Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 338 (2003) 2659–2666
CARBOHYDRATE RESEARCH
www.elsevier.com/locate/carres

Structure of the core-oligosaccharide with a characteristic D-*glycero*-α-D-*talo*-oct-2-ulosylonate-(2→4)-3-deoxy-D-*manno*-oct-2-ulosonate [α-Ko-(2→4)-Kdo] disaccharide in the lipopolysaccharide from *Burkholderia cepacia*

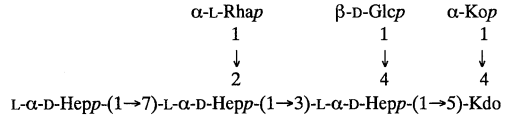
Yasunori Isshiki,[a,b,1] Ulrich Zähringer,[b] Kazuyoshi Kawahara[a,2,*]

[a] *Department of Bacteriology, The Kitasato Institute, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8642, Japan*
[b] *Division of Immunochemistry, Research Center Borstel, Parkallee 22, D-23845 Borstel, Germany*

Received 5 May 2003; accepted 25 July 2003

Abstract

The core oligosaccharide in the lipopolysaccharide (LPS) of *Burkholderia cepacia* GIFU 645^T was investigated. After mild acid hydrolysis of the LPS, a heptasaccharide was isolated and identified by chemical analyses, GLC–MS, FABMS, and NMR spectroscopy as follows:



where L-α-D-Hep stands for L-*glycero*-α-D-*manno*-heptose, Ko for D-*glycero*-D-*talo*-oct-2-ulosonic acid, and Kdo for 3-deoxy-D-*manno*-oct-2-ulosonic acid.

Keywords: *Burkholderia cepacia*; Core oligosaccharide; Lipopolysaccharide; D-*glycero*-D-*talo*-Oct-2-ulosonic acid

1. Introduction

Based on 16S ribosomal RNA base-sequence homology, the genus *Burkholderia* was proposed for seven species of bacteria[1] formally belonging to rRNA–DNA homology group II of *Pseudomonadaceae*.[2] This group includes several phytopathogens and human pathogens, such as *Burkholderia cepacia*, *B. pseudomallei*, and *B. caryophylli*. *B. cepacia* is a Gram-negative phytopathogen causing soft rot in onion and is also recognized as an important opportunistic human pathogen. A particular infectious phenomenon of this bacterium is the pulmonary infection in patients with cystic fibrosis, which is closely related to their high morbidity and mortality.[3] Despite the growing interest in this bacterium in medical and bacteriological fields and subsequent efforts to elucidate the pathophysiological events through the infection, structures and function of virulence factors of *Burkholderia,* including the lipopolysaccharide (LPS, endotoxin), remain to be elucidated.

The LPS is the major surface component of Gram-negative bacteria, which induces a variety of biological events through receptors on the immunity-mediating cells of animals and plants.[4] Structures of the O-polysaccharide chains of the *B. cepacia* LPS have been elucidated[5,6] but less information is available on the

* Corresponding author.
E-mail address: kawahara-k@kitasato.or.jp (K. Kawahara).
[1] Present address: Department of Microbiology, School of Pharmaceutical Sciences, Josai University, Sakado, Saitama 350-0295, Japan.
[2] Present address: Research Center for Biologicals, The Kitasato Institute, 6-11 Arai-Kitamato, Saitama, 364-0026, Japan. Tel.: +81-48-5933961; fax: +81-48-5933857

0008-6215/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2003.07.005

carbohydrate structure of the LPS core-region. Previously, we have studied the chemical structure of the LPS of *B. cepacia* GIFU 645^T and found a characteristic D-*glycero*-α-D-*talo*-oct-2-ulopyranosylonate-(2→4)-3-deoxy-D-*manno*-oct-2-ulosonate (Ko→Kdo) disaccharide in the core oligosaccharide.[7] Furthermore, the terminal Ko residue of the disaccharide was found to be non-stoichiometrically substituted at position 8 with 4-amino-4-deoxy-β-L-arabinopyranose (Ara4N).[8] Recently, the biological activity of this LPS has been studied by Shimamura and coworkers[9] who showed several stimulatory activities of macrophages comparable with those of *Salmonella* LPS, whereas the induction ability of IL-1β was unexpectedly weak.

In the present work, the LPS core structure was investigated in order to elucidate the chemical background of the unique biological behavior and activity of the *B. cepacia* LPS.

2. Results and discussion

2.1. Isolation and characterization of the oligosaccharides liberated by mild acid hydrolysis

The LPS was isolated by conventional phenol–water extraction from *B. cepacia* GIFU 645^T. Chemical analyses revealed that the LPS is predominantly of rough-type (Table 1). As described previously,[8] mild acid hydrolysis (0.1 M HCl, 100 °C, 1 h) was not sufficient to liberate completely the lipid A moiety owing to the stability of the linkage between the inner-core and lipid A. Nevertheless, the LPS was partially hydrolyzed under these conditions to obtain the core oligosaccharide. After removal of partially degraded LPS (LPS_{degr}) and liberated lipid A by ultracentrifugation, the water-soluble portion was subjected to GPC to yield three major oligosaccharide fractions, OS-1, OS-2 and OS-3.

Table 1
Chemical composition of the LPS and LPS degradation products from *B. cepacia* GIFU 645^T (μmol mg^{-1})

Component	LPS	LPS_{degr}	OS-1	OS-2	OS-3
L-Rha	0.27	0.15	0.57	0.48	0.42
D-Man	0.05				
D-Glc	0.23	0.17	0.48	0.42	0.32
D-Gal	0.11	0.03	0.11		
DD-Hep	0.08	0.05	0.03	0.13	0.03
LD-Hep	0.45	0.43	0.64	1.19	0.61
QuiN	0.08	0.05	0.15		
ManN	0.11	0.08	0.22		
D-GalN	0.21	0.07	0.23		
D-GlcN	0.23	0.49			
Phosphate	0.50	0.47			
14:0	0.10	0.24			
16:0	0.06	0.14			
14:0(3-OH)	0.14	0.32			
16:0(3-OH)	0.31	0.58			

Compositional analyses of the isolated oligosaccharides (Table 1) revealed the presence of L-Rha, D-Glc, L-*glycero*-D-*manno*-heptose (LD-Hep) and D-*glycero*-D-*manno*-heptose (DD-Hep) (minor component) as common sugar constituents. In addition, D-Gal, D-GalN and 2-amino-2,6-dideoxyglucose (QuiN) were determined in OS-1, thus indicating that OS-1 consists of the core-oligosaccharide substituted with an O-polysaccharide and originated from the smooth-type LPS.

FABMS of OS-2 (Fig. 1(A)) and OS-3 (Fig. 1(B)) showed that both preparations are oligosaccharide mixtures. A pseudomolecular ion at *m/z* 1357 was observed in the mass spectra of both OS-2 and OS-3 and, based on the mass number and the compositional data, was inferred to belong to a heptasaccharide composed of one residue each of Rha, Glc, Ko and Kdo, and three heptose residues. A pseudomolecular ion at *m/z* 1549 was present in the spectrum of OS-2 only and probably belongs to an octasaccharide with one additional heptose residue.

In the ^{1}H NMR spectrum of OS-2, the region of signals for anomeric protons was highly complex that precluded their assignment. In contrast, the ^{1}H NMR spectrum of OS-3 was suitable for further detailed analysis. Methylation analysis of OS-3 revealed the presence of terminal D-Glc*p*, LD-Hep*p* and L-Rha*p*, 2,7-substituted LD-Hep*p* and 3,4-substituted LD-Hep*p*. No derivatives from Ko and Kdo were detected in this analysis.

2.2. NMR spectroscopy studies of OS-3

In the ^{1}H NMR spectrum of OS-3 (Fig. 2), five anomeric signals were observed at 4.5–5.2 ppm, thus indicating that OS-3 contains five aldopyranose residues, which were designated as **A**–**E** in order of decreasing of the H-1 chemical shifts. The spectrum was assigned by 2D COSY and 1D HOHAHA experiments, and the results are summarized in Table 2. Residues **A**–**D** were shown to have the *manno* configuration on the basis of the coupling constant values of the vicinal ring-protons. The COSY spectrum demonstrated that residue **D** had a methyl group at position 6, and, hence, **A**–**C** are residues of LD-Hep*p* and **D** is a residue of L-Rha*p*. In the proton-coupled ^{1}H,^{13}C HMQC spectrum (Fig. 3), H-1,C-1 cross-peaks for each sugar residue were clearly observed. The $^1J_{C\text{-}1,H\text{-}1}$ coupling constant values of 170–175 Hz indicated that residues **A**–**D** are α-linked. Residue **E** is distinguished by relatively large $J_{1,2}$, $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$ values of ~9.5 Hz and is thus a β-linked residue of Glc*p*. Signals for H-3ax and H-3eq of Kdo were observed near δ 2.1 ppm and

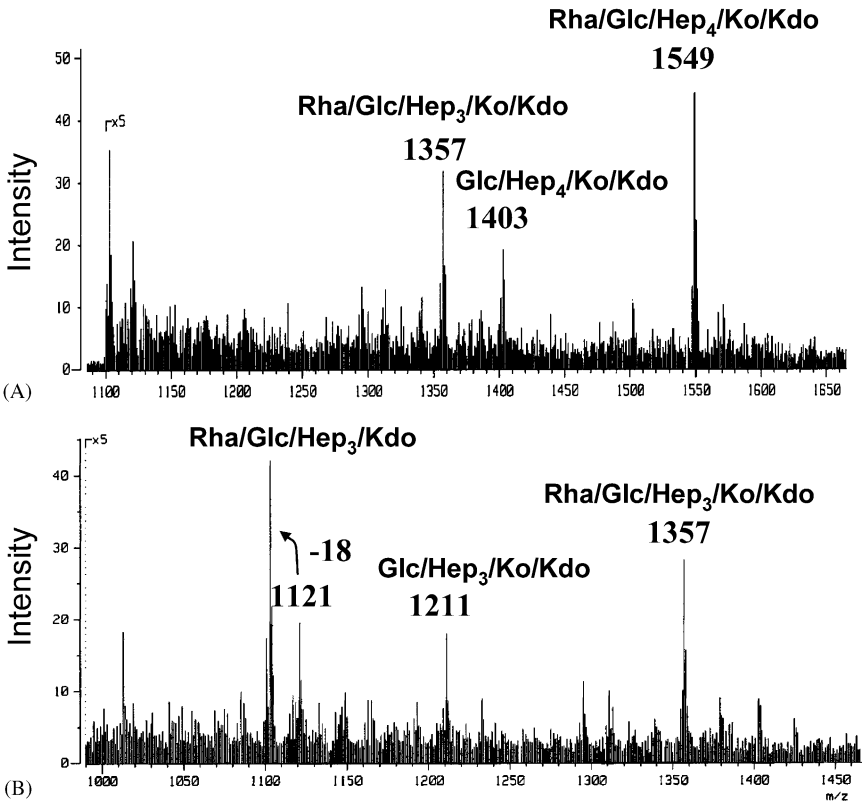


Fig. 1. Negative-ion mode FABMS of OS-2 (A) and OS-3 (B) isolated from the LPS of *B. cepacia* GIFU 645^T. Sugar composition of the respective oligosaccharides is indicated over the mass numbers.

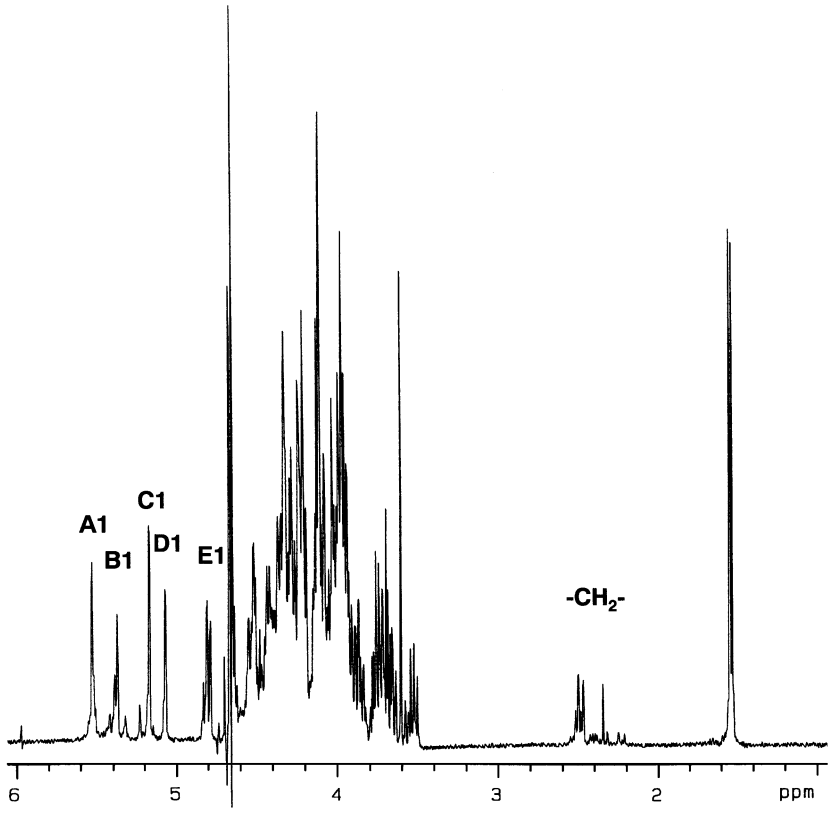


Fig. 2. ^{1}H NMR spectrum of OS-3. The spectrum was recorded at 400 MHz in D_2O at 60 °C.

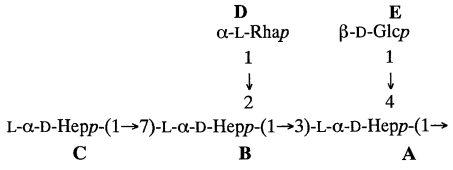
Table 2
^{1}H NMR spectral data of OS-3 (δ, ppm; $J_{n,n+1}$, Hz)

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	H-7a	H-7b
→3,4)-L-α-D-Hep*p*-(1→ **A**	5.28	4.08	4.03	4.26	4.11	3.86		3.78	
	$J_{1,2}$ 1.9	$J_{2,3}$ 2.0	$J_{3,4}$ 9.5	$J_{4,5}$ 9.9					
→2,7)-L-α-D-Hep*p*-(1→ **B**	5.13	4.31	3.78	3.86	3.68	4.18		3.73	3.73
	$J_{1,2}$ 1.2	$J_{2,3}$ 3.6	$J_{3,4}$ 10.2	$J_{4,5}$ 10.5	$J_{5,6}$ 2.5	$J_{6,7a}$ 4.8		$J_{7a,7b}$ 11.2	$J_{6,7b}$ 7.5
L-α-D-Hep*p*-(1→ **C**	4.92	3.99	3.88	3.86	3.63	4.03		3.71	3.68
	$J_{1,2}$ 1.8	$J_{2,3}$ 3.2	$J_{3,4}$ 9.8	$J_{4,5}$ 9.8	$J_{5,6}$ 2.0	$J_{6,7a}$ 5.0			
α-L-Rha*p*-(1→ **D**	4.83	3.97	3.84	3.45	4.04	1.29			
	$J_{1,2}$ 1.3	$J_{2,3}$ 3.0	$J_{3,4}$ 9.8	$J_{4,5}$ 9.5	$J_{5,6}$ 6.1				
β-D-Glc*p*-(1→ **E**	4.56	3.28	3.52	3.41	3.48	3.98	3.86		
	$J_{1,2}$ 9.5	$J_{2,3}$ 9.8	$J_{3,4}$ 9.5	$J_{4,5}$ 9.5	$J_{5,6a}$ 2.2	$J_{6a,6b}$ 12.5	$J_{5,6b}$ 8.0		

The spectrum was recorded at 400 MHz in D_2O at 60 °C.

showed a complex *J*-coupling pattern, as well as other signals for Kdo and those of Ko (Fig. 2). These finding suggested a structural heterogeneity owing to the presence of Kdo at the reducing end in multiple forms.[10]

The linkage and sequence analyses of OS-3 were performed using a NOESY experiment (Fig. 4). The NOESY spectrum showed **B** H-1,**A** H-3 and **E** H-1,**A** H-4 cross-peaks, which indicated a partial L-α-D-Hep*p*-(1→3)-[β-D-Glc*p*-(1→4)]-L-α-D-Hep*p*-(1→ trisaccharide structure. Two more partial structures, L-α-D-Hep*p*-(1→7)-L-α-D-Hep*p* and α-L-Rhap-(1→2)-L-α-D-Hep*p*, were inferred from **C** H-1,**B** H-7a,b and **D** H-1,**B** H-2 correlations, respectively. Based on these data, together with methylation analysis data, the following pentasaccharide structure was elucidated as a partial structure of OS-3:



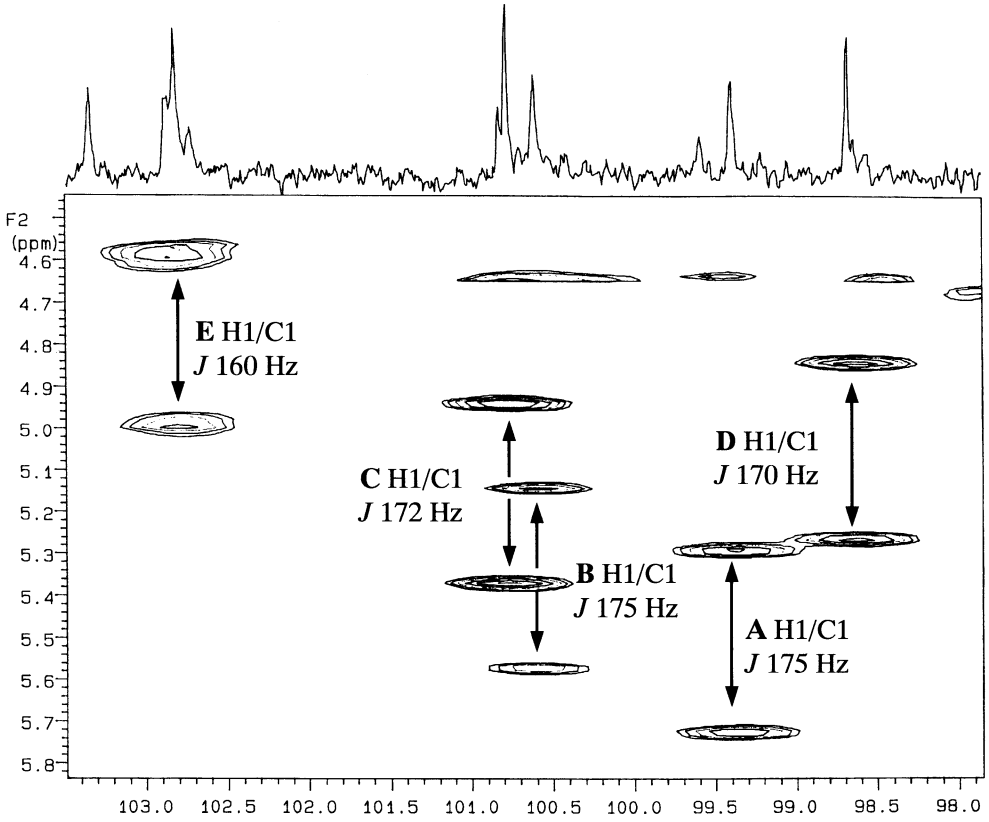


Fig. 3. Part of a proton-coupled ^{1}H,^{13}C HMQC spectrum of OS-3.

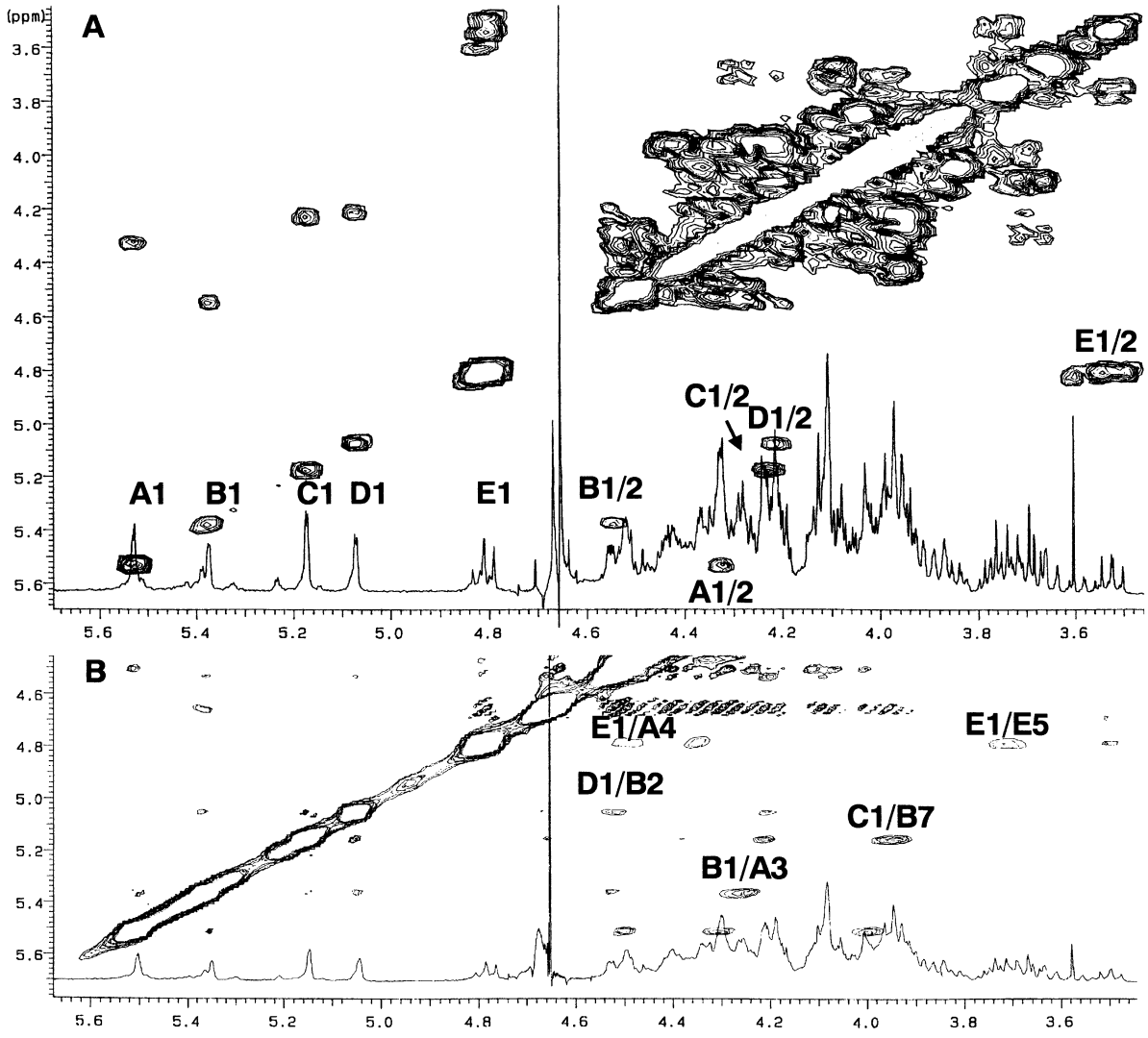


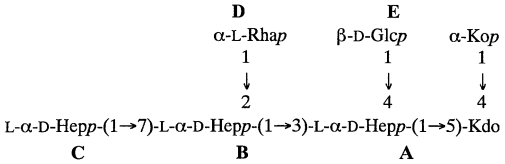
Fig. 4. Part of COSY (A) and NOESY (B) spectra of OS-3.

2.3. Structural analysis of Ko/Kdo region

LPS_{degr} was subjected to Smith degradation in order to determine the structure of the Ko/Kdo region. As a result, a single disaccharide was obtained, which was isolated by GPC on Sephadex G-10. The disaccharide was methylated and subjected to GLC–MS. The electron-impact mass spectrum (Fig. 5(A)) showed characteristic primary fragment ions at *m*/*z* 219 and 247 and secondary fragment ions generated by cleavage of methanol (-32 Da), thus indicating that the disaccharide consists of Man (from LD-Hep) and a 3-deoxyhept-2-ulosonic acid (3dHeplA; from Kdo). A methylated derivative of the carbonyl- and carboxyl-reduced disaccharide was also analyzed by GLC–MS. The observed primary fragment ions at *m*/*z* 149, 251 and 219 (Fig. 5(B)) demonstrated that Man is attached to 3dHeplA at position 5. As Smith degradation of LPS_{degr} gave a Man-(1→5)-3dHeplA disaccharide, it is concluded that a LD-Hep*p*-(1→5)-Kdo disaccharide was present in the LPS core.

2.4. Carbohydrate structure of the core region of *B. cepacia* LPS

Summarizing the data of chemical analyses, FABMS, ^{1}H NMR spectroscopy and Smith-degradation, as well as our previous results,[8] it can be concluded that the heptasaccharide (OS-3) isolated from the LPS of *B. cepacia* has the following structure:



This represents the major part of the core region of *B. cepacia* GIFU 645^T LPS, whereas the full core seems to

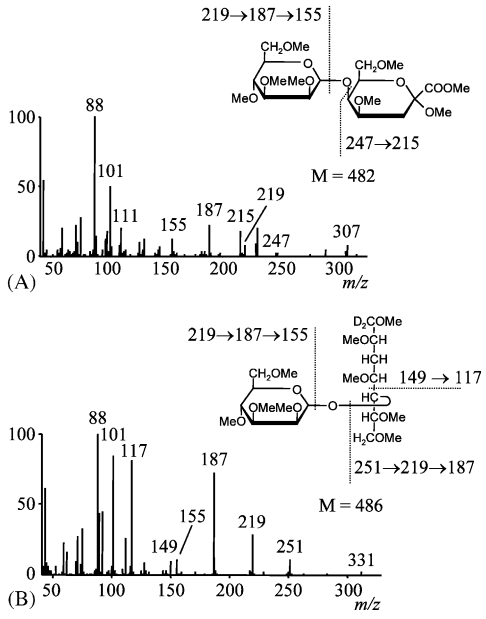


Fig. 5. Electron impact mass spectra of the permethylated Man→3dHeplA disaccharide obtained by Smith degradation of the LPS_{degr} (A) and the permethylated carbonyl- ($NaBH_4$) and carboxyl-reduced ($NaBD_4$) Man→3dHeplA disaccharide (B).

contain one more heptose residue. Based on the Ara4N→Ko→Kdo trisaccharide structure reported by us previously[8] and the structure of the inner core-lipid A moiety reported by Gronow and coworkers,[11] it is suggested that Ara4N is attached to Ko at position 8 and Kdo is linked to the lipid A backbone at position 6′.

Recently, the core structure of a taxonomically related bacterium *B. caryophylli* was established.[12] The core regions of both *B. cepacia* and *B. caryophylli* bacteria share a L-α-D-Hep*p*-(1→7)-L-α-D-Hep*p*-(1→3)-[β-D-Glc*p*-(1→4)]-L-α-D-Hep*p*-(1→5)-Kdo pentasaccharide, which is also a common core fragment of many enterobacteria.[13] However, only small amount of Ko is detectable in the LPS of *B. caryophylli*, and Kdo is substituted with another Kdo residue at position 4 rather than with Ko. For the first time, we found Ko as a constituent of the core-region in the LPS of *Acinetobacter haemolyticus* (formerly *A. calcoaceticus*) NCTC 10305.[14] The same sugar was found in the LPS from other strains of *Acinetobacter*[15] and, recently, in the LPS of *Yersinia pestis*.[16,17] Remarkably, in the LPS of *Acinetobacter* the core oligosaccharide is attached to lipid A by the α-(2→6)-ketosidic linkage of Ko. In the other Ko-containing LPS, including that of *B. cepacia*, Ko substitutes position 4 of Kdo that links the inner-core to the lipid A backbone. The substitution of Kdo with Ko is considered as a factor that renders the ketosidic linkage of Kdo stable and thus complicates cleavage of the core oligosaccharide from lipid A.

Structural data of lipid A of *B. cepacia* are limited. The lipid A backbone represents a *P*-4′-β-D-Glc*p*N-(1→6)-α-D-Glc*p*N-1-*P* disaccharide, which bears two amide-linked 3-hydroxyhexadecanoyl groups [16:0(3-OH)] (Ref. 11 and authors' unpublished data). Based on the present data, the unique biological activity of *B. cepacia* LPS[9] cannot be deduced solely from the core oligosaccharide structure. The full lipid A structure needs to be established to get more insight into the immunostimulatory activity and other biological properties of the Ko-containing LPS of *B. cepacia*.

3. Experimental

3.1. Cultivation of bacteria and extraction of LPS

B. cepacia GIFU 645^T (originally ATCC 25416^T) was cultured on Nutrient Broth No. 2 (Oxoid) at 37 °C for 24 h. After being killed by heating (100 °C, 30 min), cells were harvested by centrifugation (10,000*g*, 20 min) and washed sequentially twice each with water, EtOH and acetone. The LPS was extracted from ether-dried cells by the phenol–water procedure[18] and purified by repeated ultracentrifugation (100,000*g*, 16 h; three times) and enzymatic digestion with DNase, RNase, Trypsin, and Proteinase K.

3.2. Preparation of oligosaccharides from LPS

LPS (400 mg) was hydrolyzed with 0.1 M HCl at 100 °C for 1 h. After dialysis of the hydrolysate using tubing with a cut off size of 2000 Da, the retentate was subjected to ultracentrifugation (100,000*g*, 12 h) to give LPS_{degr} (160 mg) in the precipitate and liberated oligosaccharides (57 mg) in the supernatant. The oligosaccharides were fractionated by GPC on a column (2.4 × 100 cm) of TOYOPEARL Hw-40S (Toso, Japan) with 50 mM Py–acetate buffer pH 5.0 as eluent. Three major fractions thus obtained were designated as OS-1 (15 mg), OS-2 (15 mg) and OS-3 (9 mg).

3.3. Smith degradation of LPS_{degr}

LPS_{degr} (155 mg) was oxidized with 20 mM sodium periodate at 4 °C for 120 h, the excess of the oxidant was destroyed by adding ethylene glycol, the oxidized LPS_{degr} was reduced with $NaBH_4$ at 20 °C for 3 h, neutralized with HCl and desalted by dialysis. The lyophilized product (147 mg) was hydrolyzed with 0.1 M HCl at 100 °C for 1 h, and after neutralization with 0.05 M NaOH, the suspension was dialyzed against water. The retentate and dialysate were lyophilized to yield 90 mg of water-insoluble material and 52 mg of water-soluble material, respectively. The latter was applied to a column (2.4 × 100 cm) of Sephadex G-10

(Amersham Biosciences, Sweden) using the same eluent as above, and a disaccharide (10 mg) was collected and lyophilized. The disaccharide was permethylated according to Hakomori[19] directly or after carbonyl-reduction with $NaBH_4$. The permethylated carbonyl-reduced disaccharide was carboxyl-reduced with $NaBD_4$ in 1:1 MeOH–water mixture and remethylated.

3.4. Sugar analysis

Neutral and amino sugars were analysed by GLC as the alditol acetates after hydrolysis with 0.1 M HCl at 100 °C for 48 h and 4 M HCl at 100 °C for 16 h, respectively. GLC was performed on a Shimadzu GC-14A (Shimadzu, Japan) chromatograph equipped with a CBP1 capillary column (0.2 mm I.D. × 25 m, Shimadzu), using a linear temperature gradient from 170 to 270 °C at 5 °C min^{-1}. Kdo was estimated colorimetrically after hydrolysis with 0.1 M AcONa buffer pH 4.4.[14] The absolute configurations of the monosaccharides were determined by GLC as the peracetylated (*S*)- and (*R*)-2-butyl glycosides.[20]

3.5. Fatty acid and phosphate determination

Fatty acids were liberated by methanolysis (2 M HCl–MeOH, 120 °C, 24 h), and analyzed by GLC, using a temperature program from 150 to 250 °C at 5 °C min^{-1}. Phosphate was determined by the method of Lowry and coworkers.[21]

3.6. Methylation analysis

Methylation was performed according to Hakomori.[19] The methylated product was depolymerized by hydrolysis (2 M CF_3CO_2H, 120 °C, 2 h) or acetolysis (0.25 M H_2SO_4 in aq 95% AcHO, 80 °C, 17 h),[22] carbonyl-reduced ($NaBH_4$) and acetylated with acetanhydride–Py at 100 °C for 30 min. The partially methylated alditol acetates were analyzed by GLC–MS, using a temperature program from 150 to 320 °C at 5 °C min^{-1}.

3.7. GLC–MS and FABMS

GLC–MS in electron impact and chemical ionization modes was run on a JEOL DX-300 instrument with the same column and temperature conditions as in GLC analysis described above. Isobutane was used as reactant gas in chemical ionization MS. FABMS was performed on a JEOL EX-5500 spectrometer at an accelerating voltage of 3 keV, using 1:1 glycerol–thioglycerol mixture as matrix.

3.8. NMR spectroscopy

NMR spectroscopy was performed using a Varian XL-400 spectrometer with standard Varian software. ^1H NMR spectra were recorded at 25 or 50 °C in D_2O with acetone as internal reference (2.225 ppm). Assignment of the NMR spectra was performed using 2D COSY, 1D HOHAHA, NOESY, and proton-coupled ^1H,^{13}C HMQC experiments.

Acknowledgements

We thank A. Hatano, N. Sato, A. Nakagawa and C. Sakabe (Kitasato University) for excellent NMR spectroscopy, GLC–MS and FABMS measurements. This study was supported in part by a grant from the Japan Society for the Promotion of Science (No. 14570255).

References

1. Yabuuchi, E.; Kosako, Y.; Oyaizu, H.; Yano, I.; Hotta, H.; Hashimoto, Y.; Ezaki, T.; Arakawa, M. *Microbiol. Immunol.* **1992**, *36*, 1251–1257.
2. Palleroni, N. J.; Kunisawa, R.; Contopoulou, R.; Doudoroff, M. *Int. J. Syst. Bacteriol.* **1973**, *23*, 333–339.
3. Govan, J. R. W.; Deretic, V. *Microbiol. Rev.* **1996**, *60*, 539–574.
4. Alexander, C.; Rietschel, E. T. *J. Endotoxin Res.* **2001**, *7*, 167–202.
5. Knirel, Y. A.; Kochetkov, N. K. *Biochemistry (Moscow)* **1994**, *59*, 1325–1383.
6. Jansson, P.-E. In *Endotoxin in Health and Disease*; Brade, H.; Opal, S. T.; Vogel, S. N.; Morrison, D. C., Eds.; Marcel Dekker: New York, 1999; pp 155–178.
7. Kawahara, K.; Isshiki, Y.; Ezaki, T.; Moll, H.; Kosma, P.; Zähringer, U. *J. Endotoxin Res.* **1994**, *1* (Suppl. 1), 52.
8. Isshiki, Y.; Kawahara, K.; Zähringer, U. *Carbohydr. Res.* **1998**, *313*, 21–27.
9. Shimomura, H.; Matsuura, M.; Saito, S.; Hirai, Y.; Isshiki, Y.; Kawahara, K. *Infect. Immun.* **2001**, *69*, 3663–3669.
10. Brade, H.; Zähringer, U.; Rietschel, E. T.; Christian, R.; Schulz, G.; Unger, F. M. *Carbohydr. Res.* **1984**, *134*, 157–166.
11. Gronow, S.; Noah, C.; Blumenthal, A.; Lindner, B.; Brade, H. *J. Biol. Chem.* **2003**, *278*, 1647–1655.
12. Molinaro, A.; De Castro, C.; Lanzzeta, R.; Evidente, A.; Parrilli, M.; Holst, O. *J. Biol. Chem.* **2002**, *277*, 10058–10063.
13. Holst, O. In *Endotoxin in Health and Disease*; Brade, H.; Opal, S. T.; Vogel, S. N.; Morrison, D. C., Eds.; Marcel Dekker: New York, 1999; pp 115–154.
14. Kawahara, K.; Brade, H.; Rietschel, E. T.; Zähringer, U. *Eur. J. Biochem.* **1987**, *163*, 489–495.

15. Vinogradov, E. V.; Bock, K.; Petersen, B. O.; Holst, O.; Brade, H. *Eur. J. Biochem.* **1997**, *243*, 122–127.
16. Vinogradov, E. V.; Lindner, B.; Kocharova, N. A.; Senchenkova, S. N.; Shashkov, A. S.; Knirel, Y. A.; Holst, O.; Gremyakova, T. A.; Shaikhutdinova, R. Z.; Anisimov, A. P. *Carbohydr. Res.* **2002**, *337*, 775–777.
17. Hitchen, P. G.; Prior, J. L.; Oyston, P. C.; Panico, M.; Wren, B. W.; Titball, R. W.; Morris, H. R.; Dell, A. *Mol. Microbiol.* **2002**, *44*, 1637–1650.
18. Westphal, O.; Jann, K. *Methods Carbohydr. Chem.* **1965**, *5*, 83–91.
19. Hakomori, S. *J. Biochem. (Tokyo)* **1964**, *51*, 205–208.
20. Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1987**, *62*, 349–357.
21. Lowry, O. H.; Roberts, N.; Leiner, K.; Wu, M.; Farr, L. *J. Biol. Chem.* **1954**, *207*, 1–17.
22. Stellner, K.; Saito, H.; Hakomori, S. *Archiv. Biochem. Biophys.* **1973**, *155*, 464–472.