Glucosylated N-acetyllactosamine O-antigen chain in the lipopolysaccharide from Helicobacter pylori strain UA861

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Introduction

Infection in humans caused by the gram-negative bacterium Helicobacter pylori has been correlated with the onset of chronic gastritis and gastric carcinoma (Clovér and Blaser, 1995). The lipopolysaccharides (LPSs) from several H.pylori strains have been shown to possess elongated partially fucosylated N-acetyllactosamine O-chains which express Lewis X and Lewis Y blood-group epitopes and mimic human gastric mucosa cell-surface glycoconjugates (Aspinall et al., 1994, 1996; Aspinall and Monteiro, 1996; Monteiro, 1996). The molecular mimicry displayed by H.pylori LPSs with host molecules has been implicated in an autoimmune component of H.pylori pathogenesis (Appelmelk et al., 1996), and detection of Lewis X and Y blood-group epitopes in the LPS by monoclonal antibodies (Mabs) has also been employed as a serotyping system (Simoons-Smit et al., 1997). Recently, another example of molecular mimicry between the LPS of a Helicobacter species and a histo-blood group determinant widely present in mammalian cells has been shown in the case of H.mustelae type strain ATCC 43772 which gives rise to gastritis-like symptoms in ferrets and whose LPS carries a monofucosyl A type 1 blood-group epitope [α-D-GalNAc-(1→3)[α-D-Fuc-(1→2)]β-D-Gal-(1→3)]β-D-GlcNAc] (Monteiro et al., 1997).

The discovery of a glycosyl transferase in H.pylori UA861 (Chan et al., 1995) with the specificity of placing a terminal α-Gal unit at the O-6 position of GlcNAc prompted a detailed chemical investigation of the LPS structure from this strain for comparison with other H.pylori LPSs of which the structures were known. The structural results obtained from studies on the intact LPS from H. pylori UA861 and its derivatives are reported here.

Results and discussion

To prevent any inadvertent loss of structural units, such as acid labile fucose (Fuc) and sialic acid residues, all the outset chemical manipulations and spectroscopic analysis were performed on the intact LPS obtained from water-phenol extraction of bacterial cells. Purification of the water-soluble LPS on a column of Bio-Gel P-2 gave one fraction (LPS) at the void-volume. Sugar composition analysis (Figure 1) of LPS revealed the presence of L-Fuc, D-glucose (Glc), D-galactose (Gal), D-glycero-D-manno-heptose (α-D-Hep), D-glycero-D-manno-heptose (β-D-Hep), and N-acetyl-glucosamine (D-GlcNAc) in the approximate ratios of 1:6:9:1.5:1.4:9, respectively. Methylation linkage analysis (Table I), carried out on the intact LPS, yielded as the major components, and as putative O-chain units, terminal Glc, 3-linked Gal, 4-linked GlcNAc and 4,6-linked GlcNAc. Also detected were minor components which were attributed to the inner regions of the LPS molecule (see Table I). The FAB-MS spectrum of the methylated intact LPS (Figure 2) showed a primary glycosyl-oxonium ion at m/z 464 [Hex-HexNAc+] and a secondary ion arising from β-elimination of methanol, from the O-3 position of GlcNAc, at m/z 432 [464–32]. The loss of methanol from O-3 of GlcNAc indicated that this position was not substituted by a glucose unit and since the sole 6-linked GlcNAc, as shown later, emanates from the lipid A region (Table I) the m/z 464 ion must originate from a terminal Gal-(1→4)-GlcNAc disaccharide epitope. In accordance with methylation linkage analysis, no chains containing terminal GlcNAc were detected in the FAB-MS spectrum, and no chains with terminal epitopes of Lewis X [Gal-(1→4[Fuc-(1→3)]-GlcNAc] or Lewis Y [Fuc-(1→2)-Gal-(1→4)[Fuc-(1→3)]-GlcNAc] type, characteristic markers of other H.pylori strains (Aspinall et al., 1994, 1996; Aspinall and Monteiro, 1996), were observed, implying that the Fuc unit present in the LPS lay elsewhere in the inner regions of the molecule. The composition and linkage analysis results pointed toward the presence of an elongated O-chain polysaccharide with a backbone composed of 3-linked Gal and 4-linked GlcNAc units and with terminal Glc as side chains attached to the branched 4,6-linked GlcNAc residues. The FAB-MS spectrum showed that the O-chain was terminated by a Gal-(1→4)-GlcNAc-(1→) sequence. The minor Glc, Gal, α-D-Hep, and β-D-Hep derivatives attributed to the core region (Table I) were of the same type as found in other H.pylori strains (Aspinall and Monteiro, 1996). The nonequal molar ratio between terminal Glc and 4,6-linked

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GlcNAc pointed towards the presence of one of the terminal Glc residues being present in the core region, hence the placement of one terminal Glc unit in the core region column (Table 1). No core oligosaccharides molecules devoid of O-chain were detected.

The intact LPS was treated with mild acetic acid to remove the lipid A, through cleavage of the acid labile ketosidic linkage of the Kdo to lipid A, and fractionation on a column of Bio-Gel P-2 gave glycan PS-1. Methylation linkage analysis on PS-1 reflected the results obtained from the intact LPS with the exception of a shortfall of terminal Fuc, and the total absence of 6-linked GlcNAc which placed this unit in the lipid A region. Presumably, the alkaline treatment of NaOH hydroxide in DMSO de-O- and de-N-acylates the GlcNAc unit of the lipid A, thus rendering the 6-linked phosphate-free GlcNAc to be fully derivatized in the linkage analysis procedure, or, alternatively the typical lipid A fatty acids are completely absent. An additional treatment of PS-1
with 5% acetic acid at 100°C for 1 h gave defucosylated polysaccharide PS-2 (as shown by the disappearance of the Fuc resonances in the 1H NMR spectrum of PS-2). Methylation linkage analysis of defucosylated PS-2 (Table 1) showed only a slight increase in 4-linked GlcNAc, but the absence of 3,4-linked and 3-linked GlcNAc implied that the Fuc unit had to be attached to O-3 of the branched 3,4-linked GlcNAc.

<table>
<thead>
<tr>
<th>Linkage type</th>
<th>LPS</th>
<th>PS-1 (1% AcOH)</th>
<th>PS-2 (5% AcOH)</th>
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<tr>
<td>O-Chain region</td>
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<tr>
<td>Fuc-(1→)</td>
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<tr>
<td>Glic-(1→)</td>
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<td>4</td>
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<tr>
<td>Gal-(1→)</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>→3)-Gal-(1→)</td>
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<td>7</td>
<td>7</td>
</tr>
<tr>
<td>→4)-GlcNAc-(1→)</td>
<td>4</td>
<td>4</td>
<td>4.2</td>
</tr>
<tr>
<td>→3,4)-GlcNAc-(1→)</td>
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</tr>
<tr>
<td>→4,6)-GlcNAc-(1→)</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Core region</td>
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</tr>
<tr>
<td>→4)-Gal-(1→)</td>
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<td>1</td>
</tr>
<tr>
<td>→7)-Dd-Hep-(1→)</td>
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<tr>
<td>Lipid A region</td>
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</tr>
<tr>
<td>→6)-GlcNAc-(1→)</td>
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</tbody>
</table>

The ratios presented here are approximate values with a ±0.1 deviation. It is worth noting that the late-eluting GlcNAc permethylated alditol acetate derivatives were present in smaller ratios than expected.

The 1H NMR spectrum of the water-soluble intact LPS (Figure 3) showed three dominant anomeric resonances at δ 4.99 (J1,2 3.9 Hz), δ 4.69 (J1,2 6.9 Hz), and at δ 4.47 (J1,2 7 Hz). Due to the broad shape of the anomeric resonances in the 1D spectrum, the stated coupling constants were measured from the H-1,2 cross peaks of the respective 2D COSY experiment (Figure 4). In the upfield region of the 1H NMR spectrum, resonances from the amido-methyl protons of the GlcNAc units at δ 2.01 (s) and from the deoxy-methyl protons of the sole Fuc residue at δ 1.14 (J5,6/6′ 6.1 Hz) confirmed the presence of these units in the LPS. With a combination of 2D COSY (Figure 4) and TOCSY experiments it was possible to assign the α anomeric resonance at δ 4.99 (A) to a Glc unit (H-2 δ 3.56 (J2,3 10 Hz), H-3 δ 3.71 (J3,4 10 Hz), and H-4 δ 3.42 (J4,5 10 Hz)), the β anomeric at δ 4.69 (B) to a Gal unit (H-2 δ 3.79 (J2,3 10 Hz), H-3 δ 4.02 (J3,4 3 Hz), and H-4 δ 3.69 (J4,5 10 Hz)), and the β anomeric at δ 4.47 (C) to a sugar with the GlcNAc configuration (H-2 δ 3.60 (J2,3 10 Hz), H-3 δ 4.13 (J3,4 10 Hz), H-4 δ 3.72 (J4,5 10 Hz), H-5 δ 3.49 (m), H-6 δ 3.89 (dd) and H-6′ δ 3.99 (dd)). Also detected in the 1H NMR spectrum (Figure 3) were anomeric resonances of lower intensity which were attributed to the glycosyl units of the core region, and to one α-Fuc (δ 5.12 (J5,6 13 Hz)) residue as deduced by its disappearance after the 5% acetic acid treatment (PS-2). A 2D NOESY experiment (mixing time 150 ms) showed a strong inter-nOE connectivity between H-1 of the β-Gal at δ 4.69 and H-4 δ 3.72 from the residue with H-1 at δ 4.47. The β-anomeric resonance at δ 4.47 was then assigned to the 4-linked GlcNAc unit and the β anomeric resonance was attributed to the 3-linked Gal and the connection of β-Gal-(1→4)-β-GlcNAc (LacNAc) was established. However, only a very weak inter-nOE connectivity could be detected between H-1 (δ 4.47) of 4-linked GlcNAc and H-3 (δ 4.02) of 3-linked Gal, suggesting that the conformation of the O-chain PS does not allow these two protons to be in close proximity in the β-GlcNAc-(1→3)-β-Gal linkage. In addition, the H-1 at δ 4.99 of α-Glc showed a strong inter-nOE connectivity to H-6 (δ 3.89) and a weak inter-nOE connectivity to H-6′ (δ 3.99) of the glycosyl residue possessing the β anomeric at δ 4.47 which was earlier assigned to the 4-linked GlcNAc. Since no significant differences in neither anomeric resonances nor in ring protons chemical shifts were detected, which might have indicated that 4-linked GlcNAc and 4,6-linked GlcNAc had independent proton chemical shift pathways, and the fact that the α-Glc H-1 (δ 4.99) shows a spatial connectivity to H-6,6′ of the sugar with the H-1 at δ 4.47, it must be concluded that the 4-linked GlcNAc and the 4,6-linked GlcNAc share the same β anomeric resonance at δ 4.47. The α anomeric resonance at δ 4.99 was subsequently assigned to the terminal Glc residue which is attached to the O-6 position of the branched 4,6-linked GlcNAc unit as shown in the arrangement below:

\[
\text{α-Glc} \quad 1 \quad 6
\]

The presence of one unit of 3,4-linked GlcNAc, presumably a β-GlcNAc, shown earlier to carry one terminal α-Fuc at O-3 points towards the presence of one internal Lewis X epitope in the O-chain architecture (shown below). The exact location of this internal Lewis X moiety within the LPS molecule was not established. No double branched 3,4,6-linked GlcNAc derivative was detected.

\[
\text{......} \rightarrow \text{3)-β-Gal-(1→4)-β-GlcNAc-(1→......}
\]

Data from chemical and spectroscopic analysis indicated that the O-antigen chain in the LPS from \textit{H. pylori} UA861 was composed of an elongated, approximately eight LacNAc repeats, type 2 N-acetyllactosamine polysaccharide, [→3]-β-d-Gal-(1→4)-β-d-GlcNAc-(1→4)\text{....}-β-Gal-(1→4)-β-GlcNAc-(1→4)\text{....}, where approximately half the GlcNAc residues carried a terminal α-Glc unit as a side chain (Figure 5). The O-chain was terminated at the non-reducing end by a LacNAc epitope [β-d-Gal-(1→4)-β-d-GlcNAc]. One internal Lewis X epitope [→3)-β-d-Gal-(1→4)-β-d-GlcNAc] was found in the inner regions of the LPS. No core oligosaccharide molecules free of O-chain constituents could be isolated, and thus a detailed structural analysis of the core region was not possible. However, the partially methylated alditol acetates derivatives (Table 1), which originated from the inner regions of the LPS molecule, were of the same type as the derivatives found in other \textit{H. pylori} LPS structures, namely, type strain NCTC11637, P466, and MO19 (Aspinall and Monteiro, 1996; Aspinall et al., 1996). Therefore, it can be concluded that some similarities exist among the core structures of \textit{H. pylori} UA861, NCTC11637, P466, and MO19 (Aspinall and Monteiro, 1996; Aspinall et al., 1996).
Fig. 3. $^1$H NMR spectrum of *H. pylori* UA861 intact water-soluble LPS at 300 K showing the dominant anomeric resonances of terminal $\alpha$-Glc (δ 4.49), and for 4- and 3,4-linked $\beta$-GlcNAc units (δ 4.47). The anomeric resonance of 3-linked Gal (δ 4.69) is under the HOD peak. The upfield region of the spectrum shows the characteristic markers of -COCH$_3$ (δ 2.01) from the GlcNAc units and -C$_6$H$_3$ (δ 1.14) from the sole Fuc residue.

The most striking structural feature of the LPS from *H. pylori* UA861 is the absence of heavily fucosylated type 2 N-acetyllactosamine chains which are typical of *H. pylori* LPSs previously investigated by structural analysis (Aspinall et al., 1994, 1996; Aspinall and Monteiro, 1996). The absence of terminal Lewis X and Y blood-group determinants, and thus the lack of structures mimicking host cell-surface glycoconjugate molecules, in the LPS of *H. pylori* UA861, carries important implications with regard to pathogenic differences among *H. pylori* strains, such as possible bacterial–epithelial cell interactions, and in their role in a postulated autoimmune disease component linked with *H. pylori* strains which carry Lewis blood-group epitopes (Appelmelk et al., 1996). Serological studies that have employed MAbs specific for Lewis blood-group epitopes (Appelmelk et al., 1996) have revealed that some *H. pylori* strains do not show any reactivity with the Lewis blood-group specific MAbs. The presence of terminal Lewis X/Y structures in *H. pylori* UA861 might render this strain to be undetected to some Mabs specific for terminal Lewis blood-group epitopes. Although one would not expect *H. pylori* strains devoid of terminal Lewis epitopes, such as UA861, to cross-react with MABs raised against any terminal Lewis blood-group epitopes in fact this strain did react with MABs for Lewis X, so that the internal $\alpha(1\rightarrow3)$Fuc must be accessible to react both in ELISA and immunoblots (Sherburne and Taylor, 1995). In the case of *H. pylori* UA861, Mabs specific for the terminal LacNAc epitope [$\beta$-$\delta$-Gal-(1→4)$\beta$-$\delta$-GlcNAc] and, if not constrained by the overall polysaccharide conformation, Mabs specific for the internal Lewis X structure $\{\rightarrow4\}[\beta$-$\delta$-Gal-(1→4)$[\alpha$-$\delta$-Fuc-(1→3)]$\beta$-$\delta$-GlcNAc-(1→] are also possible candidates for serological classification of this strain.

This report amends the earlier observation that *H. pylori* UA861 produces an unusual $\alpha(1\rightarrow6)$ galactosyltransferase (Chan et al., 1995). We have now demonstrated that since UA861 carries a terminal $\alpha$-$\delta$-Glc residue at the O-6 position, the particular transferase produced by this strain is likely to be an $\alpha(1\rightarrow6)$ glucosyltransferase rather than a galactosyltransferase. This anomaly arises because of the use of UDP-galactose as the acceptor substrate in the earlier transferase assays (Chan et al., 1995). The structural studies described here must take precedence over transferase assays with chemical substrates; however, direct evidence awaits the cloning and subsequent characterization of the postulated $\alpha(1\rightarrow6)$ glucosyltransferase.

The diversity observed among *H. pylori* LPS structures may be controlled at the genetic level. The genes encoding the fucosyltransferases which control Lewis X and Lewis Y production and the glucosyltransferase described here may be present in some *H. pylori* strains and absent from others. Alternatively, all strains may contain a full complement of transferase genes and other factors may control either their expression or assembly of the
Fig. 4. $^1$H$^1$H COSY spectrum of *H.pylori* UA861 intact water-soluble LPS. The intensity level was fixed to only show the anomeric signals belonging to the O-chain glyco units.

complex carbohydrates. Diversity at both the level of individual genes (microdiversity) as well as in gene order (macrodiversity) in *H.pylori* strains has been described previously (Jiang *et al.*, 1996). The present study demonstrates additional diversity at the level of *H.pylori* LPS biosynthesis.

**Materials and methods**

*Cell growth and generation of lipopolysaccharide (LPS) and polysaccharides PS-1 and PS-2*  
*Helicobacter pylori* UA861 was isolated from endoscopic biopsy specimen obtained from a patient attending the University of Alberta Hospital by methods described previously (Taylor *et al.*, 1987). *H.pylori* UA861 cells were grown as described in Chan *et al.* (1995). Lipopolysaccharide was isolated by the water-phenol extraction of bacterial cells (Westphal *et al.*, 1956). The water-soluble LPS was purified on a column of Bio-Gel P-2 (1 cm × 1 m) with water as eluent to give one fraction (LPS) at the void-volume detected by the phenol-sulfuric acid assay (Dubois *et al.*, 1956). The LPS preparation was treated with 1% acetic acid at 100°C for 1 h, and subsequent removal of the insoluble lipid A was achieved by centrifugation (5000 × g). The supernatant was purified on a column of Bio-Gel P-2 with water (1 ml/tube) as the eluent to give glycan PS-1. Defucosylated glycan, PS-2, was generated by treating PS-1 with 5% acetic acid at 100°C for 1 h followed by fractionation on Bio-Gel P-2.

*Sugar composition and methylation linkage analysis*  
Sugar composition analysis was done by the alditol acetate method (Sawardeker *et al.*, 1967). The hydrolysis was done in 4 M trifluoroacetic acid at 100°C for 4 h followed by reduction in H$_2$O with NaBD$_4$ and subsequent acetylation with acetic
anhydride and with residual sodium acetate as the catalyst. Characterization of the alditol acetate derivatives was done by gas-liquid-chromatography–mass-spectrometry (GLC-MS) using a Hewlett-Packard chromatograph equipped with a 30 m DB-17 capillary column (180°C → 240°C at 2°C/min) and MS in the electron impact (EI) mode was recorded using a Varian Saturn II mass spectrometer. Enantiomeric configurations of the individual sugars were determined by the formation of the respective 2-((S)- and 2-((R))-butyl glycosides (Leotein et al., 1978). Methylation linkage analysis was carried out by the NaOH/DMSO/Mel procedure (Ciucanu and Kerek, 1984) and with full characterization of permethylated alditol acetate derivatives by GLC-MS in the EI mode (DB-17, isothermally at 190°C for 60 min). A fraction (1/4) of the methylated sample was used for positive ion fast atom bombardment–mass spectrometry (FAB-MS) which was carried out on a Jeol JMS-AX505H mass spectrometer with glycerol(1):thiglycerol(3) as the matrix and 3 kV as the tip voltage.

**Nuclear magnetic resonance spectroscopy (NMR)**

One-dimensional (1D) and 2D ¹H NMR experiments were recorded on a Bruker AMX 500 spectrometer at 300 K using standard Bruker software. Prior to performing the NMR experiments the samples were lyophilized thrice with D₂O (99.9%). The HOD peak was used as the internal reference at δH 4.786. 2D homonuclear correlation (COSY), total correlation (TOCSY), and nuclear Overhauser effect (NOESY) experiments were done using standard parameters (Masoud et al., 1993). The mixing time for the TOCSY and NOESY experiments was 45 and 150 ms, respectively.

**Acknowledgments**

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**Abbreviations**

DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; FAB-MS, fast atom bombardment–mass spectrometry; GLC-MS, gas liquid chromatography–mass spectrometry; LPS, lipopolysaccharide; NaBD₄, sodium borodeuteride; NaOH, sodium hydroxide; NMR, nuclear magnetic resonance; PS, polysaccharide.

**References**


