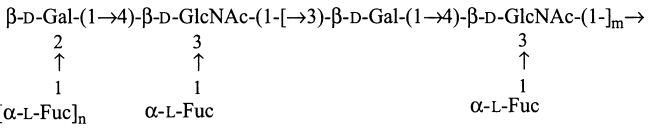
Eur. J. Biochem. **266**, 123–131 (1999) © FEBS 1999

Structural studies on lipopolysaccharides of serologically non-typable strains of *Helicobacter pylori*, AF1 and 007, expressing Lewis antigenic determinants

Yuriy A. Knirel[1,2], Nina A. Kocharova[1,2], Sean O. Hynes[3], Göran Widmalm[4], Leif P. Andersen[5], Per-Erik Jansson[1] and Anthony P. Moran[3]

[1]*Karolinska Institute, Clinical Research Center, Huddinge University Hospital, Sweden;* [2]*N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow, Russia;* [3]*Department of Microbiology, National University of Ireland, Galway, Ireland;* [4]*Department of Organic Chemistry, Arrhenius Laboratory, Stockholm University, Sweden;* [5]*Department of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark*

In contrast to other *Helicobacter pylori* strains, which have serologically detectable Lewis^x (Le^x) and Lewis^y (Le^y) antigenic determinants in the O-specific polysaccharide chains of the lipopolysaccharides, *H. pylori* AF1 and 007 were non-typable with anti-Le^x and anti-Le^y antibodies. The carbohydrate portions of the lipopolysaccharides were liberated by mild acid hydrolysis and subsequently studied by sugar and methylation analyses, ^{1}H-NMR spectroscopy and electrospray ionization-mass spectrometry. Compared with each other, and with lipopolysaccharides of strains studied previously, the lipopolysaccharides of both AF1 and 007 showed similarities, but also differences, in the structures of the core region and O-specific polysaccharide chains. The O-specific polysaccharide chains of both strains consisted of a short or long polyfucosylated poly-*N*-acetyl-β-lactosamine chains, which were distinguished from those of other strains by a high degree of fucosylation producing a polymeric Le^x chain terminating with Le^x or Le^y units:



where n = 0 or 1 in strain AF1 and 0 in strain 007, m = 0–2, 6–7 in strain AF1 and m = 0–2, 6–7 or ≈ 40 in strain 007, the medium-size species being predominant. Therefore, compared with other strains, the lack of reactivity of lipopolysaccharide of *H. pylori* AF1 and 007 with anti-Le^x and anti-Le^y may reflect the presence of a polymeric Le^x chain and has important implications for serological and pathogenesis studies. As the substitution pattern of a D-*glycero*-D-*manno*-heptose residue in the outer core varied in the two strains, and an extended DD-heptan chain was present in some lipopolysaccharide species but not in others, this region was less conservative than the inner core region. The inner core L-*glycero*-D-*manno*-heptose region of both strains carried a 2-aminoethyl phosphate group, rather than a phosphate group, as reported previously for other *H. pylori* strains.

Keywords: core oligosaccharide; *Helicobacter pylori*; lipopolysaccharide; O-antigen; O-specific polysaccharide.

Helicobacter pylori, the causal agent of chronic gastritis, is an important human gastric pathogen [1]. Infection with this bacterium is associated with a broad spectrum of clinical outcomes [2], including the development of peptic ulcers and an increased risk for the development of gastric cancer. Like the cell envelope of other gram-negative bacteria, that of *H. pylori* contains lipopolysaccharides (LPS). Fresh clinical isolates of *H. pylori* produce high molecular mass smooth-form LPS, which consists of an O-specific polysaccharide (OPS), a core oligosaccharide and lipid A [3,4].

Structural studies on the LPS of a number of *H. pylori* strains have shown that they exhibit mimicry of human cell surface glycoconjugates because of the presence of Lewis^x (Le^x) and/or Lewis^y (Le^y) blood group antigenic determinants [5–10]. These are formed by monofucosylated or difucosylated *N*-acetyl-β-lactosamine (LacNAc) units attached to the LPS core, or by the same units placed at the nonreducing end of the OPS. Hence, the OPS of *H. pylori* LPS represents a chain of fucosylated poly LacNAc [11], which in some strains is decorated with lateral glucose or galactose residues [8,9,11]. In addition, Lewis^a, Lewis^b and H type 1 antigenic determinants in the OPS chains of certain *H. pylori* strains have been found [10]. Another feature of some *H. pylori* strains is the presence of an additional chain of D-*glycero*-D-*manno*-heptose (DD-Hep) which has been reported to connect the OPS to the LPS core [7].

Although the pathogenic relevance of Lewis antigen mimicry in *H. pylori* remains unclear [12,13], serological investigations

Correspondence to P.-E. Jansson, Karolinska Institute, Clinical Research Center, Novum, Huddinge University Hospital, S-141 86 Huddinge, Sweden. Fax: +46 85 858 3820, Tel.: +46 85 858 3821, E-mail: pererik.jansson@kfcmail.hs.sll.se

Abbreviations: DD-Hep, D-*glycero*-D-*manno*-heptose; ESI-MS, electrospray ionization mass spectrometry; Hex, hexose; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; LacNAc, *N*-acetyl-β-lactosamine; LD-Hep, L-*glycero*-D-*manno*-heptose; Le^x, Lewis^x; LPS, lipopolysaccharide; OPS, O-specific polysaccharide; *P*Etn, 2-aminoethyl phosphate.

(Received 13 August 1999, accepted 1 September 1999)

using anti-Lewis antibodies have detected the expression of the respective Lewis antigens, in support of the chemical studies [10,14–17]. Moreover, serotyping of *H. pylori* strains on the basis of reactions with a panel of anti-Lewis mAb has been proposed [15]. In this paper we report chemical studies on the LPS of two new *H. pylori* strains, AF1 and 007, which are distinguished by their non-typability with anti-Le^x and anti-Le^y mAb. Despite this, the LPS were found to possess these terminal Lewis antigenic determinants.

MATERIALS AND METHODS

Bacterial strains, cultivation, isolation and degradation of LPS

H. pylori strains 007 and AF1 were isolated from gastric biopsies of patients at the Department of Microbiology, Rigshospitalet, Copenhagen, Denmark. Growth of the bacterial strains and isolation of LPS by extraction with aqueous phenol [18] were performed as described previously [3]. LPS were recovered from the water phase in yields of 3.9 and 4.0% of dried cells, respectively, and subjected to mild acid hydrolysis with 0.1 M sodium acetate buffer, pH 4.2, for 2 h at 100 °C. Precipitate was removed by centrifugation (10 000 *g*, 30 min) and the water-soluble carbohydrate portion was fractionated by gel-permeation chromatography on a column of Sephadex G-50 (Pharmacia, Uppsala, Sweden) using 0.05 M pyridinium acetate, pH 4.5, as the eluent; monitoring was performed with a Waters differential refractometer (Milford, MA). After pooling, six fractions, I–VI, were obtained for AF1 and five fractions, I–V, for 007; their relative elution volumes and yields are given in Table 1. Of these AF-II, AF-VI, 007-II, 007-III, 007-IV and 007-V were analysed using 600 MHz NMR spectroscopy and the others with 270 MHz NMR only.

Electrophoretic and serological analyses

The LPS preparations were fractionated by SDS/PAGE [19] using the gel systems and voltage conditions described previously [3]. After electrophoresis, LPS was detected by silver staining [20]. Alternatively, the fractionated LPS was electroblotted onto nitrocellulose membranes [21] and Western blots visualized with mouse mAb against Lewis antigens, anti-Le^x, -Le^y, -Le^a, -Le^b or -H type I antigen (Signet Laboratories, Dedham, MA), or against blood group determinants, anti-A, -B or -AB (Immunocor, Norcos, Ga.), diluted 1 : 1000 as primary antibody and peroxidase-conjugated goat anti-(mouse IgM) (Sigma Chemical Co.) diluted 1 : 1000 as the secondary antibody [17]. In addition, ELISA with bacterial whole cells [16] was used as described previously [17] to examine the reaction of the anti-Lewis and anti-(blood group) with *H. pylori* AF1 and 007. The specificities of the antibodies in the assay were validated by their ability to bind the respective antigen from a panel of synthetic blood group antigens and the LPS of other *H. pylori* strains of known structure [17].

Sugar analysis

Hydrolysis to liberate sugars was performed with 2 M trifluoroacetic acid (120 °C, 2 h). The hydrolysate was subsequently evaporated and monosaccharides were identified by GLC of the derived alditol acetates [22] using a Hewlett-Packard 5880 instrument with a DB-5 column. The absolute configurations of the monosaccharides were determined as described previously [23].

Table 1. Fractionation of acid-degraded LPS on Sephadex G-50. Void and full volumes of the column are 105 and 300 mL, respectively. Elution volumes are relative to the void volume and refers to the top of peaks.

	Fraction					
H. pylori strain	I	II	III	IV	V	VI
	Relative elution volume					
AF	1.03	1.31	1.76	2.08	2.24	2.44
007	1.05	1.32	1.82	2.08	2.25	
	Yield (% of LPS weight)					
AF1	< 1	28.0	3.1	< 1	3.4	7.4
007	2.0	21.6	5.2	6.2	3.8	

Methylation analysis

Methylation was carried out using methyl iodide in dimethylsulfoxide in the presence of sodium methylsulfinylmethanide [24]. Derived, partially methylated alditol acetates were identified with GC-MS using a NERMAG R10-10L mass spectrometer. Hydrolysis was performed as in sugar analysis, partially methylated monosaccharides were reduced with $NaBH_4$, converted to alditol acetates and analysed by GLC/MS on a Hewlett-Packard 5890 chromatograph equipped with a NERMAG R10-10L mass spectrometer and a DB-5 fused-silica capillary column using a temperature gradient of 130 °C (1 min) to 250 °C at 3 °C·min^{-1}. Identification was performed using published data [25,26].

Other chemical modifications

Fraction AF1-VI (0.5 mg) was treated with aqueous 48% hydrofluoric acid for 16 h at ambient temperature, the sample was evaporated in a stream of nitrogen, dissolved in water and freeze-dried to give the dephosphorylated oligosaccharide.

NMR spectroscopy

All saccharide samples were exchanged twice with ^2H_2O (99.9%), lyophilized and dissolved in ^2H_2O (99.96%). ^{1}H-NMR spectra of ^2H_2O solutions were recorded with a JEOL EX-270 instrument (Tokyo, Japan) at 75 °C or a Varian Inova 600 instrument (Palo Alto, CA) at 25 °C. Assignment of ^{1}H-NMR signals was performed using double-quantum filtered COSY and TOCSY experiments at 600 MHz. Chemical shifts were measured in p.p.m., relative to sodium 3-trimethylsilylpropanoate-d_4 as internal standard (δ_H 0.00).

Electrospray ionization-MS

ESI-MS was performed in the negative mode using a VG Quattro triple quadrupole mass spectrometer (Micromass, Altrincham, UK) with acetonitrile as the mobile phase at a flow rate of 10 μL·min^{-1}. Samples were dissolved in aqueous 50% acetonitrile at a concentration of ≈ 50 pmol·μL^{-1} and 10 μL was injected via a syringe pump into the electrospray source.

RESULTS

Serological analyses of LPS

Analysis of the isolated LPS of *H. pylori* AF1 and 007 by SDS/PAGE and silver staining confirmed the expression of high

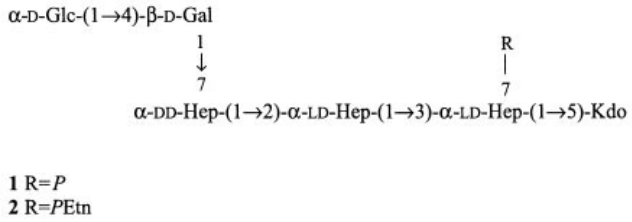


Fig. 1. Structures of the truncated core oligosaccharides 1 and 2 from *H. pylori* NCTC 11637 [5] and AF1 (fraction AF1-VI), respectively.

molecular mass LPS as reported previously [3,4]. In Western blotting, although anti-Le^x mAb reacted with control *H. pylori* LPS known to express Le^x [5], no reaction was observed with LPS of *H. pylori* AF1 and 007 (data not shown). Similarly, no reaction of these latter LPS were observed with mAb against other Lewis antigens (anti-Le^x, -Le^y, -Le^a, -Le^b or -H type I) or against blood group determinants (anti-A, -B or -AB). In case of chemical modification of LPS or selection of a particular LPS molecular species during extraction, these same mAb were tested for reaction with whole cells of *H. pylori* AF1 and 007 in an ELISA system. Confirming the results of Western blotting, no reaction of the panel of antibodies with these strains in the ELISA was observed.

Mild acid hydrolysis of LPS

Delipidation by acid treatment of LPS from the two strains of *H. pylori* under mild conditions resulted in a number of polysaccharides and oligosaccharides which were fractionated by gel-permeation chromatography on Sephadex G-50. The LPS of *H. pylori* AF1 gave six fractions designated AF1-I to AF1-VI according to their order of elution (Table 1); fractions AF1-I and AF1-IV were present in low amounts and were not studied further. The LPS of *H. pylori* 007 gave five fractions designated 007-I to 007-V which had practically the same elution times as the corresponding AF1 fractions, except for fraction 007-III which eluted later (Table 1). Further studies showed that the corresponding fractions from the two strains were also structurally similar (see below).

The fractions obtained were studied using sugar and methylation analyses, ^{1}H-NMR spectroscopy and, in addition, low-molecular mass fractions were also analysed by ESI-MS. Preliminary data suggested that the LPS of the two strains possess a structure similar to that reported for *H. pylori* serogroup O:3 LPS [7]. This structure is characterized by a core oligosaccharide occasionally substituted with an α-glucan, an α-D-*glycero*-D-*manno*-heptan (D,D-heptan) and OPS chains of polyfucosylated poly LacNAc chains, which are terminated with either the Le^x antigenic determinant, β-Gal-(1→4)-[α-Fuc-(1→3)]-β-GlcNAc, or Le^y antigenic determinant, α-Fuc-(1→2)-β-Gal-(1→4)-[α-Fuc-(1→3)]-β-GlcNAc. Sugar analysis (Table 2) showed significant amounts of D-glucose (D-Glc), D-*glycero*-D-*manno*-heptose (DD-Hep) and L-*glycero*-D-*manno*-heptose (LD-Hep) in all fractions and *N*-acetyl-D-glucosamine (D-GlcNAc) and L-fucose (L-Fuc) in fractions I to V. Fractions AF1-III and 007-III to 007-V contained relatively large amounts of Glc and DD-Hep, thus suggesting the occurrence of glucan and DD-heptan chains. The content of LD-Hep, which is a typical component of the *H. pylori* LPS inner core component [5,7], increased with decreasing molecular mass. The linkage between the O-chain and the core, and between the glucan and the core, are adapted from previous data as no structural evidence could be obtained in the present studies.

Methylation analysis (Table 3) confirmed these results and showed that in fractions enriched in DD-Hep and Glc, the former is mainly 3-substituted and the latter mainly 6-substituted. Typical OPS chain components, terminal Fuc and 3,4-disubstituted GlcNAc, were present in fractions I–IV, and 3-substituted Gal was present in fractions I–III from both strains.

Fraction AF1-VI

Methylation analysis of fraction AF1-VI revealed terminal Glc, 4-substituted Gal, 2-substituted LD-Hep and 7-substituted DD-Hep, together with a smaller amount of 3-substituted LD-Hep. Consistent with phosphorylation at position 7 [5,9], the content of 3-substituted heptose increased upon dephosphorylation up to the level of the other heptose derivatives. ESI-MS demonstrated intense singly and doubly charged pseudomolecular ions, $[M\text{-}H]^-$ and $[M\text{-}2H]^{2-}$, at *m/z* 1242.4 and 620.8, respectively. These ions correspond to a Hex_2Hep_3AnKdo*P*Etn oligosaccharide (where AnKdo is an anhydro form of 3-deoxy-D-*manno*-oct-2-ulosonic acid and *P*Etn is 2-aminoethyl phosphate), having a calculated molecular mass of 1244 Da. However, as expected, the ESI mass spectrum of dephosphorylated fraction AF1-VI showed two peaks of $[M\text{-}H]^-$ ions at *m/z* 1119.3 (major) and 1137.4 (minor), which corresponded to Hex_2Hep_3AnKdo and Hex_2Hep_3Kdo oligosaccharides with calculated molecular masses of 1121 and 1139 Da, respectively.

Table 2. Data of sugar analysis. GLC retention times for the corresponding alditol acetates are referred to fucitol pentaacetate (1.00); peak areas are given as percentage of the sum of detector response.

		Fraction from LPS of *H. pylori* strain								
		AF1				007				
Sugar	Relative retention time	II	III	V	VI	I	II	III	IV	V
L-Fuc	1.00	25	12	13	0	15	21	13	10	10
D-Glc	1.60	17	30	21	25	45	19	30	33	43
D-Gal	1.62	28	14	24	22	18	24	12	9	9
D-GlcN	1.98	13	11	12	0	16	13	11	9	8
DD-Hep	2.11	16	28	17	27	6	21	30	33	19
LD-Hep	2.17	2	5	13	27	0	2	5	6	11

Table 3. Data of methylation analysis. GLC retention times, for the corresponding alditol acetates, are referred to 2,3,4-tri-*O*-methylfucose (2,3,4-Me_3-Fuc, 1.00) and glucitol hexaacetate (2.67); peak areas are given as percentage of the sum of detector response. TR, trace amount.

		Fraction from LPS of *H. pylori* strain								
	Relative retention time	AF1				007				
Sugar		II	III	V	VI	I	II	III	IV	V
2,3,4-Me_3-Fuc	1.00	23	5			18	12	6	3	
2,4-Me_2-Fuc	1.27	2	5	7		2	3	4	4	9
2,3,4,6-Me_4-Glc	1.33	5	3	14	18	2	2	5	3	14
2,3,4,6-Me_4-Gal	1.38	6	3	1	TR	3	4	3	5	TR
2,4,6-Me_3-Glc	1.66		5			0	3	5	5	
2,3,6-Me_3-Gal	1.67		5	13	23	5	7	3	5	
2,3,6-Me_3-Glc	1.70	TR	5	6		24	2	0		2
3,4,6-Me_3-Gal	1.72	1	3							
2,4,6-Me_3-Gal	1.73	14	6			21	10	4		
2,3,4-Me_3-Glc	1.77	23	19	9		6	16	20	25	34
2,3,4,6,7-Me_5DD-Hep	1.85			7			1		2	
3,4,6,7-Me_4DD-Hep	2.19	3	6	2		2	12	7	6	2
2,3,4,7-Me_4DD-Hep	2.25	5	6	3			4	5	6	11
2,4,6,7-Me_4DD-Hep	2.26	4	17	0		5	15	19	17	3
3,4,6,7-Me_4LD-Hep	2.31	2	4	14	28		2	4	6	9
2,3,4,6-Me_4DD-Hep	2.37			9	26			2	1	TR
2,4,6,7-Me_4LD-Hep	2.38	TR	1	2	5		TR	1	1	2
3,4,6-Me_3DD-Hep	2.71	4	6	12			3	4	6	10
2,4,6-Me_3-GlcN	2.97	TR	1	1			TR	3	2	4
2,6-Me_2-GlcN	3.13	7	2			13	6	6	2	0

The presence of the 2-aminoethyl group was further confirmed by the ^{1}H-NMR spectrum of fraction AF1-VI which displayed a spin system of two CH_2 groups at δ 3.30 (CH_2N) and 4.14 (CH_2O). In the anomeric region of the ^{1}H-NMR spectrum, there were signals for H-1 of β-Gal at δ 4.52 (d, $J_{1,2}$ 7 Hz), α-Glc at δ 4.94 (d, $J_{1,2}$ 4 Hz) and two Hep at δ 5.06 (nonresolved) and 5.43 (d, $J_{1,2}$ 1 Hz). The H-1 signal for the third Hep residue was split into at least two signals at δ 5.36 and 5.45 (both nonresolved), most likely owing to its attachment to the Kdo residue which existed in multiple forms.

These data are similar to those published for the truncated core oligosaccharide **1** from LPS of the *H. pylori* type strain NCTC 11637 [5], except that 2-aminoethyl phosphate is present in fraction AF1-VI rather than a phosphate group [5]. *P*Etn also occurred in all other products derived from *H. pylori* AF1 and 007 LPS (see below). Therefore, the truncated core oligosaccharide of fraction AF1-VI has structure **2** (all monosaccharide residues are in the pyranose form; Fig. 1).

Fraction 007-V

Methylation analysis of fraction 007-V indicated the presence of terminal Fuc, terminal Glc, 4-substituted Gal, 6-substituted Glc, 6-substituted DD-Hep, 2,7-disubstituted DD-Hep, 2-substituted LD-Hep and 3-substituted GlcNAc as the main components (Table 3). The terminal Fuc residue gave a 2,4-di-*O*-methylfucose, as observed previously and this is suggested to result from incomplete methylation [5,9]. The presence of a significant amount of 6-substituted Glc showed the presence of a short (1→6)-linked glucan chain. The absence of the expected derivative of a 3,7-disubstituted LD-Hep residue was attributed to the presence of phosphate at position 7 which is difficult to remove by hydrolysis. A minor derivative from 3-substituted LD-Hep appeared on the account of partial dephosphorylation under the conditions of methylation.

In addition to the signals found in the ^{1}H-NMR spectrum of fraction AF1-VI, the anomeric region of fraction 007-V contained signals for H-1 of β-GlcNAc at δ 4.60 (d, $J_{1,2}$ 7.5 Hz), α-Fuc at δ 5.15 (d, $J_{1,2}$ 4 Hz) and a number of α-Glc residues at δ 4.95–5.02 (d, $J_{1,2}$ 3.5–4 Hz). Signals for Hep residues were observed at δ 5.01, 5.12 and 5.36–5.46. There were also characteristic signals for one *N*-acetyl group of GlcNAc at δ 2.05 (3H, s), H-6 and H-5 of Fuc at δ 1.15 (3H, d, $J_{5,6}$ 6 Hz) and 4.36 (q), respectively, and *P*Etn at δ 3.29 (CH_2N) and 4.12 (CH_2O).

ESI-MS data were in agreement with the methylation analysis and NMR spectroscopic data. The spectrum contained, as the main peak, a doubly charged pseudomolecular ion, $[M-2H]^{2-}$, at *m/z* 891.5 deriving from a $FucGlcNAcHex_2Hep_4AnKdoPEtn$ oligosaccharide with a calculated molecular mass of 1785.5 Da, and a less intense peak at *m/z* 972.4 from an oligosaccharide containing one additional Hex (Fig. 2). A number of minor peaks at *m/z* 1053.4, 1134.5, 1216.1, 1297.5, 1459.3, 1540.6 and 1621.3 arose from elongation of the glucan chain by one Glc residue up to a total of 11 residues, and those at *m/z* 810.4 and 729.3 from loss of Glc and both Glc and Gal residues, respectively. Each peak was accompanied by a smaller peak of the corresponding compound containing Kdo instead of AnKdo.

These data show that the main oligosaccharide in fraction 007-V is similar, but not identical, to oligosaccharide **3** identified as a component in a mixture from LPS of *H. pylori* NCTC 11637 [5]. The two compounds differed in the presence not only of *P*Etn, but also of 6-substituted DD-Hep instead of 7-substituted DD-Hep, and in the lack of one of the Glc residues. Therefore, structure **4** could be proposed for this oligosaccharide (Fig. 3). Higher oligosaccharide components of

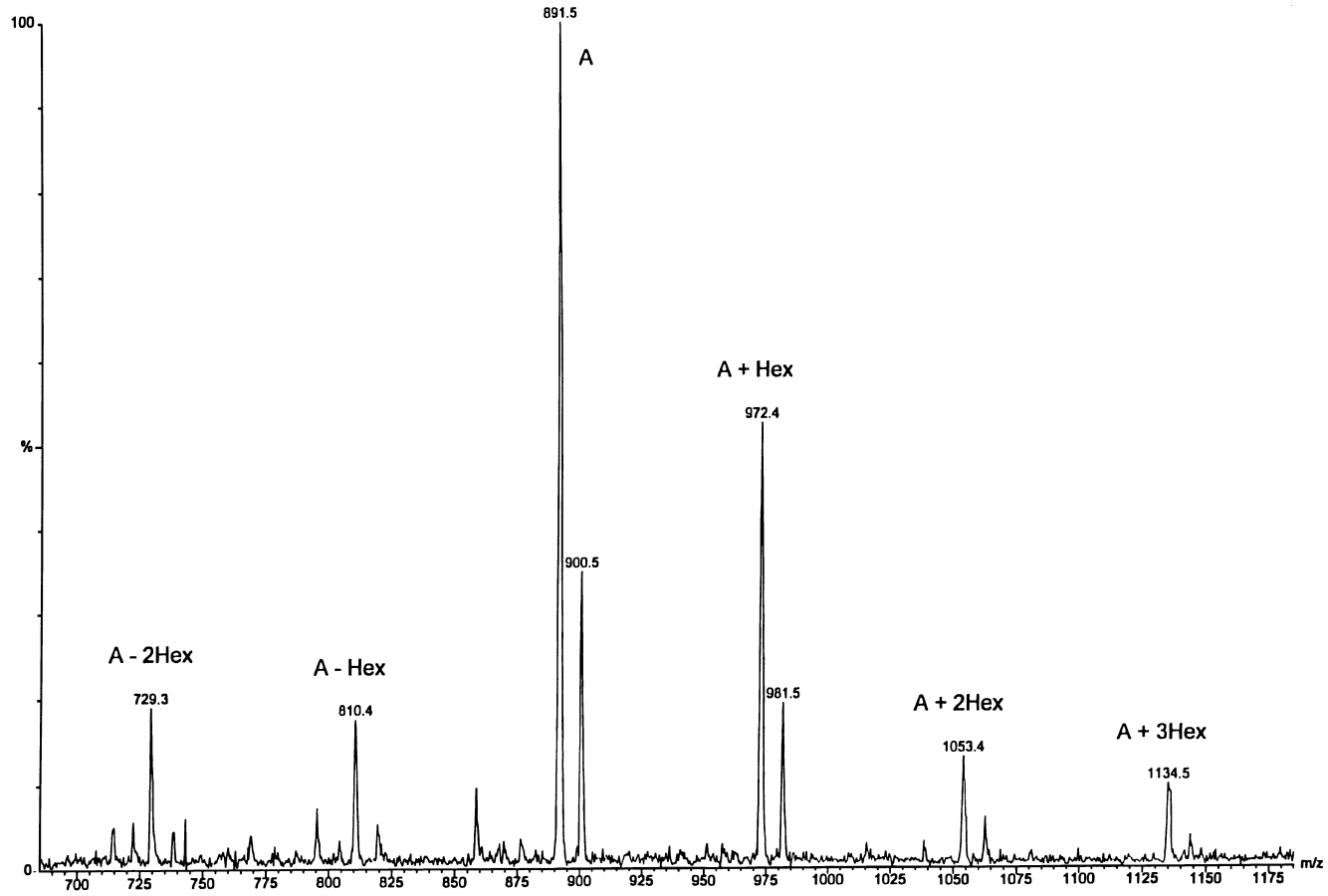


Fig. 2. The region of doubly charged pseudomolecular ions, $[M-2H]^{2-}$, in the negative mode ESI-MS spectrum of fraction 007-V from *H. pylori* 007. Compound A has structure **4** shown in Fig. 3; Hex, hexose.

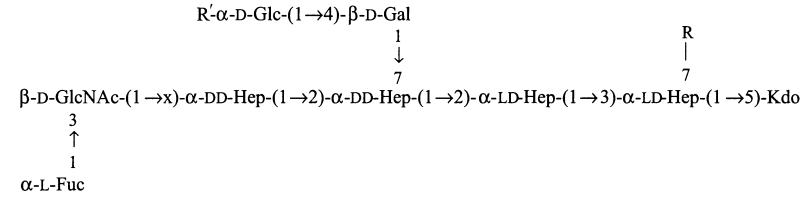
fraction 007-V had longer glucan chains. Because the three major methylated heptose derivatives were present in similar amounts, it is suggested that the terminal Glc residue in oligosaccharide **4** is the site of the glucan chain elongation, rather than a Hep residue as reported [5,7] and shown in Fig. 1. It should be mentioned that an α-Fuc-(1→3)-β-GlcNAc disaccharide fragment is present in **4** as well as in all LPS-derived products, except for fraction AF1-VI, and seems to be a core component of *H. pylori* LPS.

Fraction AF1-V

ESI-MS studies of fraction AF1-V revealed the presence of an oligosaccharide having the same molecular mass as oligosaccharide **4**, a lower content of glucan-containing higher oligosaccharides with, on average, shorter glucan chains with up to seven Glc residues and contamination by oligosaccharide **2** from fraction AF1-VI. The ^{1}H-NMR spectrum contained all the characteristic signals for, and thus confirmed the presence of, a major oligosaccharide similar or identical to **4**. However, methylation analysis revealed a difference from fraction 007-V due to the presence of 7-substituted DD-Hep rather than 6-substituted DD-Hep. Hence, structure **5** can be inferred as the main oligosaccharide present in fraction AF1-V. A significant amount of terminal DD-Hep may indicate incomplete substitution with the Fuc→GlcNAc fragment, in accordance with the relatively low content of methylated derivatives from this disaccharide; however, the occurrence of such a truncated core oligosaccharide could not be confirmed by ESI-MS data.

Fraction 007-IV

In addition to the core and glucan components present in fraction 007-V, methylation analysis of fraction 007-IV



3 R=*P*, x=7, R′=α-D-Glc-(1→3)-

4 R=*P*Etn, x=6, R′=H

5 R=*P*Etn, x=7, R′=H

Fig. 3. Structures of the core oligosaccharides 3–5 from *H. pylori* NCTC 11637 [5], 007 (fraction 007-V), and AF1 (fraction AF1-V), respectively.

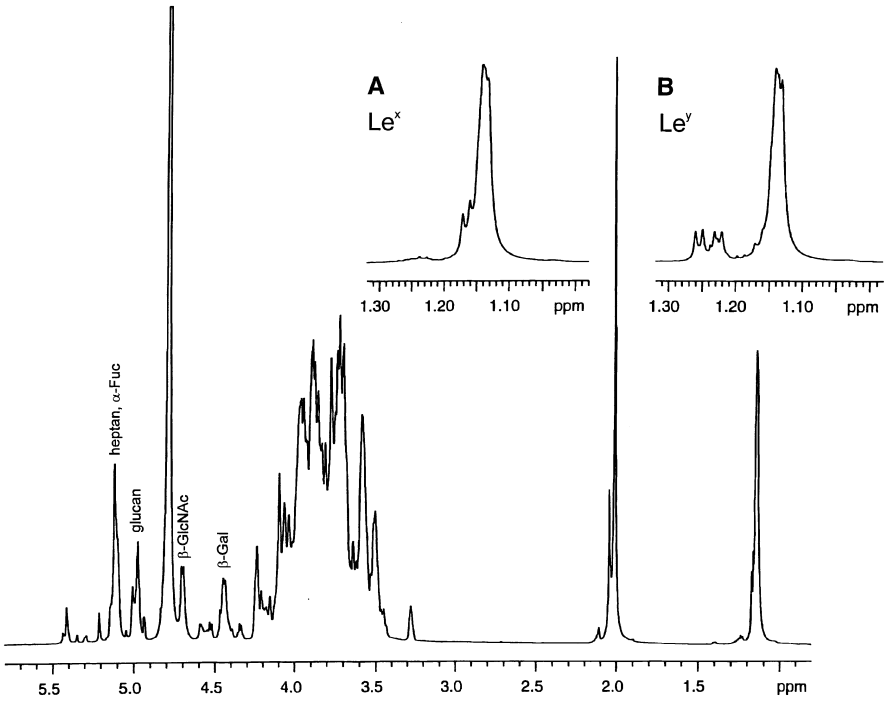


Fig. 4. ^{1}H-NMR spectrum of fraction 007-II from *H. pylori* 007. Insets, the resonance region of H-6 of Fuc residues of 007-II (A) and AF1-II (B). Apart from the H-6 region the spectrum of AF1-II is similar to that of 007-II4. Relevant chemical shift data can be found in [5–7].

revealed several other methylated monosaccharides (Table 3). Of these, 2-substituted and 3-substituted DD-Hep originated from a DD-heptan and indicated further extension of the core heptose region, and 3-substituted Glc may be a component of a glucan chain (compare with structure in Fig. 3). Additional components were also terminal Fuc, terminal Gal and 3,4-disubstituted GlcNAc which formed a single terminal OPS repeating unit having the structure of a Le^x trisaccharide.

Consistent with these findings, the ^{1}H-NMR spectrum of fraction 007-IV, compared with the spectrum of fraction 007-V, contained more signals, including those for additional residues of β-Gal (H-1 at δ 4.45), β-GlcNAc (H-1 at δ 4.56) and α-Fuc (H-6 of two Fuc residues at δ 1.16 and 1.18, both d, $J_{5,6}$ 6–6.5 Hz). Signals for H-1 of glucan and DD-heptan were at δ 4.97–5.03 and 5.10–5.14, respectively, the latter coinciding with the H-1 signal of one of the Fuc residues. Attempts to obtain an ESI mass spectrum for fraction 007-IV, as well as for higher molecular mass fractions 007-II and 007-III, failed.

Fraction 007-III

Fraction 007-III contained glucan and DD-heptan chains, the heptan somewhat longer than the glucan, as inferred in the methylation analysis from the amounts of 6-substituted Glc on the one hand, and 2-substituted and 3-substituted DD-Hep on the other, and also a short OPS chain. The methylation analysis also displayed typical core and OPS components (Table 3). The core components were similar to those found in fraction 007-IV. The OPS components were terminal Fuc, terminal and 3-substituted Gal, and 3,4-disubstituted GlcNAc. As judged by the ratio of the methylated Fuc and Gal derivatives, the OPS chain contained two repeating units, one terminal and one interior. Accordingly, the ^{1}H-NMR spectrum of fraction 007-III contained signals for H-6 of three Fuc residues at δ 1.15 (6H) and 1.17 (3H), three N-acetyl groups at δ 2.01 (3H) and 2.05 (6H) and one CH_2NH_2 group of *P*Etn at δ 3.29 (2H).

Fraction AF1-III

Sugar and methylation analyses showed that fraction AF1-III differed from fraction 007-III in the lower content of terminal Gal and the appearance of 2-substituted Gal, the ratio of 3-substituted and 2-substituted Gal was ≈ 2 : 1 (Table 3). The ^{1}H-NMR spectrum of this fraction showed signals for H-6 of Fuc at δ 1.14–1.25, N-acetyl groups at δ 2.01 and 2.05 and a CH_2NH_2 group of *P*Etn at δ 3.29 with the integral intensities indicating that these groups were present in the approximate ratio 4 : 4 : 1. From the signals for Fuc H-6, two were at lower field at δ 1.22 and 1.25 and, thus, belonged to a terminal Le^y tetrasaccharide unit. Two other Fuc H-6 signals at δ 1.14 and 1.16, which were twice as intense, were almost at the same positions as in the spectrum of fraction 007-III. These data suggest that fraction AF1-III contained short OPS chains having two or three repeating units; most terminated with a Le^y tetrasaccharide, others with a Le^x trisaccharide.

Fraction AF1-II

This fraction was distinguished by the presence of long OPS and glucan chains and the absence of a long DD-heptan chain (Table 3). The presence of 3,4-disubstituted GlcNAc and the absence of 4-substituted GlcNAc showed that all repeating units in the OPS are fucosylated, and the presence of 2-substituted Gal was indicative of the occurrence of a Le^y unit at the nonreducing end of the OPS. The ^{1}H-NMR spectrum showed the α-Glc H-1 signals from glucan at δ 4.98–5.01 and the major H-1 signals from the OPS for α-Fuc at δ 5.10, β-Gal at δ 4.44 and β-GlcNAc at δ 4.70, and for H-6 of α-Fuc at δ 1.13. Two minor signals for H-6 of Fuc at δ 1.22 and 1.25 were from the terminal Le^y tetrasaccharide (Fig. 4), as confirmed by a COSY experiment which revealed all expected characteristic Fuc H6/H5 cross-peaks at δ 1.13/4.80 (major), 1.14/4.33, 1.22/4.87 and 1.25/4.24 (all minor), from the interior repeating units (polymeric Le^x), core oligosaccharide (compare data for fraction AF1-VI), and two from the terminal Le^y unit,

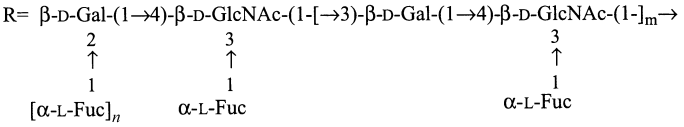


Fig. 5. Structures of the O-chain polysaccharides of *H. pylori* AF1 and 007. n = 0 or 1 in strain AF1 and 0 in strain 007, m = 0–2, 6–7 in strain AF1 and m = 0–2, 6–7 or about 40 in strain 007, the medium-size species being predominant.

respectively. One more minor signal at δ 1.16 was from the terminal Le^x trisaccharide unit (see below). The ratios of the integral intensities of the major and minor H-6 signals of Fuc and the signal for CH_2N of *P*Etn showed the presence of seven repeating units in the OPS on average, about two-thirds of the chains being terminated with the Le^y tetrasaccharide and one-third with the Le^x trisaccharide.

Fraction 007-II

Fraction 007-II, like fraction AF1-II, was the predominant OPS-containing fraction. In addition to a long-chain OPS, it contained glucan and DD-heptan chains (Table 3). Again, 4-substituted GlcNAc was absent, thus indicating complete fucosylation in the OPS chain. The absence of 2-substituted Gal suggested that the nonreducing end of the OPS has a terminal Le^x unit. Notably, DD-heptan included both 2-substituted and 3-substituted Hep residues, the latter being predominant. In the ^{1}H-NMR spectrum (Fig. 4), there were the same major OPS signals as in OPS from *H. pylori* AF1, but only one minor H-6 signal at δ 1.16, which belonged to Fuc from the terminal Le^x trisaccharide. The COSY spectrum showed the expected cross-peaks at δ 1.13/4.79 (major), 1.14/4.31 and 1.16/4.83 (both minor) from the interior repeating units (polymeric Le^x), the core oligosaccharide and the terminal Le^x unit, respectively. The ratio of the integral intensities of the Fuc minor H-6 signal, the major signal at δ 1.13 and the signal for CH_2N of *P*Etn at δ 3.28 indicated the presence of six repeating units in the OPS on average. The signals for H-1 of α-Glc and α-DD-Hep were in the regions δ 4.98–5.02 and 5.08–5.12, respectively.

Fraction 007-I

Sugar and methylation analyses of fraction 007-I (Tables 2 and 3) showed that it was a mixture of a (1→4)-linked glucan, most likely a contaminant of the LPS preparation, and a very long OPS chain. As judged by the ratio of methylated derivatives from typical OPS and core components, on average, OPS contains ≈ 40 repeating units. The (1→6)-linked glucan and (1→3)/(1→2)-linked DD-Hep were also present, but as expected, their relative contents were lower than in fraction 007-II. The degrees of polymerization of these homopolymers in fraction 007-I could be estimated as 8 and 6, respectively.

These data showed that the OPSs of *H. pylori* AF1 and 007 have the structures shown in Fig. 5.

DISCUSSION

Structural studies of carbohydrate products derived by mild acid delipidation showed that LPS of *H. pylori* AF1 and 007 are highly heterogeneous with respect to the size and the structure of different molecular species. The studies revealed an incomplete and a complete (except for the Kdo region) core oligosaccharide, the latter having extended glucan and DD-heptan regions, and a number of OPS-containing species with a chain varying from 1–3 to 6–7 or ≈ 40 trisaccharide repeating units.

The LPS core regions in *H. pylori* AF1 and 007 showed a high degree of structural similarity, but not identity, to each other and to those of strains studied previously [5–10]. The most significant difference was the presence of a 2-aminoethyl phosphate group, rather than a phosphate group, as reported previously [5–7]. The same phosphodiester group has been found by us in two other *H. pylori* LPS preparations (A. P. Moran, Y. A. Knirel & P.-E. Jansson, unpublished data), and seems to be a common core component of this bacterium. A feature that distinguished the LPS core of *H. pylori* 007 is the presence of a 6-substituted DD-Hep residue, whereas in *H. pylori* AF1, as well as in other strains that have been studied [5–7], the same position in the DD-Hep region is occupied by a 7-substituted DD-Hep residue.

An extended glucan was found in all LPS species that contained a complete core. However, we could not confirm that a DD-Hep residue was the site of attachment of the glucan chain. Moreover, our data suggested that in *H. pylori* 007 the glucan chain elongation started from the Glc→Gal disaccharide fragment of the complete core, rather than from the 7-substituted DD-Hep residue [7]. The exact site of attachment of DD-heptan remains unknown. Notably, some of the LPS species from *H. pylori* AF1, including those that contained an OPS chain, lacked any long-chain DD-heptan, a finding indicating that its presence is not a prerequisite of the attachment of OPS to the core.

Independent of chain length, OPS always showed complete substitution of LacNAc units in the main chain by a lateral Fuc group, whereas strains studied previously produced OPS with mixed repeating units both containing and lacking Fuc [5–11]. In contrast, nonreducing terminal units were different in the two strains. Although the terminal unit in *H. pylori* 007 had the same structure as other repeating units, namely a Le^x trisaccharide, in *H. pylori* AF1 most OPS chains were terminated with the Le^y tetrasaccharide. Moreover, studies with these strains confirmed the usefulness of a simple ^{1}H-NMR spectroscopic method to distinguish between the terminal Le^x and Le^y oligosaccharides.

Despite the mimicry of Le^x and Le^y blood group determinants in the OPS chains of *H. pylori* AF1 and 007 established in this study, both strains and their isolated LPS did not react with anti-Lewis mAb. Previous studies have shown that a minimal chain length is required for recognition of Le^x and Le^y by antibodies [14] and, furthermore, that loss of expression of OPS by *H. pylori* can occur under certain *in vitro* cultivation conditions [3,4]. However, expression of OPS by *H. pylori* AF1 and 007 was confirmed in electrophoretic and chemical analyses, and the calculated OPS chain length would be sufficient for recognition by the antibodies. Therefore, compared with other strains, the presence of a polymeric Le^x chain and observed differences in the core of the LPS (e.g. presence of a 2-aminoethyl phosphate group and site of attachment of the glucan chain) may influence the presentation

of Le^x and Le^y units, particularly terminal ones, for recognition by antibodies.

The lack of detection of Le^x and Le^y in *H. pylori* AF1 and 007 using antibodies has important implications for serological and pathogenesis studies. First, a proportion of *H. pylori* strains (15–20%) has been reported as untypable with anti-Lewis, particularly anti-Le^x and anti-Le^y, and have been assumed to possess different structures [15,16]. Secondly, because of these potentially false-negative serological results, expression of Le^x and Le^y blood group determinants by *H. pylori* may be more common than assumed previously [13,15]. Thirdly, the use of anti-Lewis in typing *H. pylori* strains should be applied with caution as the antibodies may be recognizing only substructures of the respective antigen [10] and antigenic determinant presentation in LPS may influence antibody reaction. Finally, because the pathogenic relevance of Lewis antigen mimicry in *H. pylori* is unclear [12,13], and the role of this mimicry in the induction of *H. pylori*-related autoimmunity is yet to be established unequivocally [13,14,27,28], the use of serological techniques alone to detect the occurrence of Le^x and Le^y in *H. pylori* strains should be reconsidered when studying the latter pathogenic phenomenon.

ACKNOWLEDGEMENTS

The authors thank Mrs G. Alvelius (Clinical Research Center) for help with mass spectrometry. This work was supported by funding from INTAS (grant 97-0695), Swedish Research Council for Natural Science (to P.-E. J. and to G. W.), Royal Swedish Academy of Sciences (grant 12572, to P.-E. J.), Irish Health Research Board (to A. P. M.), and Millennium Research Fund (to A. P. M.). Y. A. K. and N. A. K. thank the Karolinska Institute for fellowships.

REFERENCES

1. Dunn, B.E., Cohen, H. & Blaser, M.J. (1997) *Helicobacter pylori*. *Clin. Microbiol. Rev.* **10**, 720–741.
2. Hunt, R.H. (1996) The role of *Helicobacter pylori* in pathogenesis: the spectrum of clinical outcomes. *Scand. J. Gastroenterol.* **31** (Suppl. 220), 3–9.
3. Moran, A.P., Helander, I.M. & Kosunen, T.U. (1992) Compositional analysis of *Helicobacter pylori* rough-form lipopolysaccharides. *J. Bacteriol.* **174**, 1370–1377.
4. Moran, A.P. (1995) Cell surface characteristics of *Helicobacter pylori*. *FEMS Immunol. Med. Microbiol.* **10**, 271–280.
5. Aspinall, G.O., Monteiro, M.A., Pang, H., Walsh, E.J. & Moran, A.P. (1996) Lipopolysaccharide of the *Helicobacter pylori* type strain NCTC 11637 (ATCC 43504): structure of the O antigen chain and core oligosaccharide regions. *Biochemistry* **35**, 2489–2497.
6. Aspinall, G.O. & Monteiro, M.A. (1996) Lipopolysaccharides of *Helicobacter pylori* strains P466 and MO19: structures of the O antigen and core oligosaccharide regions. *Biochemistry* **35**, 2498–2504.
7. Aspinall, G.O., Monteiro, M.A., Shaver, R.T., Kurjanczyk, L.A. & Penner, J.L. (1997) Lipopolysaccharides of *Helicobacter pylori* serogroups O:3 and O:6. Structures of a class of lipopolysaccharides with reference to the location of oligomeric units of D-*glycero*-α-D-*manno*-heptose residues. *Eur. J. Biochem.* **248**, 592–601.
8. Aspinall, G.O., Mainkar, A.S. & Moran, A.P. (1997) Lipopolysaccharides from *Helicobacter pylori* and pepsinogen activation. *Ir. J. Med. Sci.* **166** (Suppl. 3), 26–27.
9. Monteiro, M.A., Rasko, D., Taylor, D.E. & Perry, M.B. (1998) Glucosylated *N*-acetyllactosamine O-antigen chain in the lipopolysaccharide from *Helicobacter pylori* strain UA861. *Glycobiology* **8**, 107–112.
10. Monteiro, M.A., Chan, K.H.N., Rasko, D.A., Taylor, D.E., Zheng, P.Y., Appelmelk, B.J., Wirth, H.P., Yang, M.Q., Blaser, M.J., Hynes, S.O., Moran, A.P. & Perry, M.B. (1998) Simultaneous expression of type 1 and type 2 Lewis blood group antigens by *Helicobacter pylori* lipopolysaccharides. *J. Biol. Chem.* **273**, 11533–11543.
11. Aspinall, G.O. & Moran, A.P. (1997) *Helicobacter pylori* lipopolysaccharide structure and mimicry of Lewis blood group antigens. In *Pathogenesis and Host Response in* Helicobacter pylori *Infections* (Moran, A.P. & O'Morain, C.A., eds), pp. 34–42. Normed-Verlag, Bad Homburg, Germany.
12. Moran, A.P. (1996) Pathogenic properties of *Helicobacter pylori*. *Scand. J. Gastroenterol.* **31** (Suppl. 215), 22–31.
13. Moran, A.P., Appelmelk, B.J. & Aspinall, G.O. (1996) Molecular mimicry of host structures by lipopolysaccharides of *Campylobacter* and *Helicobacter* spp. implications in pathogenesis. *J. Endotoxin. Res.* **3**, 521–531.
14. Appelmelk, B.J., Simmons-Smit, I., Negrini, R., Moran, A.P., Aspinall, G.O., Forte, J.G., De Vries, T., Quan, H., Verboom, T., Maaskant, J.J., Ghiara, P., Kuipers, E.J., Bloemena, E., Tadema, T.M., Townsend, R.R., Tyagarajan, K., Crothers, J.M., Monteiro, M.A., Savio, A. & De Graff, J. (1996) Potential role of molecular mimicry between *Helicobacter pylori* lipopolysaccharide and host Lewis blood group antigens in autoimmunity. *Infect. Immun.* **64**, 2031–2040.
15. Simoons-Smit, I.M., Appelmelk, B.J., Verboom, T., Negrini, R., Penner, J.L., Aspinall, G.O., Moran, A.P., Fei Fei, S., Bi-Shan, S., Rudnica, W., Savio, A. & De Graaff, J. (1996) Typing of *Helicobacter pylori* with monoclonal antibodies against Lewis antigens in lipopolysaccharide. *J. Clin. Microbiol.* **34**, 2196–2200.
16. Wirth, H.P., Yang, M.Q., Karita, M. & Blaser, M.J. (1996) Expression of the human cell-surface glycoconjugates Lewis X and Lewis Y by *Helicobacter pylori* isolates is related to *cagA* status. *Infect. Immun.* **64**, 4598–4605.
17. Marshall, D.G., Hynes, S.O., Coleman, D.C., O'Morain, C.A., Smyth, C.J. & Moran, A.P. (1999) Lack of a relationship between Lewis antigen expression and *cagA*, CagA, *vacA* and VacA status of Irish *Helicobacter pylori* isolates. *FEMS Immunol. Med. Microbiol.* **24**, 79–90.
18. Westphal, O. & Jann, K. (1965) Bacterial lipopolysaccharides. Extraction with phenol–water and further applications of the procedure. *Methods Carbohydr. Chem.* **5**, 83–91.
19. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
20. Tsai, C.-M. & Frasch, C.E. (1982) A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* **119**, 115–119.
21. Towbin, H., Staehelin, T. & Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl Acad. Sci. USA* **76**, 4350–4354.
22. Sawardeker, J.S., Sloneker, J.H. & Jeanes, A. (1965) Quantitative determination of monosaccharides as their alditol acetates by gas liquid chromatography. *Anal. Chem.* **37**, 1602–1603.
23. Leontein, K., Lindberg, B. & Lönngren, J. (1978) Assignment of absolute configuration of sugars by g.l.c. of their acetylated glycosides formed from chiral alcohols. *Carbohydr. Res.* **62**, 359–362.
24. Hakomori, S.-I. (1964) A rapid permethylation of glycolipids and polysaccharides catalyzed by methylsulfinyl carbanion in dimethylsulfoxide. *J. Biochem.* **55**, 205–208.
25. Jansson, P.-E., Kenne, L., Liedgren, H., Lindberg, B. & Lönngren, J. (1976) A practical guide to the methylation analysis of carbohydrates. *Chem. Commun. University of Stockholm* **8**, 1–75.
26. Schwarzmann, G.O.H. & Jeanloz, R.W. (1974) Separation by gas-liquid chromatography, and identification by mass spectrometry, of

the methyl ethers of 2-deoxy-2-(*N*-methylacetamido)-D-glucose. *Carbohydr. Res.* **34**, 161–168.

27. Appelmelk, B.J., Negrini, R., Moran, A.P. & Kuipers, E.J. (1997) Molecular mimicry between *Helicobacter pylori* and the host. *Trends Microbiol.* **5**, 70–73.
28. Appelmelk, B.J., Faller, G., Claeys, D., Kirchner, T. & Vandenbroucke-Grauls, C.M.J.E. (1998) Bugs on trial: the case of *Helicobacter pylori* and autoimmunity. *Immunol. Today* **19**, 296–299.