ELSEVIER

Carbohydrate Research 290 (1996) 59–65

CARBOHYDRATE RESEARCH

Note

# Structural analysis of the O-antigen-core region of the lipopolysaccharide from *Vibrio cholerae* O139

Andrew D. Cox *, Malcolm B. Perry

*Institute for Biological Sciences, National Research Council, Ottawa, Ont., Canada K1A 0R6*

Received 20 February 1996; accepted 2 May 1996

*Keywords:* Lipopolysaccharide; *O*-Antigen core; *Vibrio cholerae*

The recent identification of *Vibrio cholerae* O139 as a cause of epidemic cholera has prompted considerable investigation of this serotype. The general conclusion from these studies is that serotype O139 has evolved from serotype O1 (the only strain of *Vibrio cholerae* previously identified with epidemic-causing potential), with the only significant difference between these strains being the loss of O-chain perosamine homopolymer and the subsequent acquisition of a capsular polysaccharide. The structure of the polysaccharide capsule is well established [1,2], and recent studies in our laboratory have shown that the structure of the lipid A-core region of O139 [3] is identical to that of O1 [4]. Furthermore, serotype O139 appears to produce an SR-type LPS [5], and it has been shown immunologically that its one repeat unit shares an epitope with the capsular polysaccharide repeat unit [6]. Our recent studies provided further evidence to support this data, and in this study we present results that confirm that the capsule and LPS indeed do share the same repeating unit.

Sugar analysis on the whole LPS revealed the presence of 3,6-dideoxy-L-*xylo*-hexose (Col), D-galactose (Gal), 2-amino-2-deoxy-D-glucose (GlcN), D-glucose (Glc), L-*glycero*-D-*manno*-heptose (Hep), D-galacturonic acid (GalA), D-fructose (Fru) and 2-amino-2,6-dideoxy-D-glucose (QuiN). The presence of 3,6-dideoxy-L-*xylo*-hexose, 2-amino-2,6-dideoxy-D-glucose, D-galactose and D-galacturonic acid all being constituents of the capsular polysaccharide of this strain and not constituents of the core region was an initial indication that the LPS may contain a repeat unit similar to that of the capsular polysaccharide. The identification of 2-amino-2-deoxy-D-glucose, also a constituent of

* Corresponding author.


*PII* S0008-6215(96)00131-0

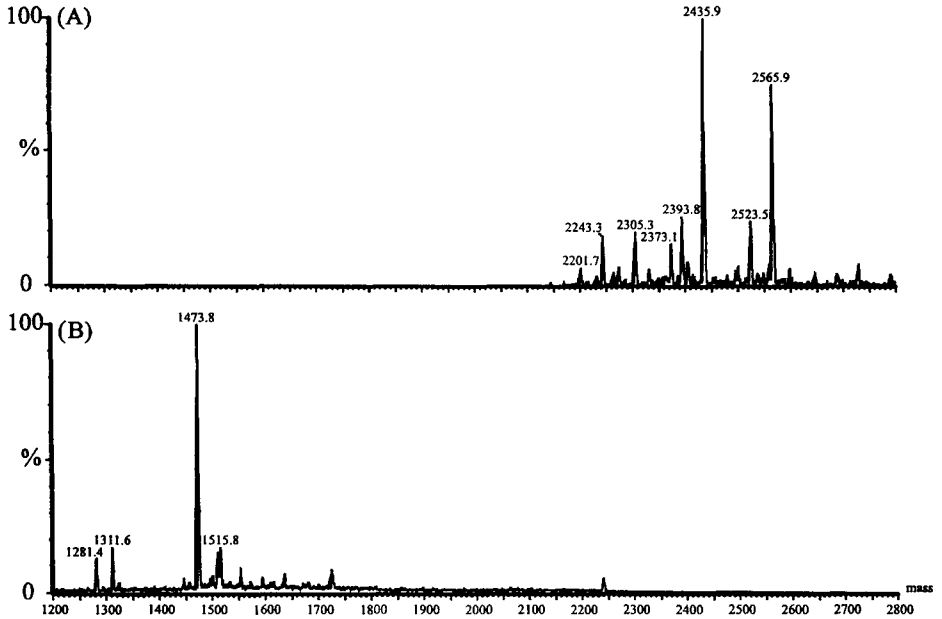


Fig. 1. Electrospray mass spectra of *Vibrio cholerae* O139 delipidated LPS (A) fraction 1 and (B) fraction 2. Peaks are represented as relative intensities of their masses in amu.

the capsule, was not considered significant as this residue is known to be present in the core-lipid A region of the LPS.

In order to remove the lipid A but to leave acid-labile residues (such as the 3,6-dideoxy-L-*xylo*-hexose sugars thought to be present in the O-antigen) attached, a mild hydrolysis was performed. After desalting, the residual product examined by $^{1}H$ NMR spectroscopy gave a poor spectrum. This sample was therefore further purified by gel-filtration chromatography. The two fractions (1 and 2) obtained were examined by electrospray mass spectrometry (Fig. 1), giving molecular ions differing by 1092 mass units, which is in close agreement with the expected mass of the capsular repeat unit.

Fraction 1 had a molecular ion of 2566 amu in good agreement with the expected size of the core plus one 'capsular repeating unit' and two smaller ions due to the consecutive loss of 130 mass units (a 3,6-dideoxy sugar) from the molecular ion, suggesting partial loss of the 3,6-dideoxy sugars on delipidation. There were also ions indicative of loss of acetate groups ($-42$ amu) and loss of a cyclic phosphate group ($-63$ amu).

Fraction 2 gave a molecular ion of 1474 amu close to the expected size of the core region, with further molecular ions due to the losses of 192 and 162 amu indicative of the loss of a heptose and a hexose unit, respectively. Clearly these data suggest the presence of R- and SR-type LPS in *V. cholerae* O139 with the one repeat unit apparently the same as the capsular repeat unit. Examination of the $^{1}H$ NMR spectra of these fractions confirmed these inferences.

Table 1
$^1$H NMR chemical shift data for *Vibrio cholerae* O139 LPS [a]

| Sugar residue [b] | H1 | H2 | H3 | H4 | H5 | H6 | | H7 | NAc | NOE |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | a, b | | | a | b | a, b | | |
| A (Hep) | 5.52 | 4.23 | 3.98 | 3.79 | nr [c] | nr | | nr | – | A1–E3 |
| B (GalA) | 5.40 | 3.69 | 3.94 | 4.28 | 4.19 | nr | | – | – | B1–I3 |
| C (Glc) | 5.27 | 3.85 | 3.78 | nr | nr | nr | | – | – | C1–D6a,b |
| D (Glc) | 5.20 | 3.56 | 3.80 | 3.53 | 3.91 | 4.16 | 3.99 | – | – | D1–E6 |
| E (Hep) | 5.16 | 4.16 | 3.89 | 4.12 | nr | 4.18 | | nr | – | |
| F (Col) | 5.11 | 4.00 | 1.91 | 3.79 | 4.31 | 1.21 | | – | – | F1–K2 |
| | | | 1.81 | | | | | | | |
| G (Hep) | 5.10 | 4.04 | nr | nr | nr | nr | | 3.96 | – | G1–A2 |
| | | | | | | | | 3.62 | | |
| H (GlcN) | 5.09 | 3.49 | 3.89 | 3.79 | nr | nr | | – | – | H1–G7a,b |
| I (QuiNAc) | 5.03 | 3.98 | 3.63 | 3.81 | 3.99 | 1.35 | | – | 2.17 | I1–G2 |
| J (Col) | 4.87 | 3.99 | 2.06 | 4.20 | 4.79 | 1.20 | | – | – | J1–M4 |
| | | | 1.86 | | | | | | | |
| K (Gal) [d] | 4.71 | 3.71 | 3.95 | 4.59 | 3.76 | 4.42 | 4.36 | – | – | K1–M3 |
| | 4.73 | 3.63 | 3.91 | 4.57 | 3.62 | 4.32 | 4.25 | | | |
| L (Glc) | 4.62 | 3.24 | 3.48 | 3.12 | 3.39 | 3.86 | 3.48 | – | – | L1–E4 |
| M (GlcNac) [d] | 4.50 | 3.84 | 4.04 | 3.68 | 3.43 | nr | | – | 2.08 | M1–B4 |
| | 4.52 | 3.78 | 4.04 | 3.49 | 3.40 | | | | 1.96 | |

[a] Measured at 300 K, pH 3.8, from internal acetone (2.225 ppm).
[b] Sugar residues A–M as in Fig. 2.
[c] nr, not resolved.
[d] Chemical shift data for residues K and M represented with substituted residue above and terminal residue below due to lack of substitution by residues F and J, respectively.

The $^1$H NMR spectrum of fraction 1 contained 13 major signals in the anomeric region (4.5–6.0 ppm), as well as several minor signals. The signals at 5.10, 4.72, 4.58 and 4.51 ppm corresponded to three, two, two and two protons, respectively. Also significant in the high-field region of the spectrum were methyl doublets at 1.35, 1.21 and 1.20 ppm, methyl singlets at 2.17, 2.08 and 1.96 ppm and unresolved signals at 2.06, 1.91, 1.86 and 1.81 ppm. A 2D $^{13}$C–$^1$H HMQC experiment revealed that only 15 of the 18 signals in the region 6.0–4.5 ppm were attributable to anomeric protons.

The $^1$H NMR spectrum of fraction 1 was assigned using COSY, TOCSY and NOESY experiments. The $^1$H NMR chemical shift assignments are shown in Table 1.

By comparison to previous NMR data for the capsule [1,2] and core region [3] of O139, it became apparent that fraction 1 consisted of the O139 core substituted by one O-antigenic repeat unit with an identical structure to the capsular repeat unit previously elucidated for this strain. Two residues of 3,6-dideoxy-L-*xylo*-hexose sugars (F and J) were identified by the characteristic chemical shifts for the H-3 methylene and H-6 methyl groups. The chemical shifts for the other sugars in this sample all correlated well with previous data for the core and capsule of this strain.

The partial removal of the 3,6-dideoxy-L-*xylo*-hexose sugars on delipidation was inferred from the presence of two sets of signals for 2-amino-2-deoxy-D-glucose (M) and galactose (K), presumably due to these residues being either terminal or substituted by

the 3,6-dideoxy sugars (J) and (F), respectively. The presence of two signals for the methyl groups of the N-acetylated 2-amino-2-deoxy-D-glucose residue (M) leaves only one methyl singlet to be assigned and therefore only one of the two remaining amino sugars in the sample is N-acetylated. The fact that the chemical shift of H-2 of the 2-amino-2-deoxy-D-glucose residue (H) in this sample is virtually unchanged from its chemical shift in the KOH treated N-deacetylated sample (3) is strong evidence that this residue is not N-acetylated. Conversely, the large downfield shift for the H-2 signal for the 2-amino-2,6-dideoxy-D-glucose residue (I) when comparing the same two samples further supports this conclusion. This assignment correlates with the data for the *Vibrio cholerae* serotype O1 core (4), which showed that the 2-amino-2-deoxy-D-glucose residue of the outer core is not acetylated.

A 1D $^{31}$P NMR spectrum revealed two signals at −3.38 and −3.74 ppm (the purified capsular polysaccharide gave a single signal at −3.38 ppm, which is our unpublished observation). The presence of two phosphorus signals was explained when the 2D $^{31}$P–$^{1}$H HMQC experiment was examined. One of the phosphorus signals correlated to the H-4, H-6a,b, H-5 and H-3 signals for the substituted D-galactose residue, and the second phosphorus signal correlated to the corresponding signals from the unsubstituted D-galactose residue. (Similar behaviour was observed in the $^{31}$P–$^{1}$H HMQC spectrum for the 'delipidated' capsular polysaccharide, which is our unpublished observation.) The chemical shifts for the protons of the D-galactose residue and the presence of the observed signals in the HMQC spectrum are clearly consistent with the presence of a cyclic phosphate moiety linked to the 4- and 6-positions of the D-galactose residue.

The sequence of monosaccharides in the LPS was established from NOE measurements, as indicated in Table 1. This data was confirmed by methylation analysis of the nonpurified delipidated sample, which also suggested the presence of a further L-*glycero*-D-*manno*-heptose residue (X) attached to heptose residue (A) at the 6-position.

An NOE signal from the heptose residue (E) to a signal at 3.65 ppm was observed. In the untreated LPS this residue (E) is attached to the 5-position of the Kdo molecule linking the core to the lipid A. However, in the delipidated sample $^{1}$H NMR signals characteristic of the axial and equatorial protons of H-3 of Kdo were absent, as was a signal in the $^{31}$P NMR spectrum for the phosphate group attached to the 4-position of Kdo, suggesting that the mildly acidic conditions of delipidation cleaved this phosphate residue, causing a subsequent rearrangement of the Kdo molecule. However attempts to identify the degradation product resulting from this rearrangement of Kdo proved unsuccessful.

The $^{1}$H NMR spectrum of fraction 2 was poorly resolved; however, it was possible to confirm the identities of the core residues in COSY and NOESY experiments to those previously characterized [3].

Fig. 2 shows the structure of the complete LPS molecule from *Vibrio cholerae* O139. This study has therefore provided a chemical–structural explanation for the immunological data which showed that the LPS and the capsule shared a common epitope.

Other bacteria have been shown to produce capsular and O-antigenic polysaccharides with identical repeating units [7–9], though to our knowledge this is the first report of such a situation in *Vibrio cholerae*. It is also often the case, as has been observed here,

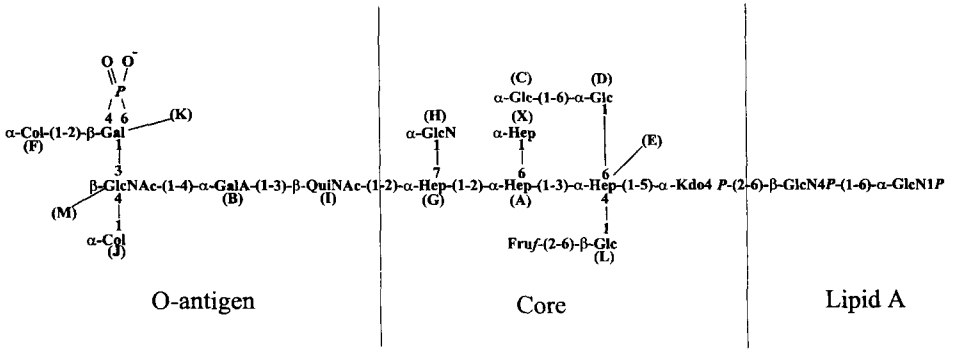


Fig. 2. Structure of the LPS of *Vibrio cholerae* O139. Sugar abbreviations are as in the text, with also Fru,D-fructose and Kdo,3-deoxy-D-*manno*-2-octulosonic acid. Specific regions of the LPS are indicated.

that when capsular and O-antigenic repeat units are identical, the O-antigen consists of only one repeating unit [10,11].

This study has, therefore, completed the structural analysis of the surface polysaccharides of this new epidemic causing strain O139. It is particularly interesting to note that, although both the capsule and O-antigen consist of the same repeating unit, only the capsule is polymerized. A genetic and mechanistic understanding of this phenomenon would be particularly rewarding in view of the considerable amount of interest in the biogenesis of this strain from serotype O1.

## 1. Experimental

*Isolation of lipopolysaccharide.*—*Vibrio cholerae* serotype O139 (NRCC#4740) was grown and isolated as described previously [3].

*Purification of lipopolysaccharide.*—LPS was delipidated according to the following procedure. LPS (200 mg) was hydrolysed at 100 °C for 4 h at pH 4.2 in a solution of 0.1 M NaOAc (20 mL). Insoluble material was removed by centrifugation, the supernatant solution was lyophilized, and the resulting oligosaccharide was purified by Sephadex G-10 gel-filtration column chromatography. Column eluents were monitored for changes in refractive index, and collected fractions (4.5 mL) were assayed colorimetrically for neutral glycoses. This desalted product was further purified by gel-filtration chromatography on a Bio-Gel P2 column, monitoring column eluants as above.

*Analytical methods.*—Glycoses were determined by GLC–MS as their alditol acetate derivatives. Samples (0.5–1.0 mg) were hydrolyzed with 2 M TFA for 90 min at 125 °C. The liberated glycoses were reduced ($NaBH_4$) and acetylated ($Ac_2O$). The absolute configurations of the LPS components were identified by GLC analysis of their acetylated (*S*)-2-butyl glycosides and by determination of the specific optical rotation of the glycans liberated by graded acid hydrolysis of the native, deacylated (anhydrous hydrazine, 37 °C, 30 min) and dephosphorylated (48% HF, 4 °C, 48 h) LPS preparations.

*Methylation analysis.*—Oligosaccharide samples (2–4 mg) were methylated with MeI in $Me_2SO$ containing an excess of potassium (methylsulphinyl)methanide. The methylated oligosaccharides were purified on a Sep-Pak $C_{18}$ cartridge. The purified methylated oligosaccharides were hydrolyzed by initial treatment with 90% (v/v) formic acid at 100 °C for 1 h, followed by overnight treatment with 0.13 M $H_2SO_4$ at 100 °C. Hydrolysis products were then reduced ($NaBD_4$), acetylated and analyzed by GLC–MS.

*Electrospray mass spectrometry.*—Samples were analyzed on a VG Quattro triple quadrupole mass spectrometer (Fisons Instruments) with an electrospray ion source as previously described [3].

*NMR spectroscopy.*—NMR spectra were obtained on a Bruker AMX 500 spectrometer using standard Bruker software. Measurements were made at pH 3.8, 300 K at concentrations $\sim 10$ mg $mL^{-1}$ in $D_2O$, subsequent to several lyophillizations with $D_2O$.

1D $^1H$ NMR spectra were measured at 500.14 MHz using a spectral width of 5.0 kHz. Acetone was used as an internal standard and chemical shifts were referenced to the methyl resonance ($\delta_H$, 2.225 ppm). Two-dimensional homonuclear proton correlation experiments (COSY), total correlation experiments (TOCSY) and nuclear Overhauser effect experiments (NOESY) were measured over a spectral width of 3.62 kHz, using data sets ($t_1 \times t_2$) of 2048 × 1024, and 16 scans were acquired. A mixing time of 200 min was employed for the NOESY experiment.

Heteronuclear 2D $^{13}C-^1H$ chemical shift correlations were measured in the $^1H$-detected mode via multiple quantum coherence (HMQC) with proton decoupling in the $^{13}C$ domain, using data sets of 2048 × 512 points and spectral widths of 4.24 and 16.6 kHz for $^1H$ and $^{13}C$ domains, respectively. 64 scans were acquired for each $t_1$ value.

$^{31}P$ NMR spectra were measured at 202.5 MHz by employing spectral widths of 41.6 kHz, and phosphoric acid (85%) was used as the external standard ($\delta_P$ 0.0 ppm). $^{31}P-^1H$ correlations (HMQC) were made in the $^1H$-detected mode by using a data matrix of 2048 × 128 points, sweep widths of 2 kHz for $^{31}P$ and 4.5 kHz for $^1H$, and a delay of 60 min.

## Acknowledgements

The authors acknowledge Mr. D.W. Griffith for large-scale production of cells, Mr. F. Cooper for GLC–MS and Mr. D. Krajcarski for ESIMS.

## References

[1] L.M. Preston, Q. Zu, J.A. Johnson, A. Joseph, D.R. Maneval, K. Hussain, G.P. Reddy, C.A. Bush, and J.G. Morris, *J. Bacteriol.*, 177 (1995) 835–838.

[2] Y.A. Knirel, L. Parades, P.-E. Jansson, A. Weintraub, G. Widmalm, and M.J. Albert, *Eur. J. Biochem.*, 232 (1995) 391–396.

[3] A.D. Cox, J.-R. Brisson, V. Varma, and M.B. Perry, *Carbohydr. Res.*, in press.

[4] E.V. Vinogradov, K. Bock, O. Holst, and H. Brade, *Eur. J. Biochem.*, 233 (1995) 152–158.

[5] P.A. Manning, U.H. Stroeher, and R. Morona, in I.K. Wachsmuth, P.A. Blake, and O. Olsvik (Eds.), *Vibrio cholerae and Cholera: Molecular to Global Perspectives*, ASM Press, Washington, DC, 1994, pp 89–90.
[6] M.K. Waldor, R. Colwell, and J.J. Mekalanos, *Proc. Natl. Acad. Sci. USA*, 91 (1994) 11388–11392.
[7] L.M. Beynon, A.J. Dumanski, R.J.C. McLean, L.L. MacLean, J.C. Richards, and M.B. Perry, *J. Bacteriol.*, 174 (1992) 2172–2177.
[8] R.C. Goldman, K. Joiner, and L. Leive, *J. Bacteriol.*, 159 (1984) 877–882.
[9] A.A. Peterson and E.J. McGroarty, *J. Bacteriol.*, 162 (1985) 738–745.
[10] M.K. Homonylo, S.J. Wilmot, J.S. Lam, L.A. MacDonald, and C. Whitfield, *Can. J. Microbiol.*, 34 (1988) 1159–1165.
[11] C. Whitfield, G. Schoenhals, and L.L. Graham, *J. Gen. Microbiol.*, 135 (1989) 2589–2599.