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Note

The structure of the lipopolysaccharide from *Klebsiella oxytoca* rough mutant R29 (O1$^-$/K29$^-$)

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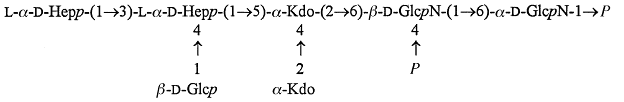
Abstract

The lipopolysaccharide from *Klebsiella oxytoca* rough mutant R29 (O1$^-$/K29$^-$) has been isolated and its complete structure has been elucidated by compositional analyses, NMR spectroscopy, and laser-desorption mass spectrometry. The carbohydrate backbone has the structure



$$\text{L-}\alpha\text{-D-Hep}p\text{-}(1\to3)\text{-}\underset{\begin{array}{c}4\\\uparrow\\1\\\beta\text{-D-Glc}p\end{array}}{\text{L-}\alpha\text{-D-Hep}p}\text{-}(1\to5)\text{-}\underset{\begin{array}{c}4\\\uparrow\\2\\\alpha\text{-Kdo}\end{array}}{\alpha\text{-Kdo}}\text{-}(2\to6)\text{-}\underset{\begin{array}{c}4\\\uparrow\\P\end{array}}{\beta\text{-D-Glc}p\text{N}}\text{-}(1\to6)\text{-}\alpha\text{-D-Glc}p\text{N-}1\to P$$

of which the GlcN residues (the lipid A backbone) are acylated by 14:(3-OH) (amide-linked) and 12:0, 14:0(3-OH)(ester-linked) fatty acids.

Keywords: Klebsiella oxytoca; Lipopolysaccharide; Core region; Structural analysis; NMR

In the genus *Klebsiella*, there are two species that frequently cause nosocomial infections which often lead to septicemia and mortality, i.e. *K. pneumoniae* and *K. oxytoca* [1]. Lipopolysaccharides (LPS) and capsules have been identified as virulence determinants in *Klebsiella* species [1,2]. The majority of O-antigenic polysaccharides have been structurally characterised [1,3], however, less is known about structural features of the core and lipid A regions of LPS. Recently, we reported the complete structure of the LPS of *K. pneumoniae* ssp. *pneumoniae* rough mutant R20 (O1$^-$/K20$^-$) [4], and the structures of the carbohydrate backbones of LPS from two rough mutants of *K. pneumoniae* ssp. *ozaenae* (O8$^-$) were published by Severn et al. [5]. We now report the complete structure of the LPS of *K. oxytoca* rough mutant R29 (O1$^-$/K29$^-$) as depicted in Fig. 1.

Compositional analyses of the LPS identified D-Glc, L-*glycero*-D-*manno*-heptose (L,D-Hep), D-GlcN, 3-deoxy-D-*manno*-oct-2-ulopyranosonic acid (Kdo), phosphate, 3-hydroxytetradecanoic acid (14:0(3-OH)), tetradecanoic acid (14:0), and dode-

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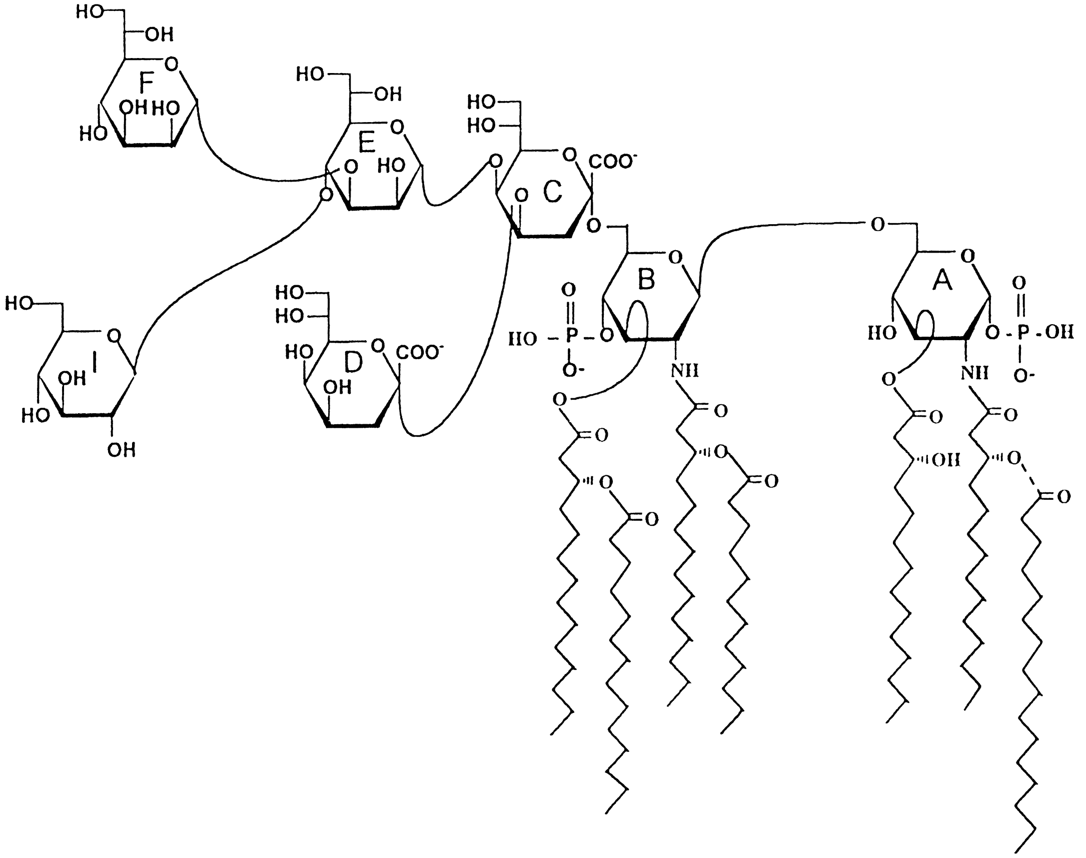


Fig. 1. The structure of the LPS from *K. oxytoca* rough mutant R29 (O1⁻/K29⁻).

canoic acid (12:0) in the molar ratio of ~1:2:2:2:2:4:1:0.5, and traces of hexadecanoic acid (16:0). The absolute configuration of 14:0(3-OH) is *R*. For the structural analysis of the carbohydrate backbone, the LPS (140 mg) was deacylated and separated using high-performance anion-exchange chromatography which yielded a homogeneous fraction that was desalted by gel-permeation chromatography on Sephadex G-50 in water and lyophilised (39.2 mg, 28% of the LPS). Its structure **1** was elucidated by NMR spectroscopy on a solution in D_2O (pD 9.5; ^1H NMR, 500 MHz; ^{13}C NMR, 90.6 MHz; ^{31}P, 145 MHz). The assigned chemical shifts, and the NOE contacts as revealed by a NOESY spectrum, are summarised in Tables 1–3. The anomeric region of the ^1H NMR spectrum contained five signals, two of which (residues **E**,**F**, compare **1**) could be assigned to heptose residues which possess the *manno*-configuration as revealed by their $J_{H-1,H-2}$ coupling constants ($\leq$ 2 Hz). Three anomeric signals (residues **A**, **B**, **I**) were assigned to the *gluco*-configurated hexoses, two of which are β- ($J_{H-1,H-2}$ 7.5–8.5 Hz, residues **B**, **I**) and one is α-linked ($J_{H-1,H-2}$ 3.5 Hz, residue **A**). The signal at 5.385 ppm was assigned to H-1 of **A**. It appeared as double doublet indicating the substitution of O-1 by a phosphate residue [6]. In the region 1.70–2.15 ppm, the characteristic high-field signals of H-3ax and H-3eq of two α-linked Kdo residues (**C** and **D**) were present.

The ^{13}C NMR spectrum (Table 2) contained seven signals in the anomeric region, representing two C-2 atoms of the Kdo residues **C** and **D** (at 101.5 and 99.6 ppm, determined by a DEPT experiment), and five C-1 atoms of two heptose and three hexose residues. Other signals established the presence of one α- and one β-linked GlcN (**A** and **B**, characteristic resonances of C-2 at 54.4 and 55.6 ppm, respectively) and two Kdo residues (characteristic high-field signals of C-3 at 34.6 (residue **C**) and 34.8 ppm (residue **D**) and carboxyl resonances at 174.7 and 174.6 ppm). Low field shifted signals indicated the substitution at O-6 of **A** (C-6 at 69.8 ppm) and **B** (C-6 at 62.5 ppm), at O-4 and O-5 of **C** (68.6 and 68.7 ppm, respectively), and at O-3 and O-4 of **E** (74.1 and 73.2 ppm, respectively). **D**, **F**, and **I** are terminal residues.

The sequence of oligosaccharide **1** was established by analysis of the interresidual NOE

Table 1
^{1}H NMR data [a] for **1**

Unit	Chemical shifts (δ)							
	H-1	H-2	H3-ax H3-eq	H-4	H-5	H-6a H-6b	H-7a H-7b	H-8a H-8b
A	5.385 [b]	3.147	3.641	3.341	3.882	3.502 4.038		
B	4.597	2.804	3.581	3.52[c]	3.502	3.191 3.422		
C			1.725 1.903	3.824	4.006	3.460	3.568	3.54 [c] 3.76 [c]
D			1.532 1.852	3.820	3.78 [c]	3.401	3.98 [c]	3.41 [c] 3.71 [c]
E	4.792	3.852	3.853	3.996	4.014	3.830	3.392 3.485	
F	5.016	3.906	3.598	3.388	3.595	3.790	3.537 3.788	
I	4.271	3.034	3.232	3.045	3.220	3.440 3.700		

[a] The spectrum was measured at 500 MHz at 283 K in D_2O relative to acetone (δ 2.225). Assignments were made by a phase-sensitive double quantum-filtered (DQF) COSY experiment. Monosaccharide units **A**–**I** are as shown in the formula.
[b] $J_{H\text{-}1,P}$ 7.6 Hz.
[c] Not resolved.

Table 2
^{13}C NMR data[a] for **1**

Unit	Chemical shifts (δ)							
	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
A	90.8	54.4	69.6	69.7	72.4	69.8		
B	99.6	55.6	72.2	73.8	74.0	62.5		
C	174.7 [b]	101.5 [c]	34.6	68.6	68.7	70.4	66.1	64.2
D	174.6 [b]	99.6 [c]	34.8	66.5	66.5	71.6	71.8	63.1
E	101.8	70.2	74.1	73.2	69.0	68.6	63.2	
F	98.7	69.9	69.3	72.4	70.7	68.8	63.8	
I	102.3	73.6	76.5	70.3	75.4	61.6		

[a] The spectrum was measured at 90 MHz in D_2O relative to dioxane (δ 67.40). Monosaccharides **A**–**I** are as shown in the formula.
[b,c] Interchangeable.

Table 3
NOE effects of oligosaccharide **1**, observed in the NOESY spectrum [a]. Shown are signals that were important for the structural determination

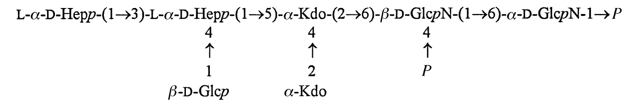
Unit	NOE signal [b]		
	From	Intraunit	Interunit
A	**A**1	**A**2	
B	**B**1	**B**3 (m), **B**5 (s)	**A**6a (w)
C	**C**3ax	**C**4 (w), **C**3eq (s)	**E**5 (w), **D**6 (s)
D	**D**3eq	**D**4 (w), **D**3ax (s)	**E**5 (w)
E	**E**1	**E**2 (m)	**C**5 (m), **C**7 (s)
	E2	**E**3 (m)	**C**5 (w), **C**7 (s)
F	**F**1	**F**2 (m)	**E**2 (w), **E**3 (w), **I**2 (w)
I	**I**1	**I**3 (m), **I**5 (s)	**E**4 (s)

[a] Spectra were recorded at 500 MHz and 283 K, the mixing time was 200 ms. Monosaccharide units **A**–**I** are as shown in the formula.
[b] w, Weak; m, medium; s, strong.

contacts (Table 3). The following connectivities were observed: between H-1 of heptose **F** and H-3 of heptose **E**, between H-1 of residue **E** and H-5 of Kdo **C**, between H-1 of *β*-Glc **I** and H-4 of heptose **E**, between H-3ax of residue **C** and H-6 of Kdo **D** {confirming the element *α*-Kdo-(2→4)-*α*-Kdo **D**–**C** [7]}, between H-3ax of residue **C** and H-5 of residue **E** {proving the *α*-(1→5) linkage between Hep **E** and Kdo **C** [8]}, and between H-1 of GlcN **B** and H-6 of GlcN **A**. Consequently, although not indicated by NOE contacts, **C** must be linked to **B**.

The ^{31}P NMR spectrum possessed two signals at 2.85 and 4.30 ppm, which were assigned by a ^{31}P,^{1}H-COSY NMR experiment to the phosphate residues at O-1 of GlcN **A** and O-4 of GlcN **B**, respectively [9].

In summary, our data establish **1** as carbohydrate backbone of the LPS of *K. oxytoca* rough mutant R29.



$$\begin{array}{ccccl}
\text{L-}\alpha\text{-D-Hep}p\text{-}(1\to3)\text{-} & \text{L-}\alpha\text{-D-Hep}p\text{-}(1\to5)\text{-} & \alpha\text{-Kdo-}(2\to6)\text{-} & \beta\text{-D-Glc}p\text{N-}(1\to6)\text{-} & \alpha\text{-D-Glc}p\text{N-1}\to P \\
4 & 4 & & 4 & \\
\uparrow & \uparrow & & \uparrow & \\
1 & 2 & & P & \\
\beta\text{-D-Glc}p & \alpha\text{-Kdo} & & &
\end{array}$$

The nature and distribution of fatty acids in the lipid A were determined by chemical analysis of LPS and LD–MS of dephosphorylated lipid A. After de-*O*-acylation of LPS with 0.25 M methanolic sodium methoxide followed by esterification of the released fatty acids with diazomethane, quantification revealed that 14:0, 12:0, and about half of the amount of 14:0(3-OH) (determined as 14:0 (3-OMe)) had been released, indicating their ester-linkage [10]. The investigation of the fatty acid content before and after esterification with diazomethane identified 14:0[3-*O*-(14:0)] as sole ester-linked acyloxyacyl structure. Fatty acid analysis of de-*O*-acylated LPS identified 14:0(3-OH) as sole amide-linked fatty acid. Thus, 12:0 and 16:0 were ester-linked to amide-bound 14:0(3-OH).

As proven by the structure of **1**, the carbohydrate backbone of the lipid A consists of β-D-Glc*p*N-(1→6)-α-D-Glc*p*N 1,4′-bisphosphate which is substituted at O-6′ by the core oligosaccharide. Thus, positions O-3, O-4, and O-3′ may be acylated. For the analysis of the fatty acid distribution, the LPS was dephosphorylated, then hydrolysed in 1% acetic acid, and the dephosphorylated lipid A was isolated by extraction with CH_2Cl_2, then dried, and, after addition of NaI, subjected to laser-desorption mass spectrometry (Fig. 2). Three pseudomolecular ions were identified, i.e. at *m/z* 1899, 1661, and 1435, representing the masses of hepta-, hexa-, and pentaacyl lipid A species, respectively. The major species, the hexaacyl lipid A contains four 14:0(3-OH), one 14:0 and one 12:0, and the heptaacyl component contains an additional 16:0. The pentaacyl lipid A possesses one 14:0(3-OH) residue less, probably that linked to O-3 of the reducing GlcN **A**. The ions at *m/z* 1417 (1661-244), 1207 (1435-228), and 862 (1090-228) originate from cleavage of either one 14:0(3-OH) or 14:0 residue. The ions marked **E** are of diagnostic value and are generated by cleavages between C-4/5 and C-5/ring oxygen of the reducing GlcN during the laser desorption process [11]. The resulting ions at *m/z* 1090 and *m/z* 862 represent the molecular masses of non-reducing GlcN residues, acylated either with four or with three fatty acids (two 14:0(3-OH), a 12:0, and one or no 14:0, respectively). Since the 14:0 residue was identified as part of the ester-bound acyloxyacyl structure, the 12:0 must consequently be ester-linked to the amide-bound 14:0(3-OH) in GlcN **B**. Therefore, 16:0 substitutes the amide-linked 14:0(3-OH) of GlcN **A**.

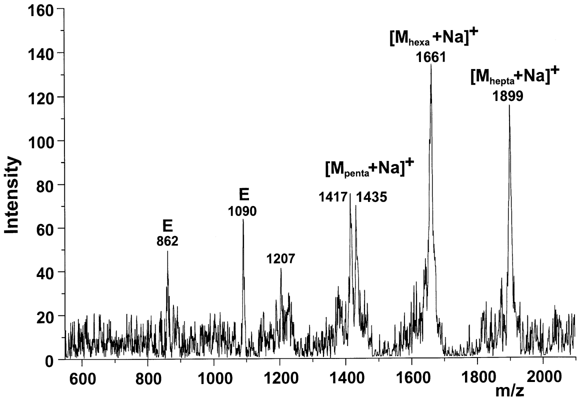


Fig. 2. Positive ion laser-desorption mass spectrum of dephosphorylated lipid A with addition of NaI. E are diagnostic ions for the identification of fatty acid distribution as explained in the text.

In conclusion, we have characterised the complete structure of LPS from *K. oxytoca* rough mutant R29 as shown in Fig. 1. The structure of its carbohydrate backbone represents a partial structure of the LPS of *K. pneumoniae* ssp. *pneumoniae* rough mutant R20 [4] and *K. pneumoniae* ssp. *ozaenae* RFK-11 [5], and is identical to that of *K. pneumoniae* ssp. *ozaenae* RFK-9 [5]. As in LPS of *K. pneumoniae* rough mutant R20, *K. pneumoniae* RFK11 [5] and *K. pneumoniae* LEN 1 [12], the lipid A backbone consists of β-D-Glc*p*N-(1→6)-α-D-Glc*p*N 1,4′-bisphosphate which represents the structural principle of lipid A of all enterobacterial species and many other LPSs [13]. Its acylation pattern differs from that of lipid A from LPS of *K. pneumoniae* rough mutant R20 [4] by amide-linked 14:0[3-*O*-(12:0)] at GlcN **B** instead of 14:0[3-*O*-(14:0)]. Heterogeneity in the secondary fatty acid pattern of acyloxyacyl structures has been observed in lipid A from various bacteria [14], but this is to our knowledge the first description of a stoichiometric replacement of a secondary fatty acid within the lipid A of a bacterial genus.

1. Experimental

The following were performed as published: cultivation of *K. oxytoca* strain R29 ($K29^-/O1^-$) and extraction of the LPS (yield: 3.4% of bacterial dry mass) [4], compositional analyses of LPS [15], determination of the absolute configuration of 14:0(3-OH) [16], analyses of ester- and amide-linked fatty acids [10], deacylation of LPS [17,18], NMR spectroscopy [4] with modifications as described in the Tables, dephosphorylation of LPS [4], hydrolysis of LPS with 1% acetic acid [4], laser-desorption mass spectrometry [4], and de-*O*-acylation of LPS with 0.25 M methanolic sodium methoxide followed by esterification of the released fatty acids with diazomethane [10].

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References

[1] P. Williams and J.M. Tomàs, *Rev. Med. Microbiol.*, 1 (1990) 196–204.

[2] J.Z. Montgomerie, *Rev. Infect. Dis.*, 1 (1979) 736–753.

[3] Y.A. Knirel and N.K. Kochetkov, *Biochemistry (Moscow)*, 59 (1994) 1325–1346.

[4] M. Süsskind, L. Brade, H. Brade, and O. Holst, *J. Biol. Chem.*, 273 (1998) 7006–7017.

[5] W.B. Severn, R.F. Kelly, J.C. Richards, and C. Whitfield, *J. Bacteriol.*, 178 (1996) 1731–1741.

[6] M. Süsskind, S. Müller-Loennies, W. Nimmich, H. Brade, and O. Holst, *Carbohydr. Res.*, 269 (1995) C1–C7.

[7] K. Bock, J.U. Thomsen, P. Kosma, R. Christian, O. Holst, and H. Brade, *Carbohydr. Res*, 229 (1992) 213–224.

[8] S. Müller-Loennies, O. Holst, and H. Brade, *Eur. J. Biochem.*, 224 (1994) 751–760.

[9] O. Holst, W. Broer, J.E. Thomas-Oates, U. Mamat, and H. Brade, *Eur. J. Biochem.*, 214 (1993) 703–710.

[10] H.-W. Wollenweber and E.Th. Rietschel, *J. Microbiol. Methods*, 11, (1990) 195–211.

[11] B. Lindner, U. Zähringer, E.Th. Rietschel, and U. Seydel, in A. Fox, S.L. Morgan, L. Larsson, and G. Odham (Eds.), *Analytical Microbiology Methods*, Plenum Press, New York, 1990, pp 149–161.

[12] I.M. Helander, Y. Kato, I. Kilpeläinen, R. Kostiainen, B. Lindner, K. Nummila, T. Sugiyama, and T. Yokochi, *Eur. J. Biochem*, 237 (1996) 272–278.

[13] E.Th. Rietschel, L. Brade, B. Lindner, and U. Zähringer, in D.C. Morrison and J. L Ryan (Eds.), *Bacterial Endotoxic Lipopolysaccharides*, Vol. I., CRC Press, Boca Raton, 1992, pp 3–41.

[14] U. Zähringer, B. Lindner, and E.Th. Rietschel, *Adv. Carbohydr. Chem. Biochem.*, 50 (1994) 211–276.

[15] W. Kaca, J. de Jongh-Leuvenink, U. Zähringer, E.Th. Rietschel, H. Brade, J. Verhoef, and V. Sinnwell, *Carbohydr. Res.*, 179 (1988) 288–299.

[16] E.Th. Rietschel, *Eur. J. Biochem.*, 64 (1976) 423–428.

[17] Y. Haishima, O. Holst, and H. Brade, *Eur. J. Biochem.*, 203 (1992) 127–134.

[18] O. Holst, J.E. Thomas-Oates, and H. Brade, *Eur. J. Biochem.*, 222 (1994) 183–194.