ELSEVIER

www.elsevier.com/locate/carres

Carbohydrate Research 335 (2001) 291–296

CARBOHYDRATE RESEARCH

Note

Structural analysis of the core region of the lipopolysaccharides from eight serotypes of *Klebsiella pneumoniae*

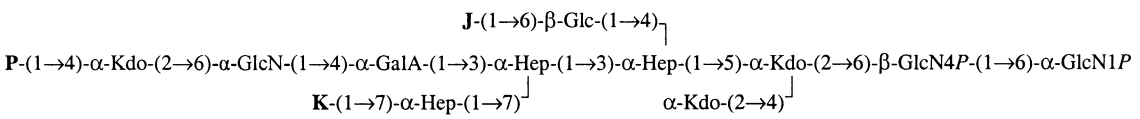
Evgeny Vinogradov,* Malcolm B. Perry

Institute for Biological Sciences, National Research Council, 100 Sussex Drive, Ottawa, Ont., Canada K1A 0R6

Received 21 March 2001; accepted 15 July 2001

Abstract

The core regions of the lipopolysaccharides (LPS) from *Klebsiella pneumoniae* serotypes O1, O2a, O2a,c, O3, O4, O5, O8, and O12 were analysed using NMR spectroscopy, ESI-MS spectroscopy, and chemical methods. All the LPSs had similar core structures, as shown below, differing only in the number and position of β-D-galacturonic acid substituents:



where **P** is H or α-Hep, **J**, **K** is H or β-GalA. LPS from all serotypes contained varying proportions of structures having additional or missing phosphate substituents. The core from serotype O1 contained a minor amount of a previously described variant with α-DD-Hep-(1→2)-α-DD-Hep-(1→6)-α-GlcN-(1→ replacing the α-Hep-(1→4)-α-Kdo-(2→6)-α-GlcN-(1→ component.

Keywords: LPS; Core; *Klebsiella pneumoniae*

1. Introduction

Klebsiella pneumoniae is a Gram-negative pathogenic bacterium associated with several serious infections, including pneumonia, bacteremia, and urinary tract infections. The lipopolysaccharide (LPS) molecule is a virulence determinant in *K. pneumoniae*. Complexes containing aggregates of capsular polysaccharide and LPS has been implicated in tissue damage that typically results from active *K. pneumoniae* lobar pneumonia. The LPS molecule is also responsible for the resistance of *K. pneumoniae* to complement-mediated serum killing.[1–3]

In view of the role of LPS in pathogenesis, the structures of the antigenic *O*-polysaccharide and core regions of the LPSs of *K. pneumoniae* serotypes are of interest. Recently we described the structure of the core part of the LPS from *K. pneumoniae* O3, which contained

Abbreviations: LPS, lipopolysaccharide; Hep, L-*glycero*-D-*manno*-heptose; DD-Hep, D-*glycero*-D-*manno*-heptose; GalA, galacturonic acid; Kdo, 3-deoxy-D-*manno*-octulosonic acid; 2,5-anhMan, 2,5-anhydromannose; Ara4N, 4-amino-4-deoxy-L-arabinose; P, phosphate; ESI-MS, electrospray ionisation mass spectrometry.

* Corresponding author. Tel.: +1-613-9900397; fax: +1-613-9529092.

E-mail address: evguenii.vinogradov@nrc.ca (E. Vinogradov).

0008-6215/01/$ - see front matter
PII: S0008-6215(01)00216-6

a previously undetected unusual structural component of -α-Hep-(1→4)-α-Kdo-(1→6)-α-GlcN in its outer region. This component was subsequently demonstrated to be present in the LPSs of other *K. pneumoniae* serotypes.[4] Herein we present the results of detailed comparative structural analyses of the lipid A–core regions of LPSs produced by eight serotypes of *K. pneumoniae*.

2. Experimental

Bacterial strains and lipopolysaccharide isolation.—*K. pneumoniae* O3:K55$^-$ was grown and LPS isolated as previously described.[4] *K. pneumoniae* strains: O1:K$^-$ (NRCC 6114); 2a:K$^-$ (NRCC 6115); O2a,c:K$^-$ (NRCC 6116); O4:K42 (NRCC 6118); O5:K57 (NRCC 6119); O8:K$^-$ (NRCC 6121); O12:K80 (NRCC 6122) were obtained from Dr C. Whitfield (Guelph University, Guelph, Ont.) were grown under the same fermenter conditions. For LPS isolation, bacterial mass obtained by Sharples centrifugation of the cultural fluid suspension (~ 400 g wet weight) was mixed with 50% aq phenol (1 L) and vigorously stirred for 30 min at 70 °C. The cooled extract was diluted with water (2 vols) and dialysed against running tap water for 5–7 days. The retentate was treated with AcOH (50 mL) and following removal of precipitated material by centrifugation, the dialysed solution was lyophilised to give LPS which was used without further purification.

NMR spectroscopy and general methods.—These methods were the same as those previously described.[4]

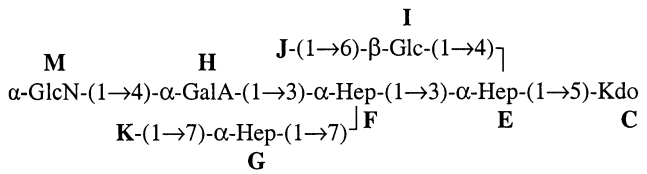
Preparation of the core oligosaccharides.—LPSs were hydrolysed with 2% AcOH (100 °C, 2 h) and core fractions were isolated as previously described.[4] Ion-exchange fractionation of core oligosaccharides was performed on a Hamilton PRP X100 anion-exchange HPLC column in a gradient of 0–100% of 1 M NaCl with UV detection at 220 nm and testing each fraction by charring spots on silica gel TLC plates after dipping in 5% H_2SO_4 in EtOH; samples were desalted by gel chromatography.

O-Deacylation of LPS.—LPS (50 mg) was dissolved in anhyd hydrazine (2 mL) and kept for 1 h at 50 °C. The cooled mixture was poured into stirred acetone (200 mL) and the precipitated material was collected by centrifugation and fractionated on a column (2.5 × 80 cm) of Sephadex G50 superfine gel (Pharmacia) using 0.05 M pyridinium acetate buffer (pH 4.6) as the eluant. The eluant was monitored by refractive index detection and collected fractions were lyophilised to give polysaccharide and core–lipid A fractions.

3. Results and discussion

LPSs from *K. pneumoniae* serotypes O1, O2a, O2a,c, O3, O4, O5, O8, and O12 were subjected to mild-acid hydrolysis with 2% AcOH followed by the separation of the products by gel chromatography to give O-specific polysaccharides and core oligosaccharide fractions, consisting mainly of oligosaccharides **1a**–**d**. These oligosaccharides do not represent complete core structures since Kdo residues **D** and **L** and heptose residue **P** were released under the conditions of acid hydrolysis. The core-derived products were fractionated by anion-exchange chromatography to give oligosaccharides **1a**–**d**, each fraction being obtained as a mixture of the products with complete and anhydro forms of Kdo (**C**) at the reducing end.

Oligosaccharides **1a**–**d** differed from each other in the number and substitution position of β-GalA residues. A detailed structural analysis of the products **1c**,**d** from *Klebsiella* serotype O3 LPS has been described in a previous communication.[4] Oligosaccharides, obtained from the other *K. pneumoniae* serotype LPSs, were identified by reference to previously established structures by the overlap of 2D NMR spectra (see Ref. 4 for NMR data). Identification of the linkage position of β-GalA residues **J** and **K** requires recording of NOESY spectra with longer than usual mixing time of 400 ms instead of 200 ms (200 ms was sufficient for the observation of all other transglycosidic NOEs). NOE from proton **J**1 to **I**6, and from **K**1 to **G**7, as well as HMBC correlations between H-1 of β-GalA **J** and C-6 of Glc **I**, and between H-1 of β-GalA **K** and C-6 of Hep **G** were observed in the respective compounds.



1, AcOH released core

Monosaccharide analysis of the oligosaccharides **1a**–**d** revealed the presence of Glc, GlcN, LD-Hep, Kdo, and GalA. GLC–MS analysis of the partially methylated alditol acetates, prepared from methylated oligosaccharides **1a**–**d** revealed the presence of derivatives of 3,4-di-*O*-substituted heptopyranose (residue **E**) and 3,7-*O*-disubstituted heptopyranose (residue **F**). Compound **1a**, obtained from serotypes O1 and O2a,c, additionally gave derivatives of nonreducing terminal glucopyranose **I** and heptopyranose **G**. Compound **1b**, isolated from serotype O1, gave derivatives of unsubstituted heptopyranose **G** and 6-*O*-substituted glucopyranose **I**. Other strains gave two oligosaccharides: **1c**-containing terminal glucopyranose **I** and 7-*O*-substituted heptopyranose **G**, and **1d**, containing 6-*O*-substituted glucopyranose **I** and 7-*O*-substituted heptopyranose **G**.

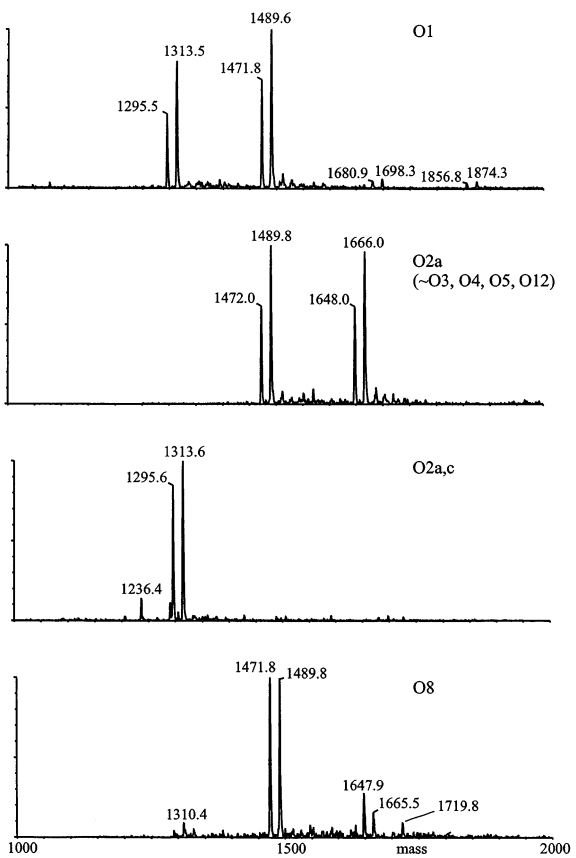
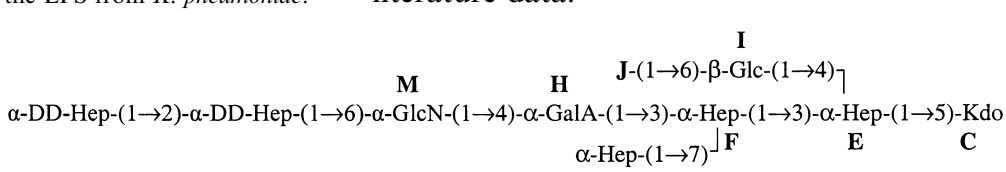


Fig. 1. Transformed negative mode ESI-MS spectra of the mixture of the core fractions of the LPS from *K. pneumoniae*.

Mixtures of the core oligosaccharides and fractions, obtained after anion-exchange separation, were characterised by negative mode ESI-MS analyses (Fig. 1). Each oligosaccharide gave two peaks in ESI-MS spectra, one corresponding to the structure with complete Kdo **C**, and the second with anhydro-Kdo at the reducing end. Serotype O1 contained structures **1a** and **1b**; serotype O2a,c—essentially structure **1a**; serotype O8—structure **1c** and a minor amount of **1d**; core fractions from all other investigated serotypes contained oligosaccharides **1c** and **1d** in roughly equal amounts. Oligosaccharides **1b** and **1c**, having the same masses, were distinguished and characterised on the basis of the NMR and methylation data.

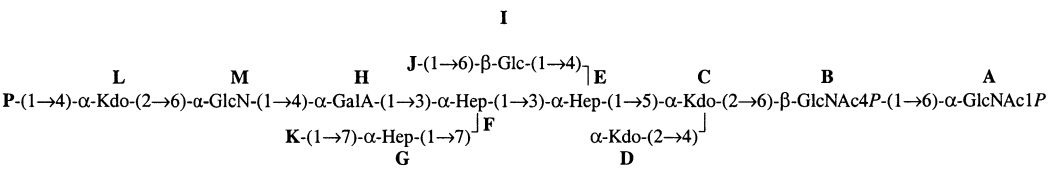
The mass spectrum of the oligosaccharide mixture obtained from serotype O1 (Fig. 1) showed small peaks at m/z 1680.9, 1698.3, 1856.8, and 1874.3, corresponding to those of the previously determined structures **2a**,**b**,[5,6] having two DD-Hep residues linked to O-6 of GlcN (**M**), lacking (**2a**) or with one β-GalA (**2b**) substituents. LPSs from other serotypes did not contain such structures. These products were not isolated in pure form and tentative conclusions about their structures are based only on literature data.



2a, **J** = H

2b, **J** = β-GalA

O-Deacylation of LPSs with hydrazine gave a complex mixture of products and their fractionation on Sephadex G-50 allowed a partial separation of the O-chain-containing derivatives from products of low-molecular mass free from O-chain residues. Products lacking O-chain were also present in the void volume fraction together with O-chain-substituted ones, probably because of micelle formation. Both the void-volume fraction and that retained on gel chromatography, on ESI-MS analysis, showed only peaks attributable to the oligosaccharides having the structure **3**, although showing different intensities. Fig. 2 and Table 3 present ESI spectra of the retained fractions. The observed peaks correspond to the oligosaccharides of structure **3**, acylated with two 3-hydroxytetradecanoyl residues in their lipid A part, an observation confirmed by fatty acid analysis. The multiplicity of the structures was due to the different degrees of phosphorylation of the basic structure (one additional or one missing phosphate residue), partial presence of the heptose residue **P**, varying amounts of galacturonic acid residues **J** and **K** (as shown for compounds **1a**–**d**), and the absence of Kdo. Missing Kdo was observed in the structures containing four heptose residues, and thus the only possibility is the absence of the residue **D**.



3, hydrazine deacylated LPS. ac = 3-hydroxytetradecanoyl

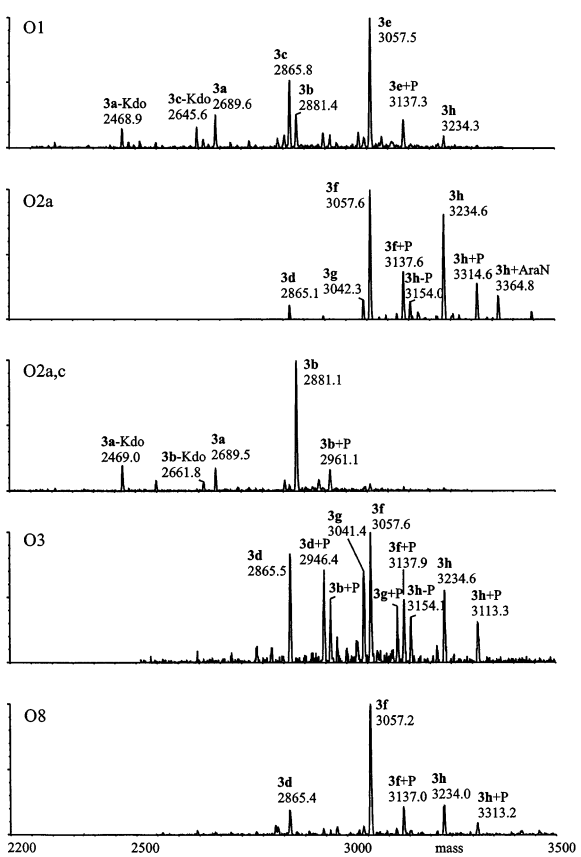


Fig. 2. Transformed negative mode ESI-MS spectra of the products of hydrazine deacylation of LPS from *K. pneumoniae*.

Additional phosphate residues in the hydrazine deacylated oligosaccharides were not located at the residues **C**, **E**–**K**, **M**, since no phosphorylated products were detected in the products of mild-acid hydrolysis. ^{13}P NMR spectra of the hydrazine deacylated LPSs contained a poorly resolved cluster of the signals of various intensities at -3 to $+5$ ppm, corresponding to the presence of phospho-mono- and diesters, but not pyrophosphates. The location of additional or missing phosphates was not determined.

The substitution position of Kdo (**L**) and heptose (**P**) residues follows from the results of deamination of the LPSs, which cleaved the glycoside bond between GlcN (**M**) and α-GalA (**H**) residues leading to the production of a mixture of the oligosaccharides **4** and **5**, the structures of which were previously described for *K. pneumonia* O3 LPS.[4]

P	L	M	
α-Hep-(1→4)-	α-Kdo-(2→6)-	2,5-anhMan	**4**
	α-Kdo-(2→6)-	2,5-anhMan	**5**

Experimental results show that all of the LPSs analysed contain compounds of the structure **3** with serotype-specific substituents

Table 1
Substituents, masses, and origin of the compounds of formula **1**

Compound	J	K	Calculated mass	Calculated mass, anhydro form	Present in serotype
1a	H	H	1314.3	1296.3	1, 2a,c
1b	β-GalA	H	1490.4	1472.4	1
1c	H	β-GalA	1490.4	1472.4	2a, 3, 4, 5, 8, 12
1d	β-GalA	β-GalA	1666.5	1648.5	2a, 3, 4, 5, 8, 12

listed in Tables 1–3. Serotype O1 LPS contains a unique minor variant structure with two Kdo and more than four heptose residues (structures **2a**,**b**), corresponding to previously described structures with heptoglycan oligomers [α-DD-Hep-(1→2)]$_n$-α-DD-Hep-(1→6)-α-GlcN replacing the fragment ***α-Hep***-(1→4)-α-Kdo-(2→6)-α-GlcN.[6]

We previously described the structure of the core part of *K. pneumoniae* O3 LPS together with the results of the preliminary analysis of other *K. pneumoniae* LPS and showed their structural similarity.[4] The results of the present work confirm these data and allow a more detailed description of the core parts of LPS from several O-serotypes of *K. pneumoniae*. It is not clear whether the structure of the core part necessarily correlates with the structure of the O-antigenic polysaccharide, thus there is the possibility that other strains of the same O-serotype may have different core structures. The analyses described here, considered together with those made on other serotype LPSs (several other strains were analysed, results not presented), showed that *K. pneumoniae* LPSs have conserved core structures, which differs from the situation observed for some other Gram-negative bacterial species.[7]

The mass spectrometric analysis of *Klebsiella* O1 LPS by Olsthoorn et al.[5] produced experimental results similar to the ones presented here, but led however to a proposed structure in which a third Kdo was attached to Kdo residue **D**:

Table 2
Substituents and masses of the compounds of formula **3**

Compound	J	K	P	Calculated mass
3a	H	H	H	2689.8
3b	H	H	α-Hep	2882.0
3c	β-GalA	H	H	2865.9
3d	H	β-GalA	H	2865.9
3e	β-GalA	H	α-Hep	3058.1
3f	H	β-GalA	α-Hep	3058.1
3g	β-GalA	β-GalA	H	3042.1
3h	β-GalA	β-GalA	α-Hep	3234.2

This latter conclusion was not supported by the data obtained from other experimental methods and now proves to be incorrect. Careful examination of the results from Ref. 5 shows that they can only be explained by the alternative presence of the DD-heptoglycan or α-Hep-(1→4)-α-Kdo- substituents at O-6 of the GlcN (**M**) residue. Our results, particularly those involving LPS deamination, show that the structures with four heptose and three Kdo residues correspond to the formula **3** and are present in all analysed serotypes of *Klebsiella*.

LPS from *K. pneumoniae* serotype O3 have been previously analysed for the presence of 4-amino-4-deoxyarabinose in the lipid A moiety.[8] It was shown that LPS from wild type serotype O3 (strain LEN-1) contains small amount of this monosaccharide substituent, whereas a polymyxin-resistant mutant contains greatly increased amounts of Ara4N. Our mass spectrometric analysis of the O-

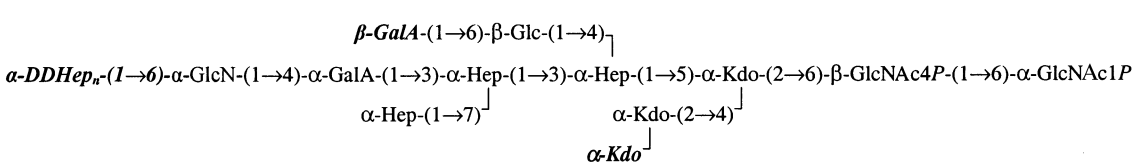


Table 3
Relative intensities of peaks in the ESI-MS spectra of the O-deacylated *Klebsiella* LPS

Compound	Serotype							
	1	2a	2a,c	3	4	5	8	12
3a	0.25		0.17					
3a−Kdo	0.14		0.19					
3b	0.25		1					
3b+P	0.10		0.16	0.5				
3b−Kdo			0.06					
3c	0.52							
3c−Kdo	0.16							
3c+P	0.12							
3d		0.24		0.81	0.8	0.82	0.19	0.76
3d+P		0.09		0.71	0.62	0.39	0.06	0.66
3e	1		0.05					
3e+P	0.22							
3e−Kdo	0.10							
3f		1		1	1	1	1	1
3f+P		0.50		0.52	0.71	0.5	0.22	0.6
3g		0.25		0.70	0.44	0.47		0.4
3g+P				0.46	0.22	0.23		0.23
3h	0.10	0.88		0.57	0.53	0.88	0.24	0.66
3h−P				0.38	0.19	0.39		0.15
3h+P		0.38		0.31	0.28	0.33	0.11	0.25
3h+Ara4N		0.24						

deacylated LPSs revealed a minor peak in the mass spectrum of the products from serotype O2a, which may be attributable to the presence of Ara4N. The overall quantity of Ara4N in the LPS was too small to permit its reliable determination by chemical methods (GLC of alditol acetates); other examined serotypes did not give any mass peaks that could be interpreted as containing an Ara4N residue.

Acknowledgements

This work was supported by Canadian Bacterial Diseases Network. The authors thank Donald Krajcarsky (NRC Canada) for the ESI-MS analysis, Anna Swierzko (Microbiology and Virology Centre, Łodz, Poland), Chris Whitfield (Guelph University, Canada) and Douglas Griffith (NRC Canada) for bacterial strains and cell growth.

References

1. Montgomerie, J. Z.; Ota, J. K. *Arch. Intern. Med.* **1980**, *140*, 525–527.
2. Trautmann, M.; Ruhnke, M.; Rukavina, T.; Held, T. K.; Cross, A. S.; Marre, R.; Whitfield, C. *Clin. Diagn. Lab. Immunol.* **1997**, *4*, 550–555.
3. Montgomerie, J. Z. *Rev. Infect. Dis.* **1979**, *1*, 736–753.
4. Vinogradov, E. V.; Cedzynski, M.; Ziolkowski, A.; Swierzko, A. *Eur. J. Biochem.* **2001**, *268*, 1722–1729.
5. Olsthoorn, M. M.; Haverkamp, J.; Thomas-Oates, J. *J. Mass Spectrom.* **1999**, *34*, 622–636.
6. Susskind, M.; Brade, L.; Brade, H.; Holst, O. *J. Biol. Chem.* **1998**, *273*, 7006–7017.
7. Holst, O. In *Endotoxin in Health and Disease*; Brade, H.; Morrison, D. C.; Opal, S. M.; Vogel, S., Eds. Chemical Structure of Core Region of Lipopolysaccharides; Marcel Dekker: New York, 1999.
8. Helander, I. M.; Kato, Y.; Kilpelainen, I.; Kostiainen, R.; Lindner, B.; Nummila, K.; Sugiyama, T.; Yokochi, T. *Eur. J. Biochem.* **1996**, *237*, 272–278.