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STRUCTURAL INVESTIGATION OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* SEROTYPE K 49

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ABSTRACT

The structure of the capsular polysaccharide from *Klebsiella* type K 49 was investigated by ^{1}H- and ^{13}C-n.m.r. spectroscopy of the original, carboxyl-reduced, and Smith-degraded polysaccharides. Methylation of the original K 49 and derivatives showed that the polysaccharide consists of a tetrasaccharide repeating-unit having D-galacturonic as a single lateral substituent. All of the sugars have the α-D-configuration. This conclusion is in agreement with measurements of spin–lattice relaxation-times for the anomeric proton. *O*-Acetyl groups are located on galacturonic acid, but do not occupy a unique position.

→3)-α-D-Gal*p*-(1→2)-α-D-Man*p*-(1→3)-α-D-Gal*p*-(1→
3
↑
1
α-D-GalAc*p*A

INTRODUCTION

The structures of the capsular antigenic polysaccharides from most of the eighty types of the Gram-negative bacterium *Klebsiella* have been elucidated, and a nearly complete account of them has been recently reported[1]. According to Nimmich's analysis[2], the capsular polysaccharide of *Klebsiella* serotype K 49 belongs to a chemotype in which K 3, K 57, and K 75 are also found. Serotype K 57 is the only one whose structure is at present known. Here we report the structure of the capsular antigen of *Klebsiella* K 49.

RESULTS AND DISCUSSION

The chemotype that comprises K 49 is characterized by the presence of only

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three types of constituent sugars, D-galactose, D-mannose, and D-galacturonic acid. According to earlier immunochemical studies by Heidelberger and Nimmich, the heavy crossreaction given by K 49 and K 63 on anti-Pneumococcus I serum[3] permitted the prediction that the sugar acid in K 49 and K 63 must belong to the D-series and be linked as lateral, nonreducing end-groups or must be attached at the 3 position. This prediction has since been verified[4] for K 63, where (1→3) linked galacturonic has been found.

The K 49 polysaccharide purified by precipitation with cetyltrimethylammonium bromide had $[\alpha]_D$ +151°. Its i.r. spectrum showed an absorption near 1735 cm^{-1}, indicative of the ester linkage of an acetate group. The presence of *O*-acetyl groups was further confirmed by the signal at 21.10 p.p.m. in the ^{13}C-n.m.r. spectrum and at ~2.10 p.p.m. in the ^1H-n.m.r. spectrum. Total acid hydrolysis indicated that the original polysaccharide contained D-mannose and D-galactose in the approximate proportion of 0.85 to 2.00 (Table I). Uronic acid estimation by the carbazole method gave 22.1% of acidic sugar. After carboxyl reduction[5], the total hydrolyzate contained mannose and galactose in the ratio 1.00 to 2.90, as estimated by gas–liquid chromatography of the alditol acetates. These figures show that K 49 is comprised of D-mannose, D-galactose, and D-galacturonic residues in the proportions 1:2:1. The ^1H-n.m.r. spectrum (Table II) of the native K 49 at 250 MHz was not well-resolved showing only three individual peaks at 5.33, 5.19, and 5.12 p.p.m. in the integrated ratio of 2:1:1. It is a generally accepted empirical rule that the signals upfield from 5.0 p.p.m. may be assigned to β linkages[6] and, as all of the signals are downfield of 5.0 p.p.m., they may be assumed to belong to α-linked sugars. None of them showed distinct splitting through coupling, and this constitutes additional evidence for the α configuration. However, it is well known that compounds having the *manno* configuration do not give rise to large coupling constants, and therefore this parameter cannot be taken as diagnostic of the anomeric form of the *manno* sugars. Another complication of the spectrum of K 49 is the presence of a signal near 4.55 p.p.m., which is at low field compared with the usual chemical shift of the ring protons, and could therefore be taken as an anomeric signal. This fifth signal in the spectral region for anomeric protons was assigned to H-5 of galacturonic acid by analogy with the study of Dutton and Folkman[7]. Additional evidence for this assignment was given by the spectrum of the Smith-degraded polysaccharide, where galacturonic acid had been eliminated and in which no signal at 4.55 p.p.m. was visible. As the peak at 5.33 p.p.m. in this spectrum now integrated for only one proton, it is evident that galacturonic acid in the native K 49 polysaccharide had its anomeric proton at 5.33 p.p.m. and was in the α configuration. Measurements of the spin–lattice relaxation-times for the anomeric proton were studied on the native K 49 polysaccharide and on the Smith-degraded derivative. Under our experimental conditions, observed values greater than 0.5 sec would agree with α configurations[8], and all of the values are of the same order of magnitude, particularly in the linear, Smith-degraded polymer. However, no conclusive evidence concerning the anomeric configurations

TABLE I

SUGAR ANALYSIS ON ORIGINAL AND MODIFIED *Klebsiella* K 49

	Molar ratios			$[\alpha]_D$ *(degrees)*
	Man	*Gal*	*GalA*	
A: Original polysaccharide	0.85	2.00	1.00	+151°
B: Carboxyl-reduced	1.00	2.90		
C: Smith-degraded B	1.00	2.1		+70°
D: Smith-degraded periodate-oxidized B	0.30	2.00		

TABLE II

N M R DATA FOR *Klebsiella* K 49 POLYSACCHARIDE AND DERIVATIVES

Compound	*^{1}H-N.m.r. data*		*T_1 values (sec)*
	δ^a	*Assignment*	
Smith-degraded K 49	5.33	α-Man	0.55
→3)-α-D-Gal-(1→2)-α-D-Man-(1→3)-α-D-Gal-(1→	5.20		
	5.20	α-Gal	0.68
Native K 49			
→3)-α-D-Gal-(1→2)-α-D-Man-(1→3)-α-D-Gal-(1→	5.33	α-Man	0.65
3	5.33	α-GalA	
↑			
1	5.19	α-Gal	0.68
α-D-GalA	5.12	α-Gal	0.64

[a]Chemical shift relative to internal acetone, δ 2.23 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (D.S.S.).

of the sugar constituents is provided by these measurements, as already shown[9] for K 24.

The complex signal of the CH_3 group of acetate centered at 2.10 p.p.m. and which integrated for ~3 protons suggested that *O*-acetyl groups occupied different positions in the polysaccharide K 49. Considering that the resonances in the anomeric region at 5.71, 5.63, and 5.36 p.p.m. disappeared upon treatment with sodium hydroxide, these signals could be asigned to hydrogen atoms attached to different acetoxylated carbon atoms of the galactosyluronic residue, in agreement with Matsuhiro *et al.*[10]. Each of these signals integrated for ~0.10 proton, and had no measurable coupling constants.

The ^{13}C-n.m.r. spectrum (Table III) of the native K 49 was well resolved, with 23 individual peaks between 174.2, C=O of the carboxyl, and 21.10 p.p.m., for methyl of the acetyl group. Four signals were clearly visible in the anomeric region, at 102.05, 101.90, 96.15, and 96.10 p.p.m. In addition, two signals in the

approximate ratio of 2 to 1 were observed at 62.40 and 62.05, respectively, corresponding to the unsubstituted primary alcohol groups of the hexoses, thus proving that neither the mannose or the galactose residues were linked through their O-6 position. The chemical-shift values observed in the anomeric region correspond to α-linked sugars, although no conclusion can be drawn for the mannosyl residue. An experiment by the gated-decoupling technique did not allow clear measurement of the $^1J_{C\text{-}1,H\text{-}1}$ couplings as the peaks were too close to each other. A better approach for the assignment of the signals was here again provided by examination of the spectrum of the Smith-degraded K 49 polysaccharide. The disappearance of the galacturonic acid residue (see below) in this modified K 49 allowed assignment of three signals remaining in the anomeric region at 102.15, 96.15, and 96.10 p.p.m., respectively, to the α-mannose and the two α-galactose residues.

Methylation analysis of the original K 49 gave only two partially methylated sugars, namely, 2,4,6-tri-*O*-methylgalactose and 4,6-di-*O*-methylmannose (Table IV) in the g.l.c. analysis of their alditol acetate derivatives. These sugars were in the relative proportions of 2.00:0.40. The analysis of the methylated, carboxyl-reduced polysaccharide showed the additional presence of 2,3,4,6-tetra-*O*-methyl-

TABLE III

^{13}C-N M R DATA FOR *Klebsiella* K 49 CAPSULAR POLYSACCHARIDE AND ITS SMITH-DEGRADED DERIVATIVE

Compound	*δ*[a] *P.p.m.*	*Assignment*
Smith-degraded K 49		
→3)-α-D-Gal-(1→2)-α-D-Man-(1→3)-α-D-Gal-(1→	102.15	α-Man
	96.15	α-Gal
	96.05	α-Gal
	80.20	C-2 Man
	75.60	C-3 Gal
	75.30	C-3 Gal
Native K 49		
→3)-α-D-Gal-(1→2)-α-D-Man-(1→3)-α-D-Gal-(1→	102.05	α-Man
3	101.90	α-Gal A
↑	96.15	α-Gal
1	96.10	α-Gal
α-D-GalA		
	80.20	C-2 Man
	79.55	C-3 Man
	75.60	C-3 Gal
	75.30	C-3 Gal

[a]Chemical shift relative to internal acetone: δ 31.07 p.p.m. downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (D.S.S.).

galactose, corresponding to galacturonic acid in a terminal, nonreducing position, thus indicating that the sugar acid was in the side chain. The presence of 4,6-di-*O*-methylmannose shows that the branching point is carried by mannose on its 2 or 3 position. The presence in low yield of the di-*O*-methylmannose derivative in the analysis of the methylated original K 49, which became quantitative in the carboxyl-reduced polysaccharide, was an indication that the mannosyl residue was part of the aldobiouronic acid and therefore that the galacturonic acid residue is directly linked on mannose as a single sugar substituent. From these results, it may be concluded that the K 49 polysaccharide is made up of tetrasaccharide repeating-units containing one terminal D-galacturonic acid residue, two D-galactose residues linked to O-3, and one branching D-mannose residue linked to O-2 and O-3, all of the sugars being pyranosidic.

When the carboxyl-reduced K 49 was submitted to periodate oxidation followed by Smith degradation, the resulting material was still polymeric and had lost the galactose coming from the galacturonic acid residue, as shown from the sugar analysis (Table I). The Smith-degraded K 49 had an optical rotation of $[\alpha]_D$ +70° which, compared with the value of +151° of the original polymer, confirms that the galacturonic acid was α-D-linked. Methylation analysis of the Smith-degraded K 49 gave only two partially methylated sugar derivatives, 2,4,6-tri-*O*-methylgalactose and 3,4,6-tri-*O*-methylmannose in the relative proportion 1.90:1.00. The fact that only tri-*O*-methyl sugar derivatives were obtained shows that the Smith-degraded polysaccharide **1** is linear and that the branching point in the original K 49 is at O-3 of the mannose residue.

$$[\rightarrow 3)\text{-}\alpha\text{-Gal-}(1\rightarrow 2)\text{-}\alpha\text{-Man-}(1\rightarrow 3)\text{-}\alpha\text{-Gal-}(1\rightarrow]_n$$

1

The ^{13}C-n.m.r. spectrum of the Smith-degraded K 49 showed 17 signals, one of them being obviously double at 62.05 p.p.m. By comparison with the spectrum of the original polymer, seven peaks had disappeared, at 174.2, 102.00, 79.55, 73.3,

TABLE IV

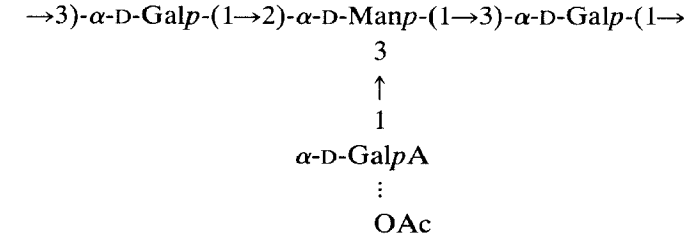
METHYLATION ANALYSIS OF ORIGINAL AND MODIFIED *Klebsiella* K 49

Methylated sugars[a]	R_T[b]	*Molar proportions, %*		
		Original	*Carboxyl-reduced*	*Smith-degraded*
2,3,4,6-Me_4-Gal	1.14		0.80	
2,4,6-Me_3-Gal	1.51	2.00	2.00	1.90
3,4,6-Me_3-Man	1.24			1.00
4,6-Me_2-Man	2.25	0.40	0.98	

[a]2,3,4,6-Me_4-Gal = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol. [b]Retention time relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, on SP 2340 at 150° isothermal.

70.7, 69.55, and 21.10 p.p.m. Five of these signals belonged to the α-linked galacturonic acid[11] and the last one to the acetyl group. The peak at 79.55, which disappeared from the spectrum of K 49 upon Smith-degradation and which does not belong to the galacturonic acid residue, must then be ascribed to C-3 of the mannose residue on which the uronic acid was branched. Removal of the substituent had the effect of shifting the C-3 upfield. After periodate oxidation of the original K 49, the ratio of uronic acid, which was 21.2%, was lowered to 7.7%. These results shows that *O*-acetyl groups do not occupy a unique position on the glycosyluronic residue, but that about one third of them must be located on O-3 of the uronic acid. To this position corresponds the signal of the hydrogen atom at 5.36 p.p.m. observed in the ^{1}H-n.m.r. spectrum of the original polysaccharide. Because of the situation of the acetyl groups on an acidic residue the method of de Belder and Norrman[12] could not be applied due to the difficulty of analysis of the products of the reaction.

From the data obtained in the present structural investigation, it may be proposed that the capsular polysaccharide from *Klebsiella* serotype K 49 is compared of tetrasaccharide repeating-units having the following structure:



O-Acetyl groups are present on this polysaccharide at least at different position the galactosyluronic residue.

Interestingly, the prediction made by Heidelberger and Nimmich on the basis of the cross reaction with anti-Pneumococcus I is confirmed here. As both K 49 and K 63 exhibit the same behavior with the antiserum, it may be suggested that, if the immunodominant sugar is the α-linked galacturonic acid, its position as a lateral substituent as in K 49 or within the main chain as in K 63 is not the factor which affects the precipitation reaction.

EXPERIMENTAL

General methods. — Gas–liquid chromatographic separations were performed on glass columns (180 × 0.15 cm) containing (A) 3% of SP-2340 on Supelcoport (100–120 mesh) at 185°; or (B) 3% of OV-225 on Chromosorb W.A.W. DMCS (100–120 mesh), using a Packard–Becker chromatograph model 417 equipped with a Hewlett–Packard 3370 B digital integrator.

Gel–filtration chromatography was conducted on a column (100 × 3 cm) of

Biogel P-2 (100–120 mesh) irrigated with the system water–pyridine–acetic acid (1000:10:4).

For g.l.c.–m.s. of partially methylated alditol acetates, an OV-225 and a SP 2340 S.C.O.T. columns (2.5, 0.25 mm from Chrompack) were used at 180° programmed at 2°/min to hold at 220°. The chromatograph was a Girdel model 3000 connected to an A.E.I. MS 30 mass spectrometer. The temperature of the source was 150° and the spectra were recorded at 70 eV with an ionization current of 100 μA.

Isolation of polysaccharide, and sugar analysis. — The capsular polysaccharide was collected and purified as described previously[13]. The purified polysaccharide showed $[\alpha]_D$ +151° (*c* 0.33, water). In the i.r. spectrum (KBr), an absorption at 1735 cm^{-1} was observed for the *O*-acyl groups. The ^{1}H-n.m.r. spectrum showed an absorption at 2.18 p.p.m., and in the ^{13}C-n.m.r. spectrum an absorption at 21.10 p.p.m. was observed, both characteristic of *O*-acetyl groups.

The sugar analysis was performed either with 72% sulfuric acid initially at room temperature and then diluted to 0.5M and for 6 h at 100°, or with M trifluoroacetic acid at 100° for 0.5–3 h. Hydrolyzates were analyzed by g.l.c. of the alditol acetate derivatives of the monosaccharides released. Uronic acids were estimated by the carbazole method and indicated a uronic acid content of 22.1% (anhydro-uronic acid).

Carboxyl reduction of the native polysaccharide. — This was performed according to Taylor and Conrad[5]. The treatment was repeated twice to achieve complete reduction. Hydrolysis of the reduced sample, and g.l.c. analysis of the sugars, showed that the reduction was nearly quantitative (Table I) and that the uronic acid consisted of galacturonic acid.

Periodate oxidation and Smith degradation. — To a solution of the carboxyl-reduced capsular polysaccharide K 49 (50 mg) in water (20 mL) was added a solution of 0.1M sodium periodate (20 mL). The solution was kept in the dark at 5° and the reaction was monitored at 223 nm. The reaction was stopped after 92 h by decomposition of the excess of periodate ions by ethylene glycol. Sodium borohydride (0.5 g) was added to the solution and the reduction allowed to proceed overnight. The excess of borohydride was decomposed with 50% acetic acid and the mixture was dialyzed. The oxidized polysaccharide was hydrolyzed overnight at room temperature with 0.5M trifluoroacetic acid. The partially hydrolyzed material was dialyzed against distilled water and the product was recovered by freeze-drying. Sugar analysis indicated that one galactose residue had been lost during this procedure. This was confirmed by methylation analysis and by examination of the ^{1}H- and the ^{13}C-n.m.r. spectra. A second periodate-oxidation experiment was performed on the Smith-degraded polysaccharide under the same condition as before and the re-oxidized material was totally hydrolyzed with 2M trifluoroacetic acid. Sugar analysis showed that a major proportion of the mannose residues had been decomposed by the second oxidation step, thus indicating that the lateral galacturonic acid was substituted at O-3 of the mannosyl residue.

When the Smith degradation was performed on the original capsular polysaccharide K 49, under the same conditions as before, the resulting oxidized material showed a decrease of the uronic acid from 22.1 to 7.5%, corresponding to the decomposition of most of the galacturonic acid side-groups.

Methylation analysis. — Methylations of polysaccharide K 49 and carboxyl-reduced K 49 were performed according to the conditions of Hakomori, followed by two consecutive treatments according to Purdie[14]. The dried material (5 mg) was dissolved in freshly distilled anhydrous dimethyl sulfoxide under ultrasonic agitation. Dimethylsulfinyl anion (2 mL) was added and the mixture was stirred under nitrogen for 4 h. Methyl iodide (4 mL) was then added and the mixture stirred for 1.5 h. Water was then added, the excess of methyl iodide was evaporated off, and the mixture was dialyzed. The product was then recovered by freeze drying. The methylated polysaccharides were hydrolyzed in 90% formic acid for 1 h and then with 2M trifluoroacetic acid for 3 h. The partially methylated alditol acetates were analyzed by g.l.c. (Table IV) then their identification confirmed by g.l.c.–m.s.

N.m.r. spectroscopy. — The ^{1}H- and ^{13}C-n.m.r. spectra were recorded with a CAMECA 250 spectrometer, with D_2O as solvent. The spectra wee recorded at 90° and chemical shifts are expressed with reference to acetone. The spin–lattice relaxation-times (T_1 values) were measured according to Freeman *et al.*[15] with the sequence $(-T-180-C-90°\text{ acquisition})_n$.

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