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# Note

## Structure of the O-specific polysaccharide chain of *Klebsiella pneumoniae* O1K2 (NCTC 5055) lipopolysaccharide. A complementary elucidation *

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The lipopolysaccharide (LPS) of *Klebsiella pneumoniae* O1K2 was isolated from dry bacteria. Mild acid hydrolysis (1% acetic acid, 90 min, 100°) of the LPS, followed by fractionation of the water soluble material by HPLC with an anion-exchange column gave six fractions named S1–S6[1]. The neutral S1 Fraction contained only galactose and was identified as the O-specific polysaccharide chain of the LPS. The structure of this fraction was reported in a previous study[2]. As demonstrated previously, the O-polysaccharide was found to be composed of two polymers having the disaccharide-repeating units **1** and **2** clustered in the same polysaccharide chain.

$$\underset{\mathbf{1}}{\left[\rightarrow 3)\text{-}\alpha\text{-D-Gal}p^{d}\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p^{b}\text{-}(1\rightarrow\right]_{n}}$$

$$\underset{\mathbf{2}}{\left[\rightarrow 3)\text{-}\alpha\text{-D-Gal}p^{c}\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}f^{a}\text{-}(1\rightarrow\right]_{m}}$$

Recently, Whitfield et al.[3] demonstrated that the O-specific polysaccharide chains of *Klebsiella pneumoniae* serotype O1 contained a mixture of two structurally distinct D-galactan polymers. The repeating unit structures of these two polymers, named by these authors D-galactan I and D-galactan II, correspond to structures **2** and **1**, respectively. These authors interpreted their results in terms of two separate galactan chains, but several results concerning their high-molecular-

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* Dedicated to Professor Jean Montreuil.

weight LPS remain unclear and could be clarified by a structure having the two D-galactan polymers clustered in the same polysaccharide chain as we have suggested: (i) No NOE connectivities between the two D-galactan polymers were detected, which led Whitfield et al.[3] to conclude that the two polymers were separate. However, this result may also be explained by the fact that the NOE connectivities between the two polymers are insignificant with regard to the whole polysaccharide chain, and not detectable. (ii) The mutant that synthesized only D-galactan I (**2**) produced a low-molecular-weight LPS. The reason for the reduced chain length of the O-specific polysaccharide of LPS could be explained by the lack of substitution of D-galactan I (**2**) by D-galactan II (**1**). The mutation could affect the *rfc* region of the gene which encode the polymerase responsible for the linking of D-galactan II to D-galactan I, giving rise to a low-molecular-weight LPS, while D-galactan II was detected only in high-molecular-weight LPS. The LPS used in our study correspond to the high-molecular-weight LPS described by Whitfield et al.[3]. (iii) Monoclonal antibodies (MAb) specific for the two galactan polymers were used by these authors. The MAb specific for D-galactan II reacted only with high-molecular-weight LPS, whereas MAb specific for D-galactan I reacted with low-molecular-weight as well as with high-molecular-weight LPS. This result could be explained by the fact that both D-galactan I and II are clustered in the same polysaccharide chain of high-molecular-weight LPS. D-Galactan II had an external position and D-galactan I was linked to the core in the LPS molecule.

In order to resolve the contradiction between our results and those reported by Whitfield et al.[3] and to demonstrate that the two galactan polymers occur in the same polysaccharide chain, the O-polysaccharide fraction was submitted to a periodate oxidation[4], followed by an hypoiodite oxidation[5]. In this experiment, only the β-D-galactofuranose residues of **2** are transformed into β-L-arabinuronic acid residues, thus providing negative charges to the molecule, whereas **1** remains unchanged. The oxidized polysaccharide was fractionated by HPLC with an anion-exchange column. As expected, > 95% of the materials were retained by the column (Fig. 1), and sugar analysis showed the presence of arabinuronic acid and galactopyranose residues, as determined by GLC–MS and $^{13}C$ NMR spectroscopy (Fig. 2, Table I). This result clearly showed that structures **1** and **2** are located in the same polysaccharide chain. However, the cluster that is linked to the core in the LPS molecular was not identified. This point has now been elucidated by a study of the $^{13}C$ NMR spectra of the other fractions obtained by mild acid hydrolysis of LPS and fractionated by HPLC (Fractions S2–S6) as described previously[1]. As reported earlier[1], all fractions contained variable contents of galactose for the same ratio of heptose to glucose. The variation of galactose content can be explained by the heterogeneity of the length of the O-polysaccharide chains linked to the core that were not cleaved by the acid hydrolysis. The S4 Fraction was further fractionated on a Bio-Gel P-4 column. Two fractions included in the Bio-Gel P-4, named S4-$P4_1$ (minor compound) and S4-$P4_2$ (major compound), were obtained. The same fractionation procedure applied to S2 and

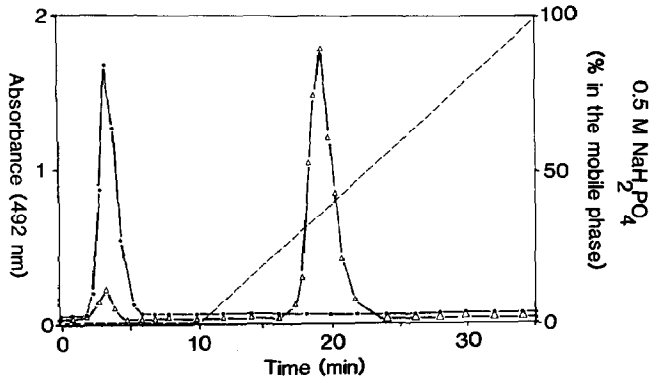


Fig. 1. Anion-exchange HPLC of native O-polysaccharide (●———●) and oxidized O-polysaccharide (△——— △); mobile phase (— — —).

S3 Fractions gave a single peak, eluted at the void volume of the column, indicating that they have a higher molecular weight than Fraction S4. As shown in Table II, the S4-$P4_2$ Fraction contained mainly glucose and heptose in the molar ratio 1:1, while galactose was absent; this fraction could be identified as the core region of the LPS. Sugar analysis of the other fractions (S2, S3, and S4-$P4_1$) showed that, together with the core constituents (glucose and heptose), they also contained galactose. As shown in Fig. 3, comparison of the $^{13}C$ NMR spectra indicated that S4-$P4_2$ Fraction shows signals for the core region (Fig. 3A), and S3 Fraction (Fig. 3B) shows the same signals as the S4-$P4_2$ Fraction, plus signals of

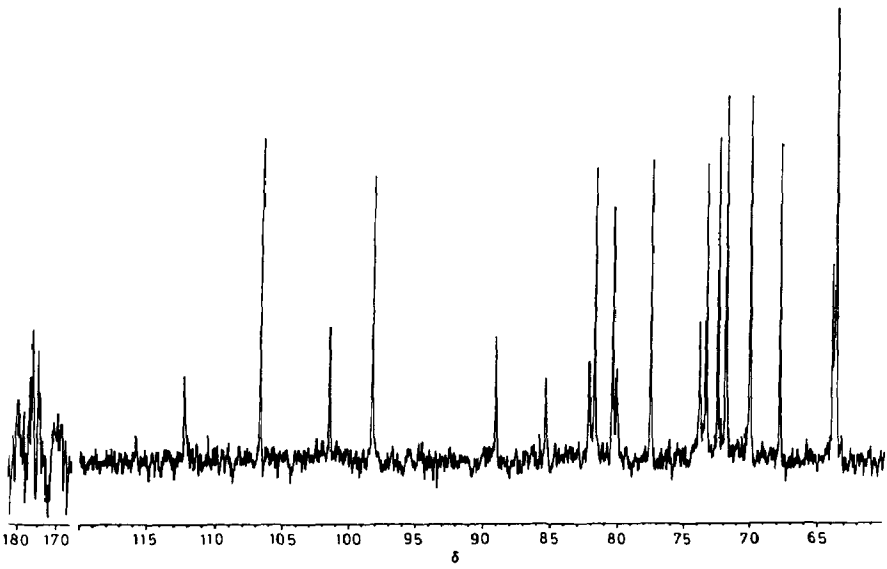


Fig. 2. $^{13}C$ NMR spectrum of oxidized O-polysaccharide.

TABLE I

$^{13}C$ NMR chemical shifts for metaperiodate- and hypoiodate-oxidized, and native [a] O-polysaccharide of *Klebsiella pneumoniae* O1K2 LPS

| Unit | Structure | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 |
|---|---|---|---|---|---|---|---|
| (*a'*) | → 3)-β-L-Ara*f*A-(1 → | 112.28 | 82.13 | 89.05 | 85.33 | 179.30 | |
| (*a*) | → 3)-β-D-Gal*f*-(1 → | (111.80) | (82.33) | (87.08) | (84.84) | (73.63) | (65.53) |
| (*b*) | → 3)-α-D-Gal*p*-(1 → | 98.22 | 70.01 | 81.70 | 71.79 | 73.31 | 63.67 |
| | | (98.27) | (70.04) | (81.79) | (71.84) | (73.34) | (63.74) |
| (*c*) | → 3)-α-D-Gal*p*-(1 → | 101.45 | 70.01 | 80.06 | 71.79 | 73.79 | 63.92 |
| | | (102.50) | (69.93) | (79.68) | (71.92) | (73.87) | (63.88) |
| (*d*) | → 3)-β-D-Gal*p*-(1 → | 106.65 | 72.42 | 80.32 | 67.80 | 77.45 | 63.67 |
| | | (106.71) | (72.46) | (80.39) | (67.84) | (77.48) | (63.70) |

[a] In parentheses.

α-D-Gal*p* (*c*) and β-Gal*f* (*a*) units contained in structure **2**. The spectrum of Fraction S2 (Fig. 3C) showed the same signals as Fraction S3, plus signals of α-D-Gal*p* (*d*) and β-D-Gal*p* (*b*) units of structure **1**, indicating that the proximal oligosaccharide linked to the core region possess structure **2**, while structure **1** can be observed only in longer O-polysaccharide chains.

These results confirm that structures **1** and **2** are clustered in the O-polysaccharide chain of the LPS and indicate that structure **1** is linked to the core region of the LPS via structure **2** as shown in **3**.

$$[\rightarrow 3)\text{-}\alpha\text{-D-Gal}p^{d}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p^{b}\text{-}(1\rightarrow]_{n}\text{-}[\rightarrow 3)\text{-}\alpha\text{-D-Gal}p^{c}\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}f^{a}\text{-}(1\rightarrow]_{m}\text{-Core}$$

**3**

## EXPERIMENTAL

*NMR spectroscopy.*—The 400-MHz $^{1}H$ NMR experiments were performed at 333 K on a Bruker AM-400 WB spectrometer, equipped with a 5-mm, $^{1}H$–$^{13}C$ mixed probe-head, operating in the pulsed FT mode and controlled by an Aspect 3000 computer. After one exchange with $^{2}H_2O$ (99.96 atom% of $^{2}H$, Aldrich, Milwaukee, IL, USA) and intermediate lyophilization, the products were analyzed

TABLE II

Comparative sugar compositions of Fractions S2, S3, S4-P4$_1$, and S4-P4$_2$

| Fraction | Gal | Glc | Hep [a] |
|---|---|---|---|
| S2 | 14.1 | 1 | 0.67 |
| S3 | 5.28 | 1 | 1.00 |
| S4-P4$_1$ | 3.7 | 1 | 1.06 |
| S4-P4$_2$ | 0.06 | 1 | 1.04 |

[a] Hep: heptose.

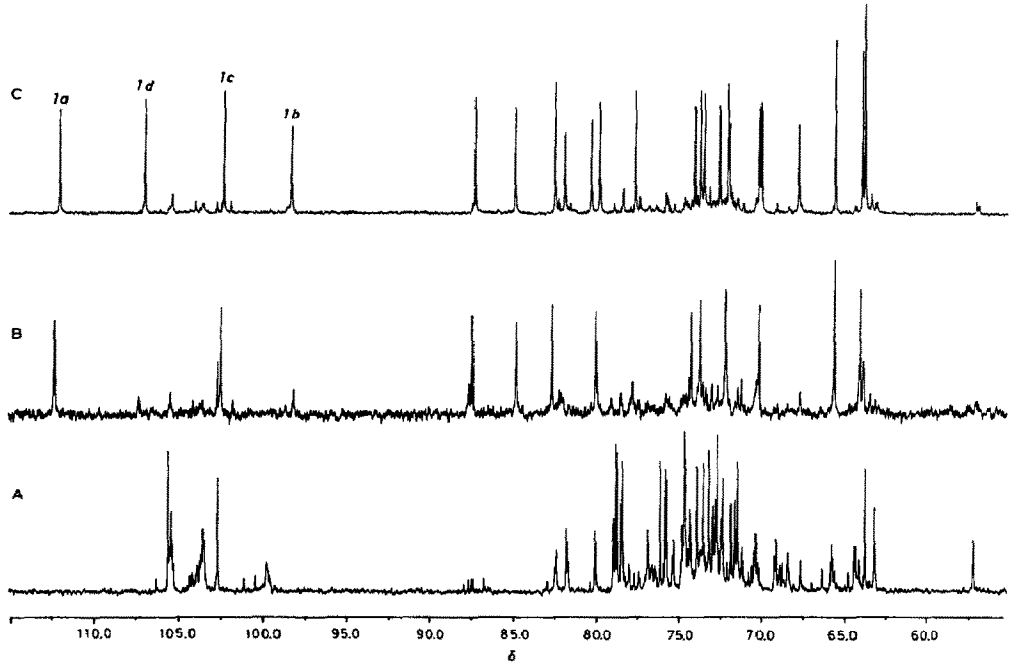


Fig. 3. $^{13}C$ NMR spectra of anomeric carbon atoms of S4-$P4_2$ (A), S3 (B), and S2 (C). See structure **3** for the location of residues *a*, *b*, *c*, and *d*.

with a spectral width of 3000 Hz for 16 K frequency domain points and time domain data, giving a final digital resolution of 0.365 Hz/point. The 90° pulse for $^1H$ was 7.1 μs. The 100-MHz $^{13}C$ NMR experiments were carried out with the standard Bruker pulse-program POWGATE with $^1H$-broad-band, composite-pulse decoupling. A 90° pulse (4.8 μs) and a 1-s recycle delay were used. The chemical shifts are given relative to 4,4-dimethyl-4-sila-(2,3-$^2H_4$)pentanoate.

*Bacterial culture and isolation of lipopolysaccharide.*—The bacterial culture of *Klebsiella pneumoniae* O1K2 (NCTC 5055) was carried out as previously described[6]. The lipopolysaccharide (LPS) was extracted by the hot phenol–water procedure[7] and purified by ultracentrifugation.

*Isolation of the O-specific polysaccharide.*—The LPS in aq 1% acetic acid was heated at 100° for 90 min. The precipitated lipid A was removed by centrifugation. The water-soluble material was fractionated by HPLC, using an anion-exchange column, into six fractions named S1–S6[1]. The O-specific polysaccharide was located in the neutral S1 Fraction.

*Oxidations.*—Periodate oxidation was performed in the dark at 4° for 4 days as described by Goldstein et al.[4] and hypoiodite oxidation as described by Schaffer and Isbell[5].

*HPLC.*—HPLC fractionation of oxidized polysaccharide was performed as previously described[1], except that the water–0.5 M $NaH_2PO_4$ gradient was as

follows: the column was washed with distilled water for 10 min, and then a linear gradient to 100% of 0.5 M $NaH_2PO_4$ was applied for 25 min.

*Determination of monosaccharide composition.*—The total neutral carbohydrate content was determined by the orcinol–$H_2SO_4$ method[8]. The molar composition of monosaccharides was determined by GLC of the per-*O*-trifluoroacetylglycosides according to Zanetta et al.[9] or of the per-*O*-trimethylsilylglycosides according to Kamerling et al.[10], as modified by Montreuil et al.[11].

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