Lipopolysaccharides of *Helicobacter pylori* Strains P466 and MO19: Structures of the O Antigen and Core Oligosaccharide Regions[†]

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ABSTRACT: Lipopolysaccharides (LPS) from phenol−water extraction of dyspeptic (P466) and asymptomatic (MO19) strains of *Helicobacter pylori* were each isolated as water-soluble material of high relative molecular mass (high M_r) and as water-insoluble gels of low M_r. Chemical and spectroscopic analyses of the soluble LPS and oligosaccharides liberated from the water-insoluble gels led to proposed structures for chains comprising the O antigen, intervening, and core regions. As in the LPS from the type strain NCTC 11637 [Aspinall, G. O., et al. (1996) *Biochemistry 35*, 000], the O antigen region of the P466 LPS is characterized by the presence of extended chains with fucosylated and nonfucosylated *N*-acetyllactosamine units, the former carrying α-L-fucopyranose units at O-3 of β-D-GlcNAc residues. This structure differs from that of the type strain in termination of the O chain by a Lewis^y (Le^y) antigenic determinant [α-L-Fuc(1→2)β-D-Gal(1→4)[α-L-Fuc(1→3)]β-D-GlcNAc] but also has internal Lewis^x (Le^x) units. The inner core region of the P466 LPS is indistinguishable from that in the type strain. In contrast, the O antigen region of the LPS from strain MO19 consists of a single Le^y epitope linked via a 3-linked β-D-Gal to an intervening region on the basis of a sequence of 3-linked D-glycero-α-D-*manno*-heptose residues which is in turn linked to an inner core identical to that in the type strain and the P466 strain. Results in this and the preceding paper show that LPS from the three *H. pylori* strains display molecular mimicry of human cell surface glycoconjugates but may vary in the expression of Le^x or Le^y determinants, the degree of O antigen chain extension, or in the presence of an additional region between the inner core and the O antigen.

Lipopolysaccharides (LPS)[1] comprise an important group of bacterial cell surface carbohydrate components which often interact specifically with surface components of an infected host. In the case of *Helicobacter pylori*, the bacterium has been variously implicated as a causative agent of gastritis, gastric and duodenal ulcers, and gastric carcinoma (Cover & Blaser, 1995). The chemical structure of LPS has remained largely undefined, although LPS is known to have low endotoxic activity, induces a low immunological response, and has been implicated in a variety of biological interactions. These include an inhibitory effect on mucus glycosylation, interference with mucosal integrity, the stimulation of pepsinogen secretion, and a role in the mediation of adherence of the bacterium laminin in the basement membrane [for a review, see Moran (1995)]. These effects, however, were noted without regard to possible structural differences in LPS. Evidence that such differences occur has been indicated by the differentiation between *H. pylori* strains in electrophoretic patterns in sodium dodecyl sulfate−polyacrylamide gel electrophoresis coupled with antigenic analyses with strain-specific antisera [see also Mills et al. (1992)]. In experiments of a different type, Borén et al. (1993) reported differences in the binding of strains of *H. pylori* to human gastric mucosal cells containing the Lewis^b (Le^b) antigen. The binding of one such strain, P466 from a patient with dyspeptic syndrome (Falk et al., 1993), to gastric mucosa was inhibited by the Le^b antigen, whereas the MO19 strain, from an asymptomatic patient, did not bind to gastric mucosa. Although LPS was not implicated in the interaction or lack of interaction of these *H. pylori* strains with gastric mucosa, the observations of Falk et al. (1993) and Borén et al. (1993) prompted a comparative examination of LPS from these strains. At that time, the first detailed structural analysis of LPS from an *H. pylori* strain, serostrain NCTC 11637 (ATCC 43504) was in progress and had revealed the presence in the LPS of O chains bearing the Le^x antigenic determinant. A preliminary account of this aspect of the LPS structure has been published (Aspinall et al., 1994), and full details of the study leading to a complete assignment of the structure of the polysaccharide component from the O antigen chain through an intervening unit to the core oligosaccharide (OS) are reported in the preceding paper (Aspinall et al., 1996). In this paper, we describe the results of studies on LPS from the *H. pylori* P466 and MO19 strains.

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[1] Abbreviations: 1D, one-dimensional; 2D, two-dimensional; COSY, correlated spectroscopy; EI, electron impact; FAB/MS, fast atom bombardment/mass spectrometry; Fuc, fucose; Gal, galactose; GC/MS, gas chromatography/mass spectrometry; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GPC, gel-permeation chromatography; Hep, heptose; DD-Hep, D-*glycero*-D-*manno*-heptose (similarly for LD-Hep); HMBC, heteronuclear multiple bond correlation spectroscopy; KDO, 3-deoxy-D-*manno*-octulosonic acid; LacNAc, *N*-acetyllactosamine [β-D-Gal*p*(1→4)D-Glc*p*NAc); LPS, lipopolysaccharide; NOESY, nuclear Overhauser enhancement spectroscopy; OS, oligosaccharide; ROESY, rotating frame nuclear Overhauser enhancement spectroscopy; TOCSY, total correlated spectroscopy.

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EXPERIMENTAL PROCEDURES

Except as listed below, experimental procedures were those described in the preceding paper (Aspinall et al., 1996).

Isolation of Lipopolysaccharides (LPS) and Liberated Core Oligosaccharides. *H. pylori* strains P466 and MO19 (Falk et al., 1993; Borén et al., 1993) were kindly provided by Dr. T. Borén (University of Umeå, Sweden), and the strains were grown in the laboratory of Dr. J. L. Penner (Department of Microbiology, University of Toronto) as described by Mills et al. (1992). LPS were isolated by hot phenol−water extraction as described previously (Aspinall et al., 1994), yielding soluble high-M_r LPS and insoluble low-M_r LPS as a gel. The soluble fractions were purified by GPC on Bio-Gel P-6 to furnish LPS used in further studies. Core oligosaccharides were liberated from the insoluble gel by heating in acetate buffer at pH 6.5 (Yamasaki et al., 1993) as reported in the preceding paper, and the solubilized OS were fractionated by GPC on Bio-Gel P-2 (Aspinall et al., 1996). In the case of OS from P466 LPS, elution with water afforded two broad carbohydrate-rich bands (Dubois et al., 1956), scanning by ^{1}H NMR within each of which showed sufficient differences to justify arbitrary separation of material into **OS-1** and **OS-2** and **OS-3** and **OS-4**, respectively.

^{1}H, ^{13}C, and ^{31}P NMR Spectroscopy. The following additional parameters were used in 2D NMR experiments: NOESY and/or ROESY, 256 × 2048 data matrix, zero-filled to 1024 data points in t_1, 128 scans per t_1 value, recycle delay of 2.0 s, mixing time of 300 ms, and with shifted sine-squared filtering in t_1 and t_2; ^{1}H−^{31}P HMBC, 512 × 512 data matrix, zero-filled to 1024 data points, and 128 scans per t_1 value.

RESULTS

The structure for the LPS from the *H. pylori* type strain NCTC 11637 (Aspinall et al., 1996) served as a reference point, and the strategies employed in its elucidation were to provide the guidelines in the examination of LPS from other *H. pylori* strains. With no formal distinction between rough- and smooth-form LPS of strains P466 and MO19, bacterial cell extracts were divided into soluble high-M_r LPS with extended outer chains and insoluble low-M_r LPS. Oligosaccharide fractions comprising inner core and developing O antigen chains were liberated and separated by GPC for detailed study. With considerable microheterogeneity in each of these fractions, compositional and linkage analysis gave only average values for constituent residues. However, since each type of constituent sugar, dHex, Hex, HexNAc, and Hep, was of different mass, FAB/MS data for pseudomolecular ions and fragment ions, in conjunction with linkage type information, provided precise estimates of composition and often of structure for the individual components of mixtures. Assuming that smaller molecules comprise the first steps in the progressive development of the mature LPS, the evidence for these defined components could be turned into an advantage in the elucidation of the structure of the complete molecule.

Characterization of Water-Soluble LPS of High M_r from H. pylori Strain P466. Water-soluble LPS was examined directly without cleavage from lipid A in order to avoid cleavage of any relatively acid-sensitive fucopyranosyl linkages. Compositional analysis showed that the main constituents were Fuc, Gal, and GlcNAc in the approximate molar ratio of 4:6:5, with smaller amounts (~2 M proportions) of sugars, Glc, DD-Hep, and LD-Hep. The identities and anomeric configurations of the principal sugar residues were defined by ^{1}H and ^{13}C NMR as those of α-L-fucose [$\delta_{H\text{-}1}$ 5.19 (minor) and 5.02 (major), each $J_{1,2}$ 3.8 Hz; δ_C 99.6 (C-1) and 15.1 (**C**H$_3$)], β-D-galactose [$\delta_{H\text{-}1}$ 4.69, $J_{1,2}$ 7.2 Hz; δ_C 103 (C-1)], and *N*-acetyl-β-D-glucosamine [$\delta_{H\text{-}1}$ 4.50, $J_{1,2}$ 7.7 Hz; δ_C 102.4, 102.6 (C-1), 175.0 (**C**O), 55.7 (C-2), and 22.5 (**C**H$_3$)].

Table 1: Methylation Linkage Analysis of LPS and Derived Oligosaccharides **OS-1**, **OS-2**, **OS-3**, and **OS-4** from *H. pylori* P466

structural units from methylation analysis	approximate molar ratios[a] LPS	**OS-1**	**OS-2**	**OS-3** (P466)	**OS-4**
O antigen region					
Fuc	5	4	2		
→3)Gal	4	3	0.5		
→2)Gal	1	1	1		
→3)GlcNAc			0.5		
→4)GlcNAc	1	1			
→3/4)GlcNAc	4	3	1		
intervening and core regions					
Glc	1	1	1	1	0.5
→3)Glc	1	1	1		
→4)Gal	1	1	1	1	0.5
DD-Hep				tr	0.5
→7)DD-Hep	1	1	1	1	0.5
→2/7)DD-Hep	1	1	1		
LD-Hep					tr
→2)LD-Hep	1	1	1	1	1
→3)LD-Hep	}1	1	1	1	1
→3)LD-Hep(P)					
→5)Kdo	nd	nd	nd	1	nd

[a] For comment on the assessment of data, see Methylations in Experimental Procedures of Aspinall et al. (1996).

Linkage analysis performed on the permethylated LPS showed the presence of terminal Fuc, 2-linked Gal, 3-linked Gal, and 4- and 3,4-linked GlcNAc residues in the approximate molar ratio of 5:1:4:1:4, with approximately 1 M proportion each of variously substituted Glc, DD-Hep, and LD-Hep residues arising from the inner region of the LPS (Table 1). An important difference in this LPS from that of the type strain NCTC 11637 was the presence of a 2-linked Gal residue and the absence, other than in trace amounts, of a terminal Gal residue. The significance of the 2-linked Gal residue arising from a Fuc(1→2)Gal unit was seen in the FAB mass spectrum of the permethylated LPS showing a fragment ion at *m*/*z* 812 of composition Fuc_2, Gal, GlcNAc which could have arisen from either an Le^b or an Le^y determinant, but the detection of a secondary fragment ion at *m*/*z* 606 from β-elimination of a terminal Fuc residue from O-3 of GlcNAc, and not at *m*/*z* 402, pointed to the Le^y epitope (Figure 1) (Egge & Peter-Katalinic, 1987). Fragment ions of higher mass were observed at *m*/*z* 1435 (Fuc_3, Gal_2, $GlcNAc_2$) and 2158 (Fuc_4, Gal_3, $GlcNAc_3$) with increments of Fuc, Gal, GlcNAc, and from the former, a secondary ion was observed with loss of a terminal Fuc residue (206 amu), indicating a regular chain extension of Le^x units in a type 2 fucosylated *N*-acetyllactosaminoglycan. For confirmation that the lactosaminoglycan backbone consisted of regular Gal(1→4)GlcNAc repeating units, the soluble LPS was heated with aqueous 5% acetic acid at 100 °C for 1 h, in which process cleavage from lipid A was accompanied by defucosylation giving **PS-1**. ^{1}H and ^{13}C NMR data for the degraded glycan showed two dominant anomeric resonances

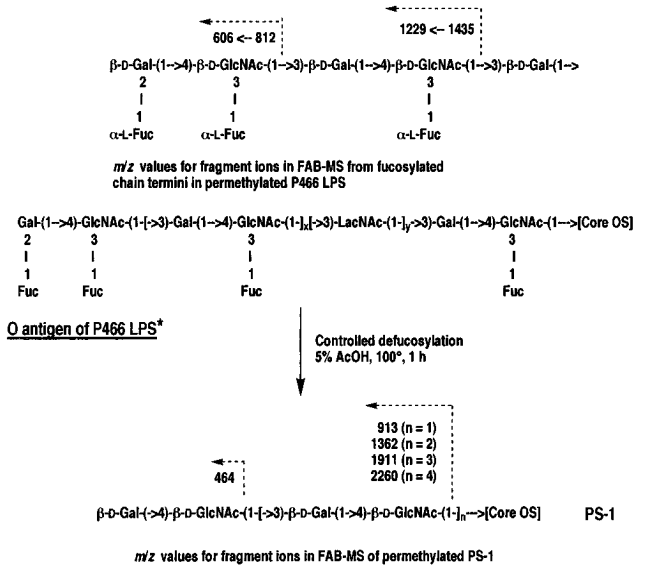

FIGURE 1: Transformations of water-soluble LPS from *H. pylori* strain P466 with analysis of positive ion FAB/MS data for the permethylated O antigen chain of LPS and of the permethylated derivative of acid-liberated and defucosylated **PS-1**. The structure shown for the O antigen chain is without regard to the sequence of the internal fucosylated and nonfucosylated *N*-acetylacetyllactosamine units. *→3)LacNAc(1- = →3)β-D-Gal(1→4)β-D-GlcNAc(1-.

for β-Gal ($\delta_{H\text{-}1}$ 4.69 and $\delta_{C\text{-}1}$ 102.3) and β-GlcNAc ($\delta_{H\text{-}1}$ 4.49 and $\delta_{C\text{-}1}$ 103.1) residues. The ^{31}P NMR spectrum of acid-liberated **PS-1** showed a single resonance at δ 3.78 at pD 6 assignable to a phosphoric monoester. Methylation linkage analysis established that 3-linked Gal and 4-linked GlcNAc residues were the main units with no detectable 3-linked GlcNAc residues. The proportion of Gal end groups indicated an average chain length of four to five disaccharide units. FAB/MS supported a regularly repeating structure for the *N*-acetyllactosaminoglycan backbone chain of the O antigen region of the P466 LPS with a series of glycosyloxonium ions at *m/z* 464, 913, 1362, 1811, and 2260 from preferential cleavage at GlcNAc residues. The proposed structure for the O antigen chain of the P466 LPS is shown in Figure 1.

Examination of Core Oligosaccharide Fractions Liberated from Water-Insoluble LPS of H. pylori Strain P466. Treatment of the water-insoluble LPS preparation in acetate buffer at pH 6.5 (Yamasaki et al., 1993) with cleavage of the ketosidic linkage to lipid A gave a mixture of oligosaccharides which was separated by GPC to give a series of fractions or subfractions, not all of which were present in sufficient quantity for detailed analysis. Compositional analyses are given in the text, and methylation linkage analyses for key fractions are summarized in Table 1.

*Characterizations of P466 Core **OS-3** and **OS-4**.* These fractions gave qualitatively similar analyses for composition and linkage types, but mass spectral data provided sufficiently different insights into sugar sequences and location of the phosphate unit to justify separate consideration. Sugar constituents of the O chain region, notably Fuc and GlcNAc, were absent. **OS-3** contained D-Glc, D-Gal, DD-Hep, and LD-Hep in the approximate molar ratio of 1:1:1:2. Linkage analysis (Table 1) showed the presence of terminal Glc, 4-linked Gal, 7-linked DD-Hep, 2-linked LD-Hep, a less than equivalent proportion combined of 3-linked LD-Hep and a 3,7-di-O-substituted LD-Hep (incompletely liberated from a phosphorylated residue), and 5-linked Kdo. ^{31}P NMR showed a single phosphoric monoester resonance at δ 3.7 (pD 7). A 2D ^{1}H−^{31}P HMBC experiment showed a connectivity from the phosphorus nucleus to a proton resonance at δ 3.60 in a region of the spectrum occupied by H-7 protons of heptose residues (Müller-Loennies et al., 1994) in accord with the indications from linkage analysis for the site of phosphorylation. ^{1}H NMR data (from COSY and TOCSY) for **OS-3** showed five anomeric resonances for α-Glc (δ 4.8 and $J_{1,2}$ 3.9 Hz) and β-Gal (δ 4.5 and $J_{1,2}$ 7.9 Hz) and for three heptose residues with the α-*manno* configuration, each as unresolved doublets, at δ 5.45 and 5.00 (double intensity). These resonances corresponded to those of the complete hexasaccharide structure shown in Figure 3. In addition, minor resonances observed in the range δ 5.45−5.00 were presumably those of the corresponding three heptose residues present in small amounts in chains deficient in outer chain residues and/or phosphorylation. A 2D ^{1}H-^{1}H ROESY experiment on **OS-3** showed inter-residue contacts from H-1 of α-Glc to H-4 (δ 3.98) of β-Gal and from H-1 of β-Gal to H-7 or H-7′ (δ 3.58) of an α-Hep residue.

FAB/MS of permethylated P466 **OS-3** (not shown) gave a pseudomolecular ion $[(M + H) - 46]^+$ at *m/z* 1429 in relatively low abundance for a complete hexasacccharide [Hex_2Hep_3Kdo] but also ions from the shortened chains of a phosphorylated tetrasaccharide [Hep_3, P, Kdo] at *m/z* 1115 $[(M + H) - 46]^+$ and, in somewhat larger abundance, from the nonphosphorylated tetrasaccharide [Hep_3, Kdo] at *m/z* 1021 [(M + H) − 46]. A relatively prominent fragment ion (Hep_3) from the nonphosphorylated molecule was seen

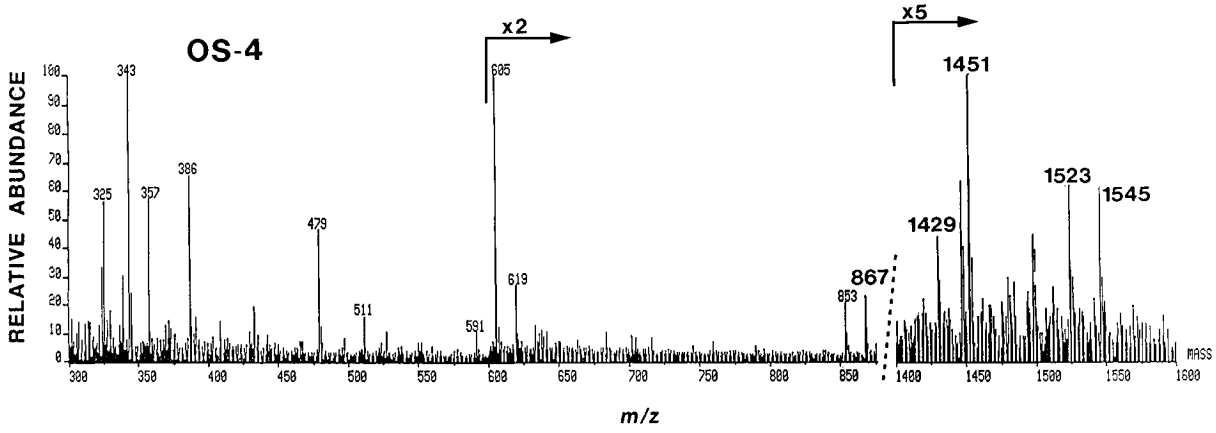

FIGURE 2: Positive ion FAB/MS spectrum of permethylated **OS-4** from P466 LPS.

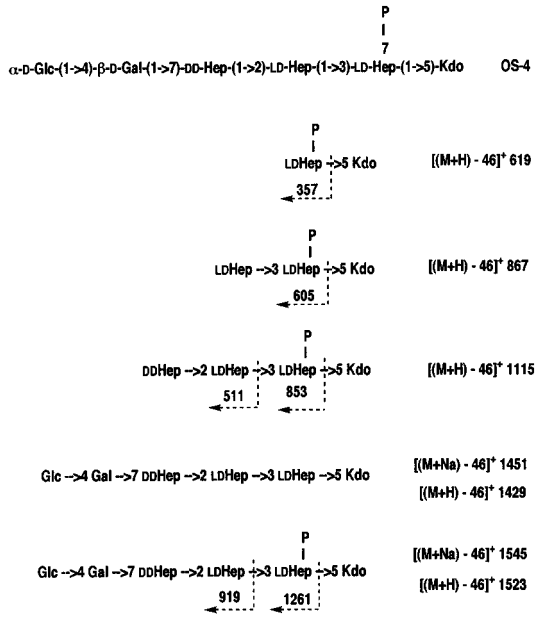

FIGURE 3: Analysis of positive ion FAB/MS data for permethylated **OS-4** from P466 LPS showing pseudomolecular ions and key fragment ions for the designated compound and accompanying minor components.

at *m/z* 759, but only traces of the corresponding phosphorylated ion were seen at *m/z* 853 [Hep_3P]. Although these tetrasaccharide derivatives were probably present as minor components of permethylated **OS-3**, the detection of terminal DD-Hep in the linkage analysis was evidence for the sequence of DD- and LD-Hep residues in the heptotriose segment. In a parallel investigation of P466 **OS-4**, compositional analysis pointed to lower relative proportions of Glc and Gal than in **OS-3**, suggesting the presence of hexasaccharide and tetrasaccharide derivatives in roughly similar amounts as the major components. This conclusion was supported by the detection in the linkage analysis (Table 2) of DD-Hep as the nonreducing terminus of the tetrasaccharide. The detection of even smaller proportions of nonreducing teminal LD-Hep residues (Table 2) pointed to the presence of tri- and disaccharide derivatives as minor components in a mixed population of related molecules in **OS-4**. This conclusion was clearly established by FAB/MS of the permethylated fraction (Figure 2 and interpretation in Figure 3), in which pseudomolecular ions and derived fragment ions were detected for phosphorylated oligosaccharides from di- through hexasaccharides, and for a nonphosphorylated tetrasaccharide. The detection of a pseudomolecular ion $[(M + H) - 46]^+$ at *m/z* 619 for the smallest phosphorylated oligosaccharide [HepPKdo] and a fragment ion at *m/z* 357 for a terminal phosphorylated Hep residue served to locate the site of phosphorylation in this family of related molecules. The high proportion of phosphorylated oligosaccharides was also reflected in the abundance of double cleavage ions $[HO\text{-}Hep(P)]^+$ at *m/z* 343 and $[HO\text{-}Hep\text{-}Hep(P)]^+$ at *m/z* 591.

*Characterizations of P466 Core **OS-2** and **OS-1**.* Compositional analysis and ^1H NMR for **OS-2** pointed to an average incremental addition to the core region of **OS-3** of Fuc_2, Glc, Gal_2, $GlcNAc_2$, and DD-Hep. Using the structure of the **OS-2** fraction from the NCTC 11637 strain as a guide, linkage analysis showed that the increment could correspond to a core extension by a single 3-linked Glc residue and the addition, as a side chain to the DD-Hep in the core, of a second DD-Hep residue from which O antigen chain development would ensue with the attachment of up to two Le^x or Le^y units. FAB/MS of permethylated P466 **OS-2** showed two groups of fragment ions, of special significance, from a mixed population of molecules. Ions of the first group were those from molecules of lower mass which were of unambiguous composition, at *m/z* 682 (Fuc, HexNAc, Hep) and 886 (Fuc, Hex, HexNAc, Hep) (A in Figure 4). These ions arose from cleavage at a Hep residue and provided evidence for attachment of the embryonic O antigen to an unbranched

Table 2: Methylation Linkage Analysis of LPS, **PS-1**, and Derived Oligosaccharide **OS-3** from *H. pylori* MO19

structural units from methylation analysis	approximate molar ratios[a] LPS	**PS-1** (MO19)	**OS-3** (MO19)
O antigen region			
Fuc	2		
→3)Gal	1	1	
→2)Gal	1		
GlcNAc		1	
→3/4)GlcNAc	1		
intervening and core regions			
Glc	1		1
→3)Glc	1		
→6)Glc	3		
→4)Gal	1		1
→3)Man-6-[$^2H_1$]		7	
DD-Hep			tr
→2)DD-Hep	0.5		
→3)DD-Hep	7		
→6)DD-Hep	1		
→7)DD-Hep			1
→ 2/7)DD-Hep	1.5		
→2)LD-Hep	1		1
→3)LD-Hep	}1		1
→3)LD-Hep[P]			

[a] For comment on the assessment of data, see Methylations in Experimental Procedures of Aspinall et al. (1996).

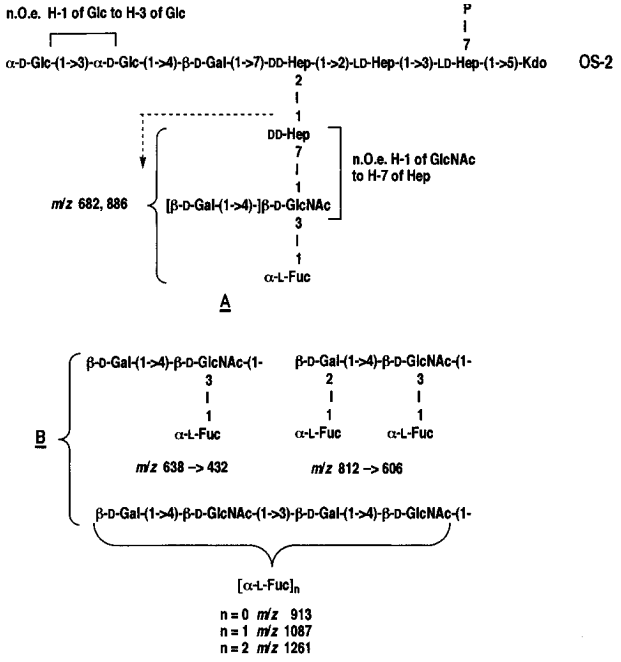

FIGURE 4: Interpretations of selected nuclear Overhauser effect interactions in the ^1H NMR spectrum of **OS-2** from P466 LPS and of FAB/MS data of the permethylated derivative showing fragment ions from regions A and B as outlined in the text.

Hep residue in the side chain (Figure 4). Support for this conclusion was obtained from a 2D $^1H-^1H$ NOESY experiment which showed an inter-residue connectivity from H-1 (δ 4.50) of GlcNAc to H-7,7′ (δ 3.59) of a Hep residue (Müller-Loennies et al., 1994). This experiment also provided confirmation of chain extension in the outer core OS region by showing a nuclear Overhauser effect contact from H-1 (δ 4.80) of a terminal Glc to H-3 (δ 3.57) of a 3-linked Glc residue (Figure 4). Glycosyloxonium ions of the second group in the FAB/MS of permethylated **OS-2** may be regarded as arising from cleavage at HexNAc residues in more extended chains and included (i) terminal Le^x and Le^y epitopes at *m*/*z* 638 (Fuc, Hex, HexNAc) and *m*/*z* 812 (Fuc_2, Hex, HexNAc) (B in Figure 4) and (ii) less fucosylated chains with two *N*-acetyllactosamine (LacNAc) units (*m*/*z* 913) and derivatives with one or two Fuc residues at *m*/*z* 1087 and 1261.

Compositional and methylation linkage analyses of P 466 **OS-1** (Table 1) indicated that in this fraction extension of the growing O chain approached that in the complete water-soluble LPS. In addition, ^1H NMR examination of **OS-1** showed the characteristic resonances of Fuc, GlcNAc, and Gal residues of the O chain, and no unexpected features were detected.

Characterization of Water-Soluble LPS from H. pylori Strain MO19. Compositional analysis of the water-soluble **LPS** showed the presence of Fuc, Gal, GlcNAc, Glc, DD-Hep, and LD-Hep in the approximate molar ratio of 2:3:1:4:10:2. Enantiomeric configurations of the sugar constituents were established by the chiral glycoside method (Leontein et al., 1978). The presence of Kdo was confirmed later in the FAB/MS of the permethylated derivative of the MO19 inner core **OS-3**.

The ^1H NMR spectrum of MO19 LPS, in combination with 2D COSY and TOCSY experiments, showed one dominant anomeric resonance at δ 5.08 (unresolved doublet) which could be attributed to the D-*glycero*-α-D-*manno*-heptose component. In addition to anomeric resonances from residues in the inner core oligosaccharides, equal intensity signals were observed for two α-Fuc (δ 5.09 and 5.12, each $J_{1,2}$ 3.8 Hz), one β-GlcNAc (δ 4.50, $J_{1,2}$ 7.7 Hz), and two β-Gal residues (δ 4.68 and 4.71, each $J_{1,2} \sim 7$ Hz). Other signals were those of H-6 of Fuc residues (3 H each at δ 1.20 and 1.32, $J_{5,6}$ 6.2 Hz) and C**H**$_3$CO (3 H, δ 1.98) of the GlcNAc residues. Proton resonances for both fucose residues from H-1 and H-6 were fully assigned by 2D COSY and TOCSY experiments to confirm the α-*galacto* configuration.

Methylation linkage analysis (Table 2) for the LPS showed derivatives from three regions of structure: (1) two terminal Fuc and one each of 2-linked Gal and 3,4-linked GlcNAc; (2) most prominently about seven 3-linked DD-Hep and three 6-linked Glc residues, the locations which will be considered later; and (3) those residues probably arising from a similarly linked core OS region to those in the NCTC 11637 and P466 LPS. An additional structural unit, not seen in the NCTC 11637 and P466 LPS, and yet to be accommodated in the MO19 LPS, was a 6-linked Hep, which was assumed, on the basis of relative proportions of the two heptoses, to be that of a DD-Hep. The FAB/MS of permethylated LPS showed the following fragment ions from the outer chain region at *m*/*z* 812 (Fuc_2, Gal, GlcNAc), 606 [812−206 (FucOH)], and 1016 (Fuc_2, Gal, GlcNAc, Hex). The former two ions were characteristic of those from an Le^y terminal unit with β-elimination of a terminal Fuc residue from O-3 of GlcNAc. In accord with compositional and linkage analysis data, no other abundant ions were observed arising from an extended chain fucosylated lactosaminoglycan chain; the next ion in the chain extension at *m*/*z* 1016 showed an increment for a Hex residue, later identified as Gal. The most striking observation from the linkage analysis was for the presence of multiple 3-linked DD-Hep residues. On the basis of proton assignments from 2D COSY and TOCSY experiments, a 2D NOESY experiment showed inter-residue connectivities from H-1 to H-3 (δ 3.85) of adjacent 3-linked DD-Hep residues indicating extended chains of these residues. Glc residues that were 6-linked were also prominent, but these data gave no direct information on their location in the overall structure. In addition to the sugar residues in the MO19 LPS probably derived from the core OS region, a 3-linked Gal residue remained to be accommodated.

The soluble **LPS** (3 mg), without prior removal of the lipid A component, was submitted to a Smith degradation in which the reduction was performed with $NaBD_4$ and afforded one fraction of MO19 **PS-1** which was separated on a column of Bio-Gel P-2. **PS-1** had a composition of [$^2H_1$]erythritol, [$^2H_1$]Man, Gal, and GlcNAc in the approximate ratio of 0.6:7:1:1; the ^1H NMR spectrum showed anomeric signals for β-Gal at δ 4.70 ($J_{1,2}$ 7.7 Hz) and β-GlcNAc at δ 4.51 ($J_{1,2}$ 7.9 Hz) and a major anomeric signal for a sugar with the α-*manno* configuration at δ 5.00 as an unresolved doublet. Linkage analysis for permethylated **PS-1** showed the presence of terminal GlcNAc, 3-linked Gal, and 3-linked Man residues in the ratio of 1:1:7, and the FAB/MS gave fragment ions for the terminal trisaccharide segment at *m*/*z* 260 (GlcNAc), 464 (GlcNAc-Gal), and 669 (GlcNAc-Gal-Man-6-^{2}H), thereby defining the sequential connection of the GlcNAc residue of the Le^y epitope via the 3-linked Gal residue to the 3-linked DD-Hep oligosaccharide unit. The erythritol moiety from the proximal terminus of **PS-1** could only arise from the oxidative−reductive degradation of a 6-linked or a 2,7-linked DD-Hep residue. The following structures may therefore be proposed for MO19 **PS-1**:

β-D-GlcNAc-(1->3)-β-D-Gal-(1-[->3)-α-D-Man-6-[^{2}H]-[(1-]$_{\sim 7}$->D-Erythritol,

and thence for the outer region of LPS

β-D-Gal-(1->4)-β-D-GlcNAc-(1->3)-β-D-Gal-(1-[->3)-D-α-D-Hep]$_{\sim 7}$-(1->6/7)-DD-Hep-->,
2 3
| |
1 1
α-L-Fuc α-L-Fuc

*Characterization of the Inner **OS** Region from Insoluble LPS from H. pylori Strain MO19.* With only limited quantities of the insoluble LPS available, an attempt to obtain oligosaccharide fractions analogous to those from the NCTC 11637 and P466 LPS yielded only one such fraction **OS-3** from the inner core region, in an amount sufficient to perform a complete structural analysis. ^1H NMR showed anomeric protons for α-Glc [δ 4.83 ($J_{1,2}$ 3.7 Hz)] and β-Gal [δ 4.41 ($J_{1,2}$ 7.6 Hz)] and an array of unresolved doublets between δ 4.9 and 5.4 assigned to Hep residues with the α-*manno* configuration. Linkage analysis (Table 2) with FAB/MS data for the permethylated **OS-3** showing a pseudomolecular ion for $[(M + H) - 46]^+$ at *m*/*z* 1544 was consistent with the same phosphorylated hexasaccharide, Glc-Gal-DD-Hep-LD-Hep-LD-Hep(P)-Kdo, inner core region as in the NCTC 11637 and P466 LPS. The presence of a phosphorylated

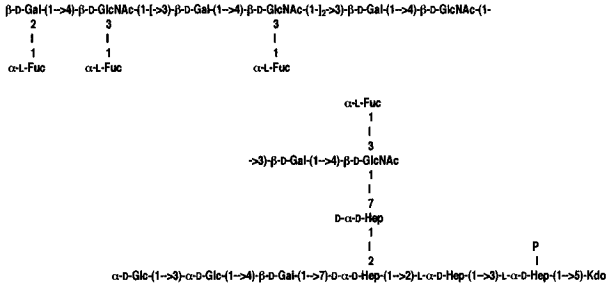

FIGURE 5: Proposed structure for the complete structure of the polysaccharide component of LPS from *H. pylori* strain P466.

tetrasaccharide, DD-Hep-LD-Hep-LD-Hep(P)-Kdo as a minor component of permethylated **OS-3** was indicated by the appearance of the corresponding pseudomolecular ion at *m*/*z* 1136 for $[(M + H) - 46]^+$. The detection of a terminal DD-Hep residue in the linkage analysis served to place this as the distal of the three Hep residues. Although insufficient material was available to ascertain the linkage site in the Kdo residue, the results allow the following structure to be proposed for the phosphorylated hexasaccharide unit in MO19 **OS-3**:

α-D-Glc-(1->4)-β-D-Gal-(1->7)-α-DD-Hep-(1->2)-α-LD-Hep-(1->3)-α-LD-Hep(P)-->Kdo

DISCUSSION

This investigation has provided the first documented evidence for structural differences between LPS from strains of *H. pylori*. The LPS from the P466 and MO19 strains, which differed in their abilities to bind to gastric mucosal surfaces bearing the Le^b antigen, carried the same Le^y epitope. In this respect, they differed from the LPS of the type strain NCTC 11637 with a terminal Le^x epitope. However, in other respects, the LPS from P466 and MO19 were markedly different. Examination of the soluble LPS and the core oligosaccharide fractions derived from the water-insoluble LPS from *H. pylori* P466 has provided clear evidence for the close structural similarity of the complete polysaccharide chain in the LPS (Figure 5) to that from the type strain NCTC 11637 (Aspinall et al., 1996) in the following respects: (i) an identical internal core OS region; (ii) the connection between the core OS and the GlcNAc at the reducing terminus of the O chain via a DD-Hep residue attached as a side chain to the core, as shown in **OS-2**; (iii) the extension of the core OS region with an α-D-Glc residue; and (iv) the presence of an O chain based on a type 2 fucosylated *N*-acetyllactosaminoglycan. The difference of greatest significance between the P466 strain and that of the type strain lies in the termination of the extended fucosylated *N*-acetyllactosaminoglycan chains by the Le^y in place of the Le^x blood group antigen. Other differences are the lower degree of chain extension (four to five repeating units) in the O chain compared with nine repeating units in that of the type strain LPS and the absence of evidence of 6-linked α-D-Glc residues in the developing O chain structure.

In contrast to the P466 strain, the LPS from the MO19 strain was of a very different type in carrying a single Le terminal epitope, that of Le^y, but with no extended fucosylated *N*-acetyllactosaminoglycan. The core OS region of the MO19 LPS as in the **OS-3** fraction was, within the limits of detection, identical to those of the P466 and type strains. The outer region of the LPS consisted of a single difucosylated *N*-acetyllactosamine unit, the Le^y epitope, joined via a 3-linked β-D-Gal residue to a region of structure or "third domain" consisting of a chain of 3-linked DD-Hep residues as the most prominent feature of the LPS. The limited quantity of this LPS available was insufficient to establish the formal connection of this outer chain to core OS region. However, on the basis of linkage analysis, five types of sugar residues, in addition to those already placed in the outer O chain + third domain and the inner core OS regions of polysaccharide structure, remained to be accommodated. Two types of units were those of α-D-Glc residues, a single 3-linked Glc residue, which probably arose from extension of the outer region of core OS as in the P466 and type strain LPS, and ~three 6-linked Glc residues, probably mutually linked as in similar short chains in **OS-2** from the type strain (Aspinall et al., 1996) and other *H. pylori* LPS (unpublished results). Three types of DD-Hep residues, in addition to that in **OS-3** and those in the 3-linked oligosaccharide, were single units of 2-, 6-, and 2,7-linked residues. The absence of 7-linked DD-Hep residues in the LPS implies that this residue in **OS-3** occurs as one of two branched residues in the LPS. The other 2,7-linked unit is presumably that to which the 6-linked Glc residues are attached in the LPS. Either this second branched unit or the 6-linked DD-Hep residue could be the residue to which the whole outer chain is linked, and it gave rise to an erythritol terminus in **PS-1** from Smith degradation of the LPS. The known structural features of the MO19 may therefore be summarized in Figure 6 which shows (a) the single Le^y chain terminal epitope linked via a β-D-Gal residue to the 3-linked oligomer of D-α-D-Hep residues, (b) an incompletely defined region showing three DD-Hep residues and three Glc residues, and (c) the inner core region based on the liberated oligosaccharide **OS-3** extended by an additional Glc residue. This LPS contains in the third domain a structural region of a type not previously encountered.

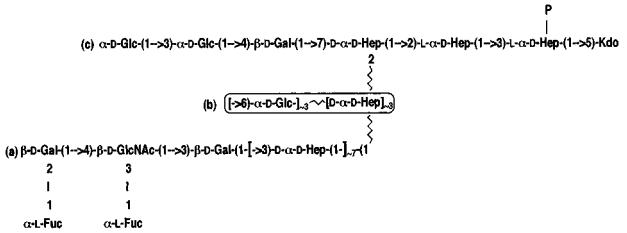

FIGURE 6: Proposed partial structure for the polysaccharide component of LPS from *H. pylori* strain MO19 as outlined in the text showing (a) the outer O antigen chain region, (b) an incompletely defined intervening region, and (c) the inner core region.

As with the LPS from the *H. pylori* type strain NCTC 11637, the most significant conclusion from the present investigation concerns the elaboration of O antigen chains with Lewis epitopes in mimicry of structures expressed in human cell surface glycoconjugates. The Lewis antigen epitopes in termination of the O antigen chains of *H. pylori* vary in structure, but it is now clear that there are variations elsewhere in the structure of the LPS. The P466 and MO19 LPS provide a unique example of two LPS with identical inner cores and O chain terminal epitopes but entirely different intervening regions. Although found in normal cells, e.g. from granulocytes, the expression in abundance of these tumour-associated carbohydrate antigens is limited to malignant cells (Hakomori, 1989). The further implica-

tions of these surface structures in interaction between the bacterium and the human host will require biological experiments with chemically defined LPS structures from different strains of the organism. In contrast to the oligomeric Le^x epitopes in the type strain LPS, the P466 LPS contains an Le^y terminus with internal Le^x units and the possibility may be raised that these separate units in the same O chain might interact with different components in the human host. The MO19 LPS would be expected to display interactions dependent only on the Le^y terminus. Related evidence bearing on these considerations has been obtained by Dr. B. J. Appelmelk of the Free University in Amsterdam (unpublished results). Using a panel of monoclonal antibodies specified with respect to synthetic neoglycoconjugates, two anti-Le^x Mabs appear to differentiate between terminal and internal (possibly polymeric) Le^x units. In recognizing the P466 LPS, the latter Mab would seem to be specific for nonterminal Le^x. An anti-Le^y Mab in the panel recognizes the Le^y termini in both P466 and MO19 LPS.

The structural conclusions from these studies on LPS from *H. pylori* are difficult to reconcile with the well-documented biosynthetic pathways for LPS in typical enteric bacteria such as *Salmonella* [for a recent review, see Whitfield (1995)]. These pathways require the independent synthesis of the core OS region by stepwise chain extension and the assembly of oligosaccharide monomers on a lipid carrier to furnish the regular repeating units of the O antigen chain. The presence of the Le^y epitope at the terminus of the fucosylated *N*-acetyllactosaminoglycan in the P466 LPS, and externally to the third domain in the MO19 LPS, clearly implies the operation of very different assembly processes.

In proposing any overall biosynthetic scheme for future experimental verification, attention may be drawn to the following present structural conclusions that are difficult to reconcile with regular transfer of preformed oligosaccharide blocks from the lipid carrier: (i) The detection of a Fuc-(1→3)GlcNAc unit as the smallest O chain-related fragment in the development in the **OS-2** fractions from tne NCTC 11637 and P466 strains would point to stepwise glycosylation in the synthesis for this first Le^x unit. (ii) The lack of fucosylation at each LacNAc unit as chain extension proceeds would imply that fucosylation is not a mandatory event in the incorporation of uniformly complete Le^x units. (iii) The generation of the Le^y epitope in the P466 and MO19 LPS would require a specific Fuc(1→2)Gal transferase. This latter glycosylation step might be regarded as a "capping" process which disallows further chain extension with Le^x units. The activity of such a glycosyltransferase would be highly competitive with others in the case of the MO19 LPS with the single Le^y epitope, less competitive in the case of P466 with O chains of four to five repeating units, but not detected in the case of the type strain with the most extended O chains of nine repeating units.

The synthesis of the MO19 LPS is of an entirely different type with the assembly of the 3-linked D-*glycero*-α-D-*manno*-heptoglycan segment. The glycosyl donor for DD-Hep residues must be present in each of the *H. pylori* strains, but creation of this linkage, absent in the other strains, would require a separate transferase. The initiation of attachment of the Le^y epitope in the MO19 LPS is also different from those for the type strain and the P466 strain in requiring glycosyl transfer to a Gal residue at the terminus of the heptoglycan domain rather than directly to a DD-Hep residue.

In summary, the structural differences between the LPS of the P466 and MO19 imply that the LPS may be involved in the binding of the former strain to gastric mucosa. We consider that the lack of binding of the MO19 strain to the mucosa is more likely to be due to the absence of the extended fucosylated lactosaminoglycan chain in the O chain than to the presence of the heptan in the third domain. It is possible that the binding of the P466 strain is through a specific Le^x−Le^x interaction (Kojima et al., 1994), of the multiple Le^x units in the subterminal region of the Le^y-terminated O chain in the LPS, with Le^x units expressed in normal human gastric tissue (Sakamoto et al., 1989).

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