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A RE-INVESTIGATION OF THE STRUCTURE OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* K10

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ABSTRACT

The capsular polysaccharide of *Klebsiella* K10 was investigated by methylation analysis and ^{1}H-n.m.r. spectroscopy, and the deacetylated bacteriophage-degraded polysaccharide by 1D- and 2D-n.m.r. spectroscopy. The repeating unit was shown to be a branched hexasaccharide

$$\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow$$
$$\begin{array}{c} 3 \\ \uparrow \\ 1 \\ \alpha\text{-D-Gal}p \end{array}$$

OAc substituents were located on the terminal and 2-linked galactopyranosyl residues by Prehm methylation of a low-molecular-weight fraction obtained by bacteriophage degradation.

INTRODUCTION

The structures **1** and **2** reported[1,2] for the repeating unit of the capsular polysaccharide of *Klebsiella* K10 differ in the sequence, glycoside linkages, and anomeric configurations of some of the monosaccharide residues. We now report the results of a re-investigation of the structure of the repeating unit.

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α-D-Gal*p*
1
↓
4
β-D-Glc*p*
1
↓
4
β-D-Glc*p*A
1
↓
2
→4)-β-D-Gal*p*-(1→3)-α-D-Man*p*-(1→4)-α-D-Gal*p*-(1→

1[1]

→4)-β-D-Glc*p*A-(1→2)-α-D-Man*p*-(1→3)-β-D-Gal*p*-(1→6)-α-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→
3
↑
1
α-D-Gal*p*

2[2]

DISCUSSION

The serological test strain for *Klebsiella* K10, used in the present study, was obtained from the same source as those which were used previously[1,2]. The bacteria were grown on a sucrose-rich medium and the polysaccharide was isolated and purified by standard procedures. The presence of Man, Gal, Glc, and GlcA in the K10 polysaccharide was confirmed, and the D configuration of each was established by g.l.c. of the derived acetylated (−)-2-octyl glycosides[3]. The ^{1}H-n.m.r. spectrum of the K10 polysaccharide recorded at 95° was complex and characterised by broad peaks. The presence of OAc groups in non-stoichiometric amounts at more than one site in the repeating unit [evident from signals at δ 2.14, 2.17 (major), and 2.20] was responsible for the complexity of the spectrum. The *O*-deacetylated polysaccharide gave greatly simplified ^{1}H-n.m.r. spectra, and the data are shown in Table I. There were six resonances for anomeric protons, for which the $J_{1,2}$ values and chemical shifts indicated four α- and two β-linkages. These findings contrast with the three α- and three β-linkages reported[1,2]. A sample of the K10 polysaccharide studied previously gave, with the exception of a small peak for acetate at δ 2.17, ^{1}H-n.m.r. spectra identical to those given by the deacetylated polysaccharide now described.

TABLE I

H-1 CHEMICAL SHIFTS AND COUPLING CONSTANTS FOR *Klebsiella* K10 POLYSACCHARIDE

Chemical shift[a] *(p.p.m.)*	$J_{1,2}$ *(Hz)*	*Chemical shift (p.p.m.)*	$J_{1,2}$ *(Hz)*	*Assignment*
Acid form		*Sodium salt*		
5.679	3.7	5.655	n.o.[b]	→2)-α-Gal
5.285	3.0	5.274	4.1	α-Gal
5.168	4.0	5.169	3.9	→6)-α-Glc
5.136	n.o.	5.137	n.o.	→2,3)-α-Man
4.590	7.9	4.521	7.8	→4)-β-GlcA
4.487	7.7	4.481	7.7	→3)-β-Gal

[a]Relative to acetone at 2.23 p.p.m. [b]Not observed.

Methylation analysis. — The polysaccharide was methylated by the modified Hakomori method[4] using potassium dimsyl[5]. The alditol acetates derived from the methylated polysaccharide with and without prior reduction were analysed by g.l.c.–m.s. and showed (Table II) that the polysaccharide was composed of 6-linked Glc, 4-linked GlcA, and terminal, 2- and 3-linked Gal, and 2,3-linked Man.

Bacteriophage-mediated degradation of Klebsiella *K10 polysaccharide.* — A bacteriophage which infects *Klebsiella* K10 bacteria was isolated from sewage and was propagated on its host strain. A partially purified, concentrated solution of the bacteriophage was used to depolymerise the K10 polysaccharide. The depolymerised material was dialysed (mol. wt. cut-off, 3500) in order to remove products of lower molecular weight, and those in the retentate were fractionated on Sephacryl S400 and Sephadex G50 to give polysaccharide fractions with $M_r \sim 10^4$ (*A*) and 4–10 × 10^3 (*B*). Deacetylated fraction *A*(*Ad*) gave a ^{1}H-n.m.r. spectrum

TABLE II

METHYLATION ANALYSIS OF *Klebsiella* K10 POLYSACCHARIDE

Methylated sugar[a] *(as alditol acetate)*	T[b]	*Molar ratio*[c]	
		I	*II*
2,3,4,6-Gal	1.00	0.90	1.00
2,3,4-Glc	1.34	0.81	0.99
3,4,6-Gal	1.36	0.79	0.90
2,4,6-Gal	1.39	1.00	1.00
4,6-Man	1.63	0.98	1.00
2,3-Glc	1.78		0.70

[a]2,3,4,6-Gal = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, *etc.* [b]Retention time relative to that of 2,3,4,6-Gal. [c]I, methylated polysaccharide; II, methylated, carboxyl-reduced polysaccharide.

at 40° similar to that given by the deacetylated K10 polysaccharide. The ^{13}C-n.m.r. spectrum of *Ad* contained six resonances for anomeric carbons with $J_{C\text{-}1,H\text{-}1}$ values indicative of four α- and two β-linkages (Table III).

2D-N.m.r. studies of Ad. — Assignment of most of the ^{1}H resonances for the repeating unit of *Ad* were made from COSY[7] and 1- and 2-step RELAY COSY[8] experiments. The residues in the repeating unit were labelled **a**–**f** in order of decreasing chemical shift of the resonances of their anomeric protons. Following the cross-peaks, the majority of the proton resonances were readily assigned. The ^{1}H resonances for H-5 of the β residues **e** and **f** were assigned from the expected intramolecular n.O.e. contacts shown in the NOESY[9] experiment between H-1 and H-3 and H-1 and H-5 of **e** and **f**. The ^{13}C resonances for residues **a**–**f** were assigned from a heteronuclear shift-correlated experiment[10] (HETCOR). A DEPT[11] experiment of *Ad* showed that one of the methylene carbons resonated ~8 p.p.m. downfield from the others. On the basis of the results of the methylation analysis, this resonance was assigned to C-6 of the Glc residue. The H-6,6′ resonances for this residue followed from the HETCOR experiment. Both the ^{13}C and ^{1}H data indicated residues **a**–**d** to be α, and **e** and **f** to be β. Comparison of the ^{1}H- and ^{13}C-n.m.r. data for residues **a**–**f** with literature values for methyl glycosides[12–14] permitted the sugar residues in the repeating unit to be identified, as indicated in Table III, and their linkage positions to be established. In agreement with the results of methylation analysis, C-2**a**, C-6**c**, C-2**d**, C-3**d**, C-4**e**, and C-3**f** experienced significant deshielding.

The sequence of the residues **a**–**f** in the repeating unit was established by a NOESY[9] experiment. The inter- and intra-residue n.O.e. contacts observed are

TABLE III

N.M.R. DATA[a] FOR K10 DEACETYLATED PHAGE PRODUCT (*Ad*)

Atom	*Unit* **a** →2)-α-*Gal*	*Unit* **b** α-*Gal*	*Unit* **c** →6)-α-*Glc*	*Unit* **d** →2,3)-α-*Man*	*Unit* **e** →4)-β-*GlcA*	*Unit* **f** →3)-β-*Gal*
H-1	5.689 (3.5)[b]	5.275 (2.9)	5.182 (4.1)	5.127 (n.o.)	4.612 (7.9)	4.487 (7.9)
C-1	97.25 (174)[c]	101.85 (173)	97.25 (174)	95.08 (171)	103.27 (165)	104.11 (162)
H-2	3.968 (10.2)	3.816 (10.3)	3.623 (10.0)	4.253 (2.4)	3.420 (9.6)	3.646 (9.9)
C-2	73.63	69.64	72.07	79.60	73.07	70.06
H-3	3.903 (2.3)	3.854	3.782 (10.0)	4.115 (10.6)	3.776 (9.6)	3.774 (3.7)
C-3	68.39	70.39	73.74	76.84	76.42	77.25
H-4	4.052 (<1)		3.573 (10.0)	3.948	3.933 (9.6)	4.179
C-4	69.89		70.14	66.88	76.67	65.29
H-5					4.112 (10.6)	3.691
C-5					74.99	75.75
H-6			4.200			
C-6			69.31			
H-6′			3.880			

[a]Chemical shifts with acetone as internal reference (2.23 and 31.07 p.p.m. for ^{1}H and ^{13}C, respectively). [b]$^{3}J_{H,H}$ in Hz. [c]$^{1}J_{C,H}$ in Hz.

TABLE IV

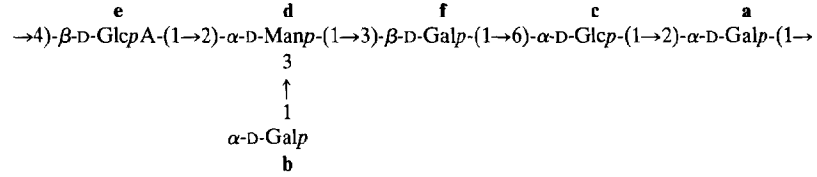
N.O.e. CONTACTS FOR FRACTION *Ad*

Anomeric proton	*N.O.e. contact to*
a	3.968 (**a**, H-2), 5.182 (**c**, H-1), 3.933 (**e**, H-4)
b	3.816 (**b**, H-2), 4.115 (**d**, H-3)
c	3.623 (**c**, H-2), 3.968 (**a**, H-2)
d	4.253 (**d**, H-2), 4.612 (**e**, H-1) 3.774 (**f**, H-3), 4.179 (**f**, H-4)
e	3.776 (**e**, H-3), 4.112 (**e**, H-5) 4.253 (**d**, H-2)
f	3.774 (**f**, H-3), 3.691 (**f**, H-5) 4.200 (**c**, H-6)

presented in Table IV. The α-residues **a–d** showed characteristic intramolecular n.O.es. to H-2 and, as mentioned earlier, the β-residues **e** and **f** showed the expected intramolecular n.O.e. contacts. Inter-residue n.O.es. of anomeric protons and the relevant protons of the adjacent residues were observed clearly. Intense cross-peaks were noted also between the anomeric protons of **a** and **c**, and also of **d** and **e**. Such uncommon n.O.es. have been observed[15–17] for other α-D-hexose residues glycosylated at position 2.

Frequently[15,18], for a 3-glycosylated Gal, the n.O.e. to H-3 is accompanied by a strong n.O.e. to H-4 and, in the absence of supporting evidence, may lead to an incorrect assignment of the linkage. Although the Man residue **d** showed n.O.e. contacts to H-3 and H-4 of the Gal residues **f**, the extent of the downfield shift noted for C-3**f** (Table III) and the methylation data for the polysaccharide (Table II) confirmed the linkage assignment.

The combined chemical and n.m.r. evidence presented permits the structure of the hexasaccharide repeating-unit of the deacetylated K10 polysaccharide to be written as **3**.



3

The location of the OAc groups was investigated by Prehm methylation[19] of fraction *B* (see above). The gas chromatogram of the derived partially methylated alditol acetates was complex and confirmed the presence of OAc groups at more than one site and in non-stoichiometric proportions. The results establish that two-thirds of the terminal Gal and ~90% of the 2-linked Gal residues are acetylated.

The former residue is acetylated at O-6, whereas, in the latter residue, some of the 3- and 6-positions are acetylated. Some di-*O*-acetylation was also detected in the latter residue. The ^{1}H-n.m.r. data accord with the results of methylation analysis and indicated 1.5 OAc groups per repeating unit. The ^{1}H-n.m.r. spectrum of fraction *A*, on the other hand, indicated the presence of only 0.9 OAc group per repeating unit and showed only 40% of the 2-linked Gal residues to be acetylated. Thus, the fraction (*B*) of lower molecular weight derived from the bacteriophage degradation of the K10 polysaccharide is more highly *O*-acetylated than the fraction (*A*) of higher molecular weight.

In a study[20] of the depolymerisation of *Klebsiella* capsular polysaccharides by bacteriophages, it was established that Gal was the reducing terminus produced during the depolymerisation of K10 polysaccharide. The detection of 0.2 mol of 2,3,4,6-tetra-*O*-methylglucose amongst the products of hydrolysis of methylated fraction *B* indicates that the bacteriophage used in this work also possessed galactosidase activity and that it is the Gal residue linked to O-6 of the Glc residue that is hydrolysed.

The structure **3** established above for the repeating unit of the deacetylated K10 polysaccharide is different from structures **1** and **2**. Structure **2** most resembles structure **3**, but incorrectly shows the 6-linked α-D-glucopyranosyl residue to be linked to O-4 of a β-D-galactopyranosyl residue rather than to O-2 of an α-D-galactopyranosyl residue. The anomeric configuration for the 4-linked β-D-galactopyranosyl residue in **2** was established by a study of the oxidation of the acetylated polysaccharide with chromium trioxide. This approach[21,22] can give incorrect results[23] and it has been suggested[24] that polysaccharides whose anomeric configurations were established by this method should be re-investigated.

EXPERIMENTAL

General methods. — Analytical g.l.c. was performed with a Hewlett–Packard 5890A gas chromatograph with flame-ionisation detection, a DB-17 bonded-phase capillary column (15 m × 0.25 mm, 0.25-μm film), and helium as the carrier gas. The following temperature programmes were used: for acetylated aldononitriles, 180° (2 min) → 240° at 5°/min; for methylated alditol acetates, 180° (1 min) → 250° at 2°/min. G.l.c.–m.s. was performed on a Varian Vista 6000 gas chromatograph coupled directly to a Delsi Nermag R10-10C quadrupole mass spectrometer, using a 30-m DB-17 capillary column and an ionisation potential of 70 eV.

Hydrolyses were carried out with 2M trifluoroacetic acid at 100° for 16 h. Acetylated aldononitrile derivatives were prepared as described[25]. Alditol acetates were prepared conventionally. Carboxyl reduction was achieved by treating the sample with refluxing methanolic 3% hydrogen chloride for 16 h and reducing the resulting methyl esters with sodium borohydride in anhydrous methanol.

Preparation of the polysaccharide. — The capsular polysaccharide was prepared from *Klebsiella* 919 (K10) which was grown on sucrose-rich nutrient

agar[26]. The acidic polysaccharide was separated from the cells by ultracentrifugation and purified by precipitation with cetyltrimethylammonium bromide (yield, 440 mg/L nutrient medium). A portion (50 mg) of the capsular polysaccharide was deacetylated by treatment with 0.1M NaOH for 4 h at 40°.

Methylation analysis. — K10 polysaccharide (H^+ form) was methylated by the modified Hakomori method[4] using potassium dimsyl[5]. The methylated alditol acetates derived from the methylated polysaccharide, with and without prior reduction of the uronic acid, were examined by g.l.c.–m.s.

Enrichment of bacteriophage. — A bacteriophage which infects *Klebsiella* K10 bacteria was isolated from Vancouver sewage. The purified phage was enriched as follows. Phage suspension (1.0 mL) and an overnight culture of *Klebsiella* K10 (1.0 mL) were added to salt-medium (SM) buffer (1 mL) containing 1% of maltose. The resulting solution was incubated at 35° for 10 min, molten 0.7% Luria Broth agar (10 mL) was added, and the mixture was poured into a petri dish (75 cm^2) containing a 1.5% agar base. After incubation (overnight at 35° and at room temperature for 24 h), the phage was harvested by scraping the 0.7% agar layer off the plate and transferring it to a centrifuge bottle containing SM buffer (20 mL), mixing vigorously, and centrifuging at 8,000 r.p.m. at 4° for 15 min. The supernatant solution, which was decanted and stored over chloroform, contained 1×10^{13} plaque-forming units per mL.

Degradation of the K10 polysaccharide with bacteriophage. — To a solution of the polysaccharide (800 mg) in water (80 mL) was added enriched phage solution (20 mL), and the mixture was shaken at 37°. After 5 days, the mixture was freeze-dried. A solution of the residue in water (20 mL) was dialysed (mol. wt. cut-off, 3500) against distilled water (6 × 50 mL). The retentate was freeze-dried and the residue was eluted with 0.1M aqueous pyridinium acetate from columns of Sephacryl S400 and Sephadex G50 to give fractions *A* ($M_r \sim 10^4$) and *B* (M_r 4–10 × 10^3). A portion (80 mg) of fraction *A* was deacetylated as described for the native polysaccharide, to afford fraction *Ad* which was used for all the 2D-n.m.r. experiments.

N.m.r. 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 p.p.m. for ^{13}C). Spectra were recorded at 40°, 80°, or 95° on a Bruker WH-400 or AM-400 spectrometer, equipped with an Aspect 3000 computer and an array processor, using standard Bruker software.

^1H-Homonuclear shift-correlated experiments (COSY[7], 1- and 2-step RELAY COSY[8]) and homonuclear dipolar-correlated (NOESY[9]) experiments were performed at 40° using a spectral width of 1040 Hz. Data matrices of 256 × 1024 data points were collected for 96 or 112 transients for each t_1 delay. The matrices were zero-filled in the t_1 dimension and transformed in the magnitude mode by use of the sine-bell window function in both dimensions. Digital resolution in the resulting 512 × 1024 matrices was 2.0 Hz per point. Relaxation delays of 1.1

to 1.5 s were used. For the 1- and 2-step RELAY COSY experiments, fixed delays of 0.036 s were used. The mixing delay in the NOESY experiment was 0.3 s.

A ^{13}C–^{1}H shift-correlated experiment[10] was recorded at 40° with proton decoupling in the F_1 domain. The initial matrix of 256 × 2048 data points was zero-filled to 512 × 2048 points, and processed with Gaussian functions and a magnitude calculation to give a final resolution of 8.4 Hz/point in the F_2 domain and 6.3 Hz/point in the F_1 domain.

Location of the OAc groups. — Fraction *B* was methylated by the Prehm method[19] and the partially methylated alditol acetates derived after reduction of the methoxycarbonyl groups were examined by g.l.c. G.l.c.–m.s. analysis of the alditol acetates identified 2,3,4,6-Me_4Glc (x), 2,3,4,6-Me_4Gal (x), 2,3,4-Me_3Glc (xxx), 3,4,6-Me_3Gal (x), 2,4,6-Me_3Gal (xxx), 2,3,4-Me_3Gal (xx), 4,6-Me_2Man (xxx), 2,3-Me_2Glc (xxx), 2,6-Me_2Gal (x), 4,6-Me_2Gal (x), 3,4-Me_2Gal (x), 2,4-Me_2Gal (x), and 3/4-MeGal (x) in the methylated polysaccharide.

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