. The structure of **PS** is almost identical to that reported[17] for the repeating unit of the capsular antigen of *Klebsiella* K13. In the latter polysaccharide, the terminal β-D-Gal carries a 3,4-*O*-(1-carboxyethylidene) substituent and the bacteriophage-mediated depolymerisation also involved hydrolysis of the 3-linked β-Glc residue.

EXPERIMENTAL

General methods.—Analytical GLC was performed with a Hewlett–Packard 5890A gas chromatograph fitted with flame-ionisation detectors, He as carrier gas, and a 3392A recording integrator. A J & W Scientific fused-silica DB-225 bonded-

phase capillary column (30 m × 0.25 mm; film thickness, 0.25 μm) was used for acetylated aldononitriles (230°), partially methylated alditol acetates (210°), and acetylated (−)-2-octyl glycosides (220°). The identity of each derivative was confirmed by GLC–MS on a Hewlett–Packard 5988A instrument, using the appropriate column, and with an ionisation energy of 70 eV and an ion-source temperature of 200°. Analytical GPC of PS 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. GPC on a column (2.5 × 90 cm) of Bio-Gel P4 was used to separate the products of the bacteriophage-mediated degradation of **PS**, using the same mobile phase.

Samples were hydrolysed under nitrogen with 4 M trifluoroacetic acid for 1 h at 125° in a sealed vial. Carboxyl reduction was achieved by treating samples with refluxing methanolic 3% HCl for 16 h and reducing the resulting methyl esters with $NaBH_4$ in anhyd MeOH. Acetylated aldononitriles were prepared by the method of McGinnis[7]. The dp of oligosaccharides was determined by the method of Morrison[13]. The absolute configuration of the sugars was determined by the method of Leontein et al.[8]. The polysaccharide and the oligosaccharides which had been reduced with $NaBH_4$ were methylated by the Hakomori procedure[9] as modified by Phillips and Fraser[10]. Base-catalysed degradation[11] of the methylated **PS** was carried out with potassium dimsyl. Samples isolated after 1, 2, 4, and 6 h were treated with trideuteriomethyl iodide.

Preparation of E. coli *K35 polysaccharide* (**PS**).—An authentic culture (A104a) of *E. coli* O9:K35:H^- was obtained from Dr. I. Ørskov (Copenhagen) and the bacteria were propagated on Mueller–Hinton agar at 37°. The harvested slime was treated with 1 vol of aq 2% phenol and the mixture was stirred for 16 h at 4°. The polysaccharides were separated from the cells by ultracentrifugation and **PS** was isolated by precipitation with cetyltrimethylammonium bromide.

Bacteriophage-mediated degradation of **PS**.—A bacteriophage which could be propagated on *E. coli* K35 bacteria was isolated from Grahamstown sewage and purified by successively picking single plaques. The bacteriophage titre was increased by successive tube and flask lyses in Luria–Bertani broth until a dialysed solution containing 10^{11} plaque-forming units per mL was obtained. **PS** was dissolved in the bacteriophage solution which was incubated at 37° for 3 days, then concentrated, and dialysed (mol wt cut-off 3500) against distilled water. The diffusates were combined and freeze-dried, and a solution of the residue in water was treated several times with Amberlite IR-120 (H^+) resin prior to GPC, to afford **P1** (55 mg) and **P2** (35 mg).

NMR spectroscopy.—Samples were deuterium-exchanged by freeze-drying solutions in D_2O and then dissolved in 99.99% D_2O (0.45 mL) containing a trace of acetone as internal reference (δ 2.23 for ^1H and 31.07 ppm for ^{13}C). The ^1H NMR spectra of **P1** and **P1**-alditol were recorded at 30° with a Bruker WM-500 spectrometer, whereas the 1D and 2D NMR experiments on **PS** were recorded at 70° with a Bruker AMX-400 spectrometer equipped with an X32 computer using

UXNMR software. The parameters used for the 2D experiments were as follows. COSY: 512 × 2048 data matrix, zero-filled to 1024 data points in t_1; 64 scans per t_1 value; 0.7-s recycle delay; spectral width, 1401 Hz; unshifted sine-bell filtering in t_1 and t_2; HOHAHA; spectral width and recycle delay as above; 256 × 2048 data matrix, 128 scans per t_1 value; shifted sine-square filtering in t_1 and t_2; 50 mlev cycles; phase sensitive NOESY: 256 × 2048 data matrix; spectral width, 1401 Hz; 112 scans per t_1 value; 1.0-s recycle delay; shifted sine-squared filtering in t_1 and t_2; and a mixing delay of 0.3 s.

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