Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 340 (2005) 1253–1257
Carbohydrate RESEARCH

Note

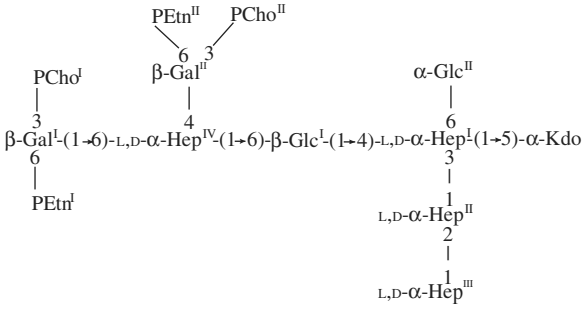
Structural analysis of the core oligosaccharide from *Pasteurella multocida* strain X73

Frank St. Michael, Jianjun Li and Andrew D. Cox*

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

Received 21 October 2004; accepted 15 February 2005

Abstract—The structure of the core oligosaccharide region of the lipopolysaccharide from the *Pasteurella multocida* strain X73 was elucidated. The lipopolysaccharide was subjected to a variety of degradative procedures. The structure of the purified oligosaccharide was established by monosaccharide and methylation analyses, NMR spectroscopy and mass spectrometry. The following structure illustrates a similar structure to the recently identified oligosaccharide from another *P. multocida* strain VP161, but with additional symmetrical substitution of the terminal galactose residues with phosphoethanolamine moieties,



where based on the NMR data all sugars were found in pyranose ring forms and Kdo is 3-deoxy-α-D-*manno*-2-oct-2-ulosonic acid, L,D-α-Hep is L-*glycero*-D-*manno*-heptose, PEtn is phosphoethanolamine and PCho is phosphocholine.

Keywords: *Pasteurella multocida*; LPS; Core oligosaccharide; Mass spectrometry; NMR

Pasteurella multocida (*Pm*) is a Gram-negative bacterium and multi-species pathogen that causes serious diseases in food animals and humans.[1] Structural analyses on the lipopolysaccharide (LPS) of the genome strain Pm70[2] and a virulent serotype A:1 strain VP161[3] have revealed a conserved inner core oligosaccharide (OS) structure with variable outer core OS structures. This study was carried out on another virulent serotype A strain X73 and structural analysis of the purified LPS products revealed a similar OS structure to that found for strain VP161 but with an additional symmetrical phosphorylation pattern to the outer core OS. Sugar analysis of the purified LPS and 8K pellet material from strain X73 revealed a similar profile to strain VP161 containing the sugars glucose (Glc), galactose (Gal) and L-*glycero*-D-*manno*-heptose (LD-Hep) in the approximate ratio of 2:1:4, respectively. *O*-Deacylated LPS (LPS-OH) was prepared from fermenter-grown cells and analysed by CE–MS (Table 1). A similar mass profile to that observed for strain VP161 was observed, but

* Corresponding author. Tel.: +1 613 991 6172; fax: +1 613 952 9092; e-mail: andrew.cox@nrc-cnrc.gc.ca

0008-6215/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2005.02.014

Table 1. Negative-ion CE–ESIMS data and proposed compositions of *O*-deacylated LPS (LPS-OH) and core OS from *P. multocida* strain X73[a]

Strain	$[M-3H]^{3-}$	$[M-4H]^{4-}$	Observed molecular ion	Calculated molecular ion	Relative intensity	Lipid A size	Core OS size	Proposed composition
X73	992.0	744.0	2979.5	2977.6	0.1	952	2025.6	2PCho, 3Hex, 4Hep, 2Kdo, Lipid A-OH
LPS-OH	999.0	749.0	2999.5	2999.5	0.2	952	2047.5	2PCho, 4Hex, 4Hep, Kdo-P, Lipid A-OH
	1033.0	774.0	3101.0	3100.7	0.2	952	2148.7	PEtn, 2PCho, 3Hex, 4Hep, 2Kdo, Lipid A-OH
	1040.0	780.0	3123.5	3122.6	0.4	952	2170.6	PEtn, 2PCho, 4Hex, 4Hep, Kdo-P, Lipid A-OH
	1074.0	805.0	3224.1	3223.7	0.3	952	2271.7	2PEtn, 2PCho, 3Hex, 4Hep, 2Kdo, Lipid A-OH
	1081.0	810.0	3245.0	3245.6	1.0	952	2293.7	2PEtn, 2PCho, 4Hex, 4Hep, Kdo-P, Lipid A-OH
	1122.0	841.0	3368.5	3368.7	0.2	952	2416.7	3PEtn, 2PCho, 4Hex, 4Hep, Kdo-P, Lipid A-OH
X73 Core OS	$[M-3H]^{3-}$	$[M-2H]^{2-}$						
	—	901.3	1804.6	1805.2		—	—	2PCho, 3Hex, 4Hep, Kdo
	—	962.8	1927.6	1928.2		—	—	PEtn, 2PCho, 3Hex, 4Hep, Kdo
	—	982.8	1967.6	1967.6		—	—	2PCho, 4Hex, 4Hep, Kdo
	—	1024.3	2050.3	2051.3		—	—	2PEtn, 2PCho, 3Hex, 4Hep, Kdo
	—	1044.3	2090.6	2090.6		—	—	PEtn, 2PCho, 4Hex, 4Hep, Kdo
	736.8	1106.3	2214.0	2213.7		—	—	2PEtn, 2PCho, 4Hex, 4Hep, Kdo

[a] Average mass units were used for calculation of molecular weight based on proposed composition as follows: Hex, 162.15; Hep, 192.17; Kdo, 220.18; PEtn, 123.05; PCho, 165.05; Lipid A-OH, 952.00. Relative intensity is expressed as relative to the largest triply charged ion in the spectrum for the LPS-OH molecule.

with the major species now being a triply charged ion of *m*/*z* 1081.0, which would correspond to two additional PEtn residues compared to the most prominent species (*m*/*z* 999.0) from VP161 LPS-OH. MS/MS analyses revealed that all lipid A molecules were the basal species consisting of a disaccharide of *N*-acylated (3-OH C 14:0) glucosamine residues, each residue being substituted with a phosphate group with a molecular weight of 952 amu as indicated by doubly and singly charged ions of *m*/*z* 475.5 and 951.5. This MS/MS analysis therefore indicated that the additional PEtn residues in strain X73 reside in the core OS region of the LPS molecule, and the expected mass of the core OS molecules is indicated in Table 1.

MS analysis on the core oligosaccharides alone revealed a series of ions consistent with the compositions inferred from the LPS-OH data, indicating the presence of additional PEtn residues when compared to strain VP161. The location of the PEtn residues was determined from MS/MS experiments in positive-ion mode. Fragmentation of the doubly charged ion of *m*/*z* 1108 corresponding to the largest glycoform produced several product ions including *m*/*z* 183.5^{+} (PCho), 450.5^{+} (PCho–Hex–PEtn), 547.5^{2+} (2PCho, 2PEtn, 2Hex, Hep) and 916.5^{2+} (M−Kdo–Hex). The doubly charged ion at *m*/*z* 547.5^{2+} was of particular interest when compared to the doubly charged ion observed at *m*/*z* 424^{2+} in strain VP161, as it would indicate a glycoform with a mass 246 amu higher, which is consistent with the presence of two PEtn residues. MS/MS/MS analysis on the doubly charged ion at *m*/*z* 1108^{2+} revealed the product-ion spectrum shown in Figure 1a. The fragmentation pattern indicated in Figure 1a enabled the PEtn residues to be localised to the terminal Hex–PCho moieties on the Hep^{IV}, and a subsequent MS/MS/MS experiment (Fig. 1b) fragmenting the ion *m*/*z* 450.5 confirmed that this ion corresponded to the PCho–Hex–PEtn moiety as originally inferred.

In order to confirm and extend these inferences, NMR experiments were performed on the fractionated core OS sample that contained both PEtn residues (as determined by MS, data not shown). Apart from the two terminal galactose residues, all assignments were identical to those obtained on the core OS from strain VP161. Chemical shifts for the H-1 and H-2 resonances of the galactose residues were as observed for the VP161 sample, but the H-3 and H-4 resonances were shifted slightly downfield from 4.17 and 4.12 to 4.21 and 4.17 ppm, respectively. Inter-NOE contacts from the galactose residues were as observed previously, although the intra-NOE contact to the H-5 residue was now observed at 3.94 ppm as opposed to 3.80 and 3.76 ppm for strain VP161 (Table 2). In order to identify the locations of the two PEtn residues, a ^{31}P–^{1}H HSQC experiment was performed that identified a resonance at 4.07 ppm as the point of attachment of each PEtn residue. A sub-

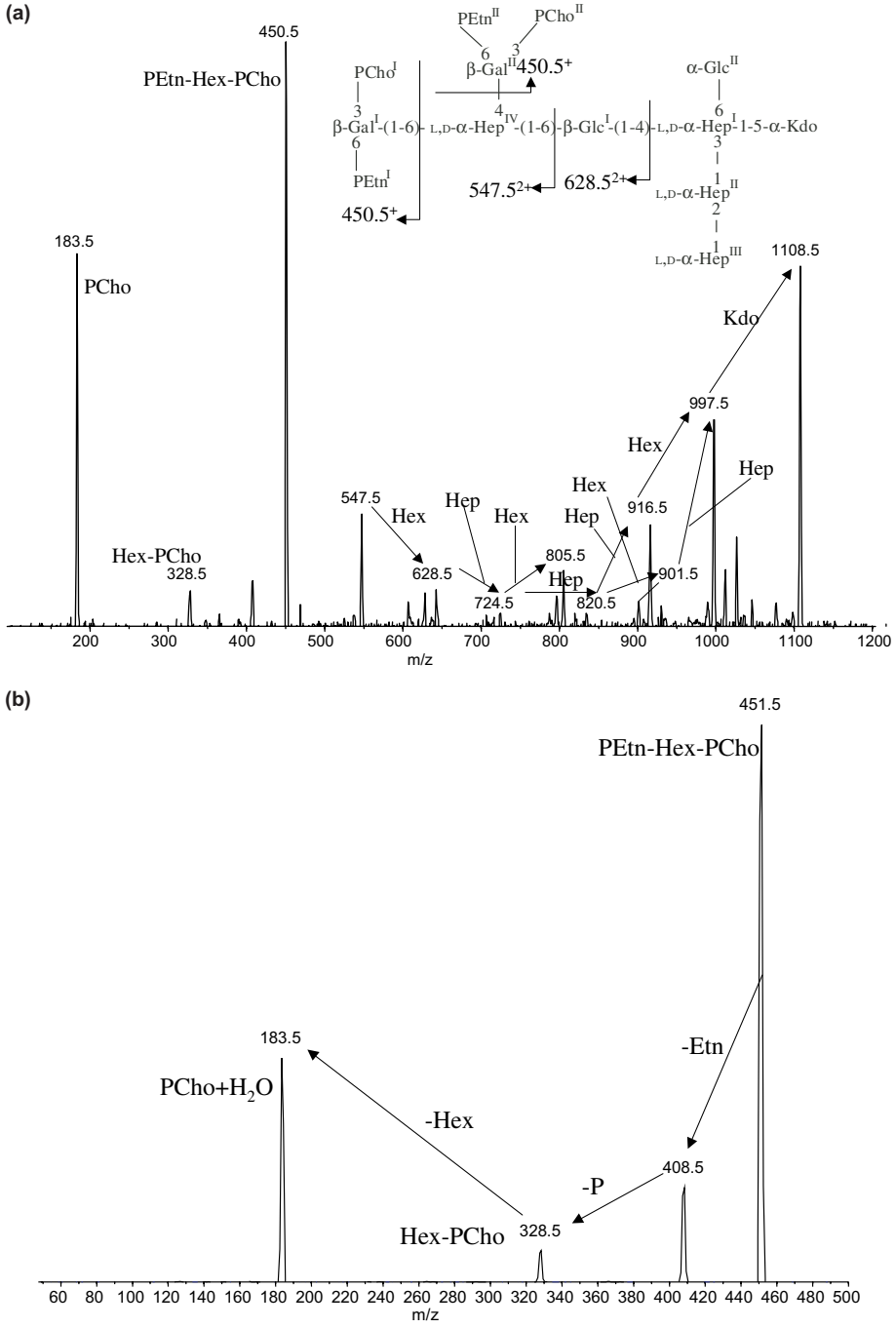


Figure 1. Positive-ion capillary electrophoresis–electrospray mass spectra (CE–ESIMS) of the core OS from *Pm* strain X73. (a) Product-ion spectrum from *m/z* 1108^{2+}, (b) Product-ion spectrum from *m/z* 451.5^{+} utilising a high orifice voltage.

sequent ^{31}P–^{1}H HSQC–TOCSY experiment identified the H-5 resonance of the galactose residues at 3.94 ppm (Fig. 2). The assignment of 4.07 ppm as the H-6 resonance of the galactose residues was confirmed by a ^{13}C–^{1}H HMQC experiment, which identified as a characteristic positive peak for a $-CH_2$ moiety, the downfield chemical shift of the C-6 atoms of each Gal residue at low-field values (~65 ppm), which is consistent with phosphorylation. Finally a ^{13}C-^{1}H HMQC–TOCSY experiment identified cross-peaks between the C-6 and H-5 resonances and vice-versa, confirming the 6-positions of the two galactose residues as the points of attachments of the additional two PEtn moieties found in the strain X73 core OS (Fig. 3).

This study has, therefore, identified a similar core OS structure from the LPS of a second serotype A strain.

Table 2. ^{1}H and ^{13}C NMR chemical shifts for the terminal region of the core OS from *Pasteurella multocida* strain X73[a]

Residue	H-1	H-2	H-3	H-4	H-5	H-6	H-7	NOE connectivities	
								Inter	Intra
Hep^{IV}	4.95 (99.9)	4.18 (70.0)	3.96 (70.9)	4.18 (77.4)	3.93 (70.2)	4.32 (79.9)	3.97 3.75 (63.5)	4.09 Glc^{I}H-6 3.77 Glc^{I}H-6	4.18 H-2
β-Gal^{I}	4.72 (103.4)	3.70 (70.7)	4.20 (78.2)	4.17 (68.2)	3.94 (74.0)	4.07 4.07 (65.4)	—	4.18 Hep^{IV} H-4	4.20 H-3 3.93 H-5
β-Gal^{II}	4.70 (104.5)	3.73 (70.7)	4.21 (78.1)	4.17 (68.2)	3.94 (74.0)	4.07 4.07 (65.4)	—	4.32 Hep^{IV} H-6	4.21 H-3 3.93 H-5
$PEtn^{I}$	4.13 (62.7)	3.31 (40.8)							
$PEtn^{II}$	4.13 (62.7)	3.31 (40.8)							
$PCho^{I}$	4.38 (60.4)	3.68 (66.8)	3.22 (54.7)						
$PCho^{II}$	4.38 (60.4)	3.68 (66.8)	3.21 (54.7)						

[a] All other residues have identical chemical shifts to strain VP161.

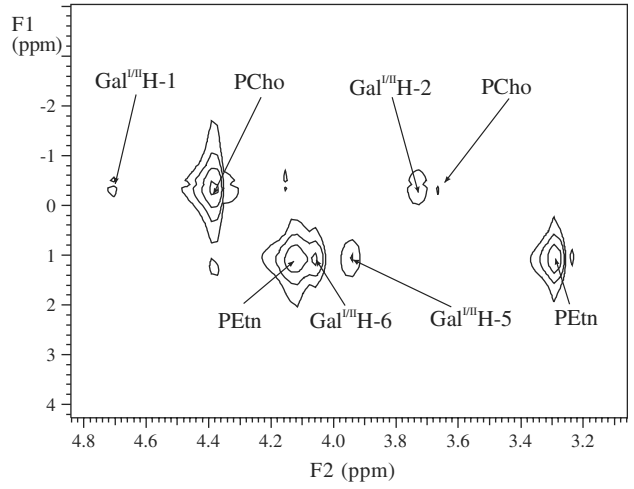


Figure 2. Region of the 2D-^{31}P–^{1}H HMQC–TOCSY NMR spectrum of the core OS from *Pm* strain X73 showing correlations between the ^{31}P resonances (*x*-axis) and ^{1}H resonances with a mixing time optimised at 10 Hz for the ^{31}P–^{1}H coupling between PEtn and the galactose residues. The spectrum was recorded in D_2O at pH 7.0 and 25 °C.

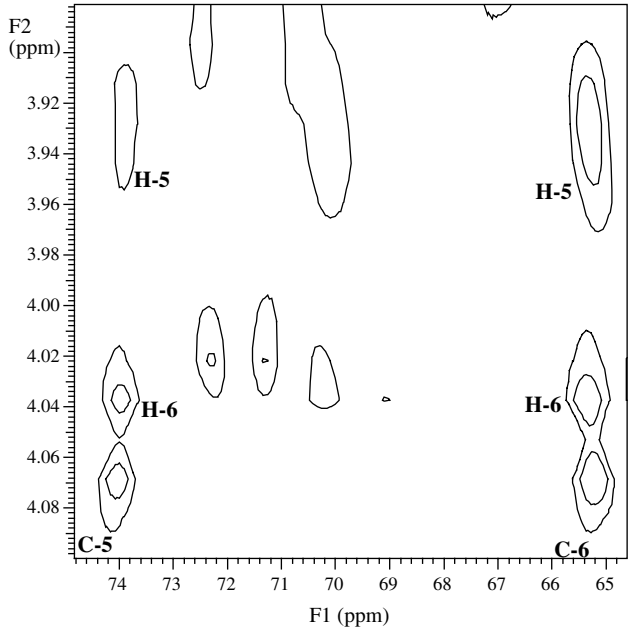


Figure 3. Region of the 2D-^{13}C–^{1}H HSQC–TOCSY NMR spectrum of the core OS from *Pm* strain X73 showing correlations between the ^{13}C resonances (*y*-axis) and ^{1}H resonances indicating the correlations between the C-5, C-6, H-5 and H-6 resonances of the galactose residues. The spectrum was recorded in D_2O at pH 7.0 and 25 °C.

Further studies will extend LPS structural analyses to other serotypes to examine if this novel terminal structure is present in other serotypes. The identification of PEtn at the 6-position of a six-carbon sugar is somewhat unusual. PEtn is commonly found at the 6-position of heptose residues in both *Neisseria meningitidis*[4] and *Haemophilus influenzae*.[5] Recent studies in our laboratory have identified PEtn at the 6-position of a terminal GalNAc residue in several *Haemophilus influenzae* strains (unpublished observations), and a recent poster also found a PEtn residue at the 6-position of a 3-linked galactose molecule in *Citrobacter* sp. PCM1443 (serotype O39) with similar chemical shifts to those observed here.[6]

1. Experimental

1.1. Growth of bacteria, isolation and fractionation of LPS

P. multocida strain X73 (NRCC#6235) was grown and isolated as described previously.[3] Briefly, *P. multocida* cells (~210 g wet wt) were freeze-dried, yielding ~56 g. Freeze-dried cells were washed with organic solvents

(1 × EtOH, 2 × acetone, 2 × light petroleum ether) to remove lipids and other lipophilic components to enhance the efficiency of the LPS extraction.[7] The washed cells (10 g from ~42 g) were extracted by the hot phenol–water method,[8] and the aq phases were combined and dialysed against running water for 48 h. The retentate was freeze-dried yielding ~0.57 g, made up to 2% solution in water and treated with DNase and RNase at 37 °C for 4 h, followed by proteinase K treatment at 37 °C for 4 h. Small peptides were removed by dialysis. After freeze-drying, the retentate (~0.42 g) was made up to 2% solution in water, centrifuged at 8000*g* for 15 min (yielding an '8K pellet' of ~265 mg), followed by further centrifugation of the supernatant at 100,000*g* for 5 h. The pellet, containing purified LPS, was redissolved and freeze-dried (yielding ~3 mg). LPS-OH and core OS were isolated and fractionated as described previously.[3] Briefly, 8K pellet material (~15 mg) and LPS (~3 mg) were treated with anhyd. hydrazine with stirring at 37 °C for 1 h to prepare LPS-OH yielding ~10 mg from the 8K preparation and ~1 mg from the LPS. The core OS was isolated by treating the 8K pellet material (~115 mg) with 1% HOAc (10 mg/mL, 100 °C, 1.5 h) with subsequent removal of the insoluble lipid A by centrifugation (5000*g*). The lyophilised supernatant was subsequently further purified down a Bio-Gel P-2 column with separate fractions lyophilised yielding ~40 mg.

1.2. Analytical methods

Sugars were determined as their alditol acetate derivatives[9] by GLC–MS as described previously.[3] Methylation analysis was carried out by the NaOH–Me_2SO–CH_3I procedure[10] and analysed by GLC–MS as described previously.[3]

1.3. Mass spectrometry and NMR spectroscopy

All mass spectrometry and NMR experiments were performed as described previously.[3]

Acknowledgements

The authors would like to thank Perry Fleming for cell growth, Lisa Morisson for mass spectrometry and Professor Ben Adler and Dr. Marina Harper for providing strain X73.

References

1. Adlam, C.; Rutter, J. M. *Pasteurella and Pasteurellosis*; Academic: London, 1989, p 341.
2. St. Michael, F.; Vinogradov, E.; Li, J.; Cox, A. D. *Glycobiology*, **2005**, *15*, in press.
3. St. Michael, F.; Li, J.; Vinogradov, E.; Larocque, S.; Harper, M.; Cox, A. D. *Carbohydr. Res.* **2005**, *340*, 59–68.
4. Kogan, G.; Uhrin, D.; Brisson, J. R.; Jennings, H. J. *Carbohydr. Res.* **1997**, *298*, 191–199.
5. Cox, A. D.; Masoud, H.; Thibault, P.; Brisson, J.-R.; van der Zwan, M.; Perry, M. B.; Richards, J. C. *Eur. J. Biochem.* **2001**, *268*, 5278–5286.
6. Toukach, F. V.; Katzenellenbogen, E.; Kocharova, N. A.; Bushmarinov, I. S. *Abstr. of Paper*, 22nd International Carbohydrate Symposium, Glasgow, 2004; Poster 458.
7. Masoud, H.; Moxon, E. R.; Martin, A.; Krajcarski, D.; Richards, J. C. *Biochemistry* **1997**, *36*, 2091–2103.
8. Westphal, O.; Jann, K. *Methods Carbohydr. Chem.* **1965**, *5*, 88–91.
9. Sawardeker, D. G.; Sloneker, J. H.; Jeanes, A. *Anal. Chem.* **1965**, *37*, 1602–1604.
10. Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1994**, *131*, 209–217.