Lipopolysaccharide of the *Helicobacter pylori* Type Strain NCTC 11637 (ATCC 43504): Structure of the O Antigen Chain and Core Oligosaccharide Regions[†]

Gerald O. Aspinall,*[,‡] Mario A. Monteiro,[‡] Henrianna Pang,[§] Evelyn J. Walsh,[||] and Anthony P. Moran[||]

Department of Chemistry, York University, North York, Toronto, Ontario M3J 1P3, Canada, Carbohydrate Research Centre, Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario M5S 1A8, Canada, and Department of Microbiology, University College, Galway, Ireland

Received August 9, 1995; Revised Manuscript Received November 20, 1995[⊗]

ABSTRACT: Smooth- and rough-form lipopolysaccharides from phenol−water extraction of cells from *Helicobacter pylori* type strain NCTC 11637 were isolated as the water-soluble component of high-M_r and water-insoluble low-M_r gel. Structural investigations were performed on the intact water-soluble smooth-form lipopolysaccharide, various oligosaccharides formed as chemical and enzymic degradation products, and three oligosaccharide fractions liberated by acetic acid hydrolysis from the water-insoluble rough-form lipopolysaccharide. A structure is proposed for the complete polysaccharide component of the smooth-form lipopolysaccharide comprising the O antigen chain, an intervening region, and the inner core oligosaccharide on the basis of ^{1}H and ^{13}C NMR experiments, fast atom bombardment/mass spectrometry, and methylation linkage analysis of permethylated oligo- and polysaccharide derivatives. The most striking feature of the O antigen region in the lipopolysaccharide is the presence of extended chains with fucosylated and nonfucosylated *N*-acetyllactosamine (LacNAc) units that mimic human cell surface glycoconjugates in normal human granulocytes. The chains are terminated by di- or trimeric Lewis^x (Le^x) determinants, which are also found in tumor-associated carbohydrate antigens in many adenocarcinomas.

Helicobacter pylori (formerly *Campylobacter pylori*) is an important and widespread human pathogen which is implicated as a causative agent of gastritis, gastric and duodenal ulcers, and gastric carcinoma (Cover & Blaser, 1995). The characterization of the bacterial surface structures is incomplete, and little is known structurally of lipopolysaccharides (LPS),[1] one of the key components of the outer membrane of the bacterium.

H. pylori LPS has low endotoxic activity, induces a low immunological response, and has been implicated in a variety of biological interactions [for a review, see Moran (1995)]. These include an inhibitory effect on mucus glycosylation, interference with mucosal integrity, inhibition of the interaction of mucin with its receptor (Piotrowski et al., 1993), the stimulation of pepsinogen secretion, and a role in the mediation of adherence of the bacterium to laminin in the basement membrane (Valkonen et al., 1994). These effects, however, have been noted without regard to structural differences in LPS. Evidence for such differences has been indicated by an ability to differentiate *H. pylori* strains on the basis of electrophoretic patterns in sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) coupled with antigenic analyses with strain-specific antisera (Mills et al., 1992). In considering such structural variations, account must be taken of possible modifications of LPS expression during in vitro cultivation. The modifications include loss of O antigen chains on repeated passages on solid medium and its reversal when grown in a fluid medium (Moran & Walsh, 1993a,b).

This, the first of two papers presenting evidence for differences in LPS structures from *H. pylori* strains, gives a complete account of the polysaccharide component, O antigen chains through to the inner core oligosaccharide (OS) regions, of LPS from the *H. pylori* type strain NCTC 11637 (ATCC 43504). We report the results of studies on both water-insoluble rough-form LPS (R-LPS), supposedly of exclusively low M_r on the basis of SDS−PAGE and for which compositional analysis has been reported (Moran et al., 1992), and water-soluble smooth-form LPS (S-LPS) of relatively uniform size (SDS−PAGE), for which a preliminary account of the O antigen structure has been published (Aspinall et al., 1994c). The overall approach to the structural investigations is based on that used in studies on LPS from *Campylobacter* species in which insoluble gels from phenol−water extractions of bacterial cells comprise low-M_r LPS with core OS linked to lipid A, as for

[†] This work was supported by research grants from the Natural Sciences and Engineering Research Council of Canada (to G.O.A.), the Medical Research Council of Canada (for the Carbohydrate Research Centre), and the Irish Health Research Board (to A.P.M.).

* Address correspondence to this author at the Department of Chemistry, York University, North York, Toronto, Ontario M3J 1P3, Canada.

[‡] York University.

[§] University of Toronto.

[||] University College, Galway.

[⊗] Abstract published in *Advance ACS Abstracts,* February 1, 1996.

[1] Abbreviations: 1D, one-dimensional; 2D, two-dimensional; COSY, correlated spectroscopy; EI, electron impact; ES/MS, electrospray/mass spectrometry; FAB/MS, fast atom bombardment/mass spectrometry; Fuc, fucose; Gal, galactose; Galol, galactitol; GC/MS, gas chromatography/mass spectrometry; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GPC, gel-permeation chromatography; Hep, heptose; D-Hep, D-*glycero*-D-*manno*-heptose (similarly for L-Hep); HMQC, Heteronuclear multiple quantum correlated spectroscopy; HPLC, high-performance liquid chromatography; Kdo, 3-deoxy-D-*manno*-octulosonic acid; LPS, lipopolysaccharide; OS, oligosaccharide; PAGE, polyacrylamide gel electrophoresis; R-LPS, rough-form LPS; SDS, sodium dodecyl sulfate; S-LPS, smooth-form LPS; TOCSY, total correlated spectroscopy; ud, unresolved doublet.

0006-2960/96/0435-2489$12.00/0

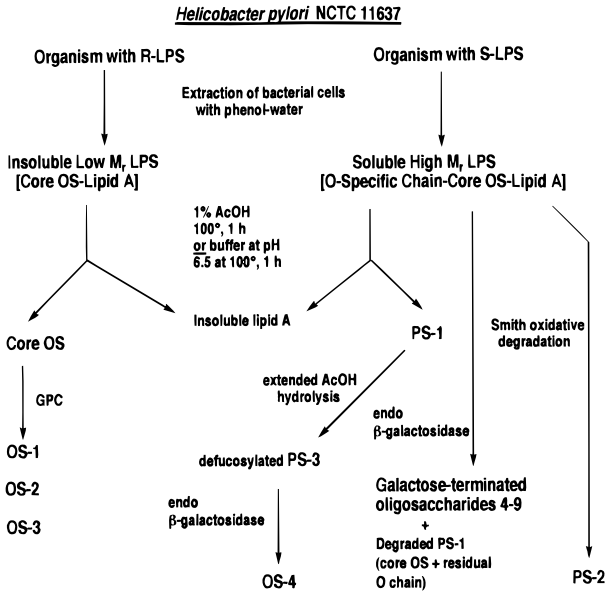

FIGURE 1: Transformations of rough- and smooth-form LPS preparations from *H. pylori* type strain NCTC 11637.

Campylobacter jejuni serotype O:19 (Aspinall et al., 1994a). The aqueous phases from the extracts frequently furnish polysaccharides either as the O antigen component of high-M_r LPS, as for *C. jejuni* serotype O:19 (Aspinall et al., 1994b), or as separate carbohydrate polymers not associated with lipid A, as for *Campylobacter coli* serotype O:30 (Aspinall et al., 1993). Figure 1 shows the general strategy adopted for the examination of the *H. pylori* LPS components. We present first the full details of the previously summarized studies of the S-LPS (Aspinall et al., 1994c) together with the very recent isolation of an oligosaccharide (**OS-4**) remaining after chemical and enzymic degradation of the parent LPS. Mutually supportive evidence came from studies on oligosaccharides (**OS-1**, **OS-2**, and **OS-3**) from the R-LPS, of presumed low M_r, but in this preparation showing progressive chain extensions from the inner core OS region leading to O chain initiation, and studies on **OS-4** from degradation of the S-LPS. From these observations, a structural model has been developed, and a structure is proposed for the complete polysaccharide chain from the nonreducing terminus of the O antigen chain through an intervening region to the Kdo-terminated core OS resulting from cleavage of the ketosidic linkage to lipid A.

EXPERIMENTAL PROCEDURES

Bacteria and Growth Conditions. H. pylori NCTC 11637 was obtained from the National Collection of Type Cultures (Public Health Laboratory Service, London, England). For production of S-LPS, *H. pylori* was grown in a liquid medium of brain heart infusion and 2% fetal calf serum (Moran & Walsh, 1993b), whereas for production of R-LPS, *H. pylori* was grown on blood agar under microaerobic conditions as described previously (Moran et al., 1992).

*Isolation of Lipopolysaccharides and Liberated Polysaccharides (**PS-1** and **PS-3**) from S-LPS and Liberated Core Oligosaccharides (**OS-1, OS-2,** and **OS-3**) from R-LPS.* As outlined in Figure 1, S- and R-LPS were isolated as described previously (Moran et al., 1992). Gel-permeation chromatography (GPC) of the soluble high-M_r LPS on a column (1 × 105 cm) of Bio-Gel P-6 with detection by the phenol−sulfuric acid assay (Dubois et al., 1956) afforded a single fraction (S-LPS) which was used in subsequent experiments. S-LPS was heated in aqueous 1% acetic acid at 100 °C for 1 h to cleave the ketosidic linkage to lipid A. Lipid A was precipitated by centrifugation, and after concentration, the supernatant solution was purified by GPC on Bio-Gel P-6 by elution with water to give **PS-1**. Further treatment of **PS-1** in aqueous 5% acetic acid at 100 °C for 2 h followed by GPC on Bio-Gel P-6 afforded fucose-free polysaccharide, **PS-3**.

R-LPS obtained as a water-insoluble gel was heated at 100 °C for 1 h in 0.1 M acetate buffer (pH 6.5) to cleave the ketosidic linkage to lipid A with minimum hydrolysis of fucosidic bonds (Yamasaki et al., 1993). The aqueous supernatant solution was concentrated and fractionated by GPC on Bio-Gel P-2 (1 × 105 cm) with elution with water to give two fractions **OS-1** and **OS-2**, between which base line separation was not achieved, and a fully separated fraction **OS-3**.

Analytical and Spectroscopic Methods. GC was performed on a Hewlett-Packard model 5890A chromatograph. Separations were achieved using capillary columns with the following programs: DB-23 (30 m × 0.25 mm) (A) isothermally at 220 °C, (B) isothermally at 190 °C, and (C) at 190 °C (10 min), to 250 °C at 2 °C/min and (D) DB-17 (15 m × 0.25 mm) isothermally at 190 °C. GC/MS was carried out on a KRATOS analytical Profile instrument in the EI (70 eV; source temperature, 220 °C) mode.

FAB/MS spectra were recorded with a VGZAB-SE instrument equipped with an Ion Tech saddle field gun. Permethylated glycans in methanol (1−2 μL) were loaded onto the target with a matrix (1 μL) of thioglycerol or 3:1 thioglycerol−glycerol. The samples were bombarded with xenon atoms (1.2 mA anode current, 8 keV anode potential), spectra were recorded with a VG 11-250 data system under the multichannel analyzing mode, and four to five scans were acquired. Resolution was set at 1500−2000 (10% valley definition), and CsI was used as calibrant. In some cases, the resolution was set lower in the high-mass range; thus, the average mass to charge was observed for the molecular ion. The molecular ions and fragment ions are reported as the nominal mass of the ^{12}C-containing component (corresponding to the mass of integral atomic weights). The observed mass is about 1.5 amu higher in the 2000 mass range.

The interpretations of positive ion mass spectra of permethylated derivatives were as previously described (Dell et al., 1990). Spectra of permethylated derivatives from glycans with reducing Kdo termini showed that the major component of the molecular ion cluster corresponded to that calculated for $[M + H]^+$ or other adduct ions. Related ions at $[M + H]^+ - 46$ and $[M + H]^+ - 116$ were sometimes present, and their co-occurrence aided in the identification of molecular ions.

Electrospray/mass spectrometry (ES/MS) and HPLC/ES/MS were performed on a PE-Sciex API-III instrument. The orifice voltage was set at 80 V. HPLC was carried out with a C_8 RP-300 column (100 × 2.1 mm) with H_2O and MeOH as eluent.

Sugar Analyses. Glycose analyses as alditol acetate derivatives (Sawardeker et al., 1967) were performed by GC using program A. Enantiomeric configurations were determined by the conversion of aldoses into 2-(*R*)- and/or 2-(*S*)-

butyl glycosides (Leontein et al., 1978) and GC analysis of acetylated derivatives using program C. The presence of Kdo was confirmed by methanolysis (0.75 M CF_3CO_2H at 100 °C for 6 h), followed by acetylation and GC/MS using program D.

Methylations. Samples (0.3−0.5 mg) were methylated in Me_2SO by the method of Ciucanu and Kerek (1984) followed by passage through Sep-Pak C_{18} cartridges (York et al., 1986). Portions of the permethylated derivatives were retained for FAB/MS and/or ES/MS, and the remaining portions were hydrolyzed in aqueous 4 M CF_3CO_2H at 100 °C for 4 h, followed by reduction with $NaBD_4$ and acetylation. The partially methylated alditol acetates were analyzed by GC/MS using programs B and C.

The quantitative aspects of methylation analyses are often presented only as approximate molar ratios which reflect the natural microheterogeneity of permethylated oligo- and polysaccharides shown in their FAB/MS data. The detection in these spectra of homologous series of oligoglycosyl-oxonium ions of individually precise composition may identify the nature of the microheterogeneity and may also aid in sequence determination. Departures from integral molar ratios of constituents are therefore to be expected, and the following are typical examples: (i) half molar ratios which reflect the occurrence in similar abundance of the corresponding units in homologous fragment ions; (ii) tr = trace which denotes the presence of a minor (<0.2 M) constituent to which specific attention may be drawn in sequence determination, e.g. a newly exposed end group in a structurally incomplete segment of an oligosaccharide unit; and (iii) <1 which denotes a residue no longer fully represented in an attenuated chain (the converse of ii).

Determination of the linkage site of the Kdo residue was carried out by the procedure of Tacken et al. (1986) by methylation of the $NaBD_4$-reduced **OS-3**, followed by reduction with Super-Hydride, hydrolysis as before, and analysis of the derived partially methylated alditol acetates, including a 3-deoxyoctitol derivative in addition to the previously characterized derivatives using program B.

Chemical and Enzymic Degradations. The Smith degradation was performed as described by Pritchard et al. (1988) by oxidation with $NaIO_4$, desalting by GPC on Bio-Gel P-6, reduction with $NaBD_4$, decationization, concentration with methanol, and mild hydrolysis with 1 M CF_3CO_2H at room temperature for 2 h. The products were separated by GPC on a column of Bio-Gel P-2. O-Dephosphorylation was carried out by treatment with aqueous 48% HF at 4 °C for 48 h. Excess HF was removed in a vacuum desiccator over KOH, and the products were fractionated by GPC on Bio-Gel P-2.

Digestions with *Bacteroides fragilis endo-β*-D-galactosidase (Boehringer Mannheim) were performed in 0.1 M sodium acetate buffer at pH 5.8 for 12 h at 37 °C. The enzyme was denatured by heating the solution at 100 °C, and after removal of the precipitate by centrifugation, the solution was desalted and fractionated by GPC on a column of Bio-Gel P-2.

^1H, ^{13}C, and ^{31}P NMR Spectroscopy. 1D and 2D NMR experiments were carried out on a Bruker ARX 400 spectrometer in the Fourier transform mode. The glycan samples were exchanged several times with D_2O (99.8%) and dissolved in D_2O (99.9%). Chemical shifts were measured relative to internal acetone (δ 2.225 for ^1H NMR and δ 31.4 for ^{13}C NMR). Chemical shifts for ^{31}P NMR were measured with reference to external orthophosphoric acid (δ 0.0).

The following parameters were used in 2D NMR experiments: $^1H-^1H$ COSY and TOCSY, 512 × 1024 data matrix, zero-filled to 1024 points in t_1, spectral width of 2048 Hz, 64 scans per t_1 value, 1.5 s for the recycle delay, mixing time of 80 ms for TOCSY, unshifted sine-bell filtering in t_1 and t_2 for COSY, but shifted sine-squared filtering in t_1 and t_2 for TOCSY as for all phase sensitive experiments; $^1H-^{13}C$ HMQC, 256 × 2048 data matrix, zero-filled to 1024 data points in t_1, 64 scans per t_1 value, recycle delay of 1.0 s, fixed delay of 3.5 ms, and pulse angle of 60°.

RESULTS

Characterization of the O Antigen Chains in S-LPS. S-LPS was almost completely water-soluble. In preliminary investigations (Aspinall et al., 1994c), it was noted that treatment of the water-soluble LPS from the smooth-form organism under standard conditions with dilute acetic acid, to cleave the ketosidic linkage of the Kdo terminus to lipid A, resulted in some liberation of fucose. In order to avoid inadvertent loss of fucose residues, the key experiments for the characterization of the O antigen chain were, therefore, repeated on untreated LPS. Glycose analyses by the alditol acetate method (Sawardeker et al., 1967) showed that the main constituents were Fuc, Gal, and GlcNAc in the approximate molar ratio of 5:10:9, with smaller amounts (≤2 M proportions) of sugars, Glc, DD-Hep, and LD-Hep which had been shown previously to be constituents of R-LPS (Moran et al., 1992). The previously assumed absolute configurations of the two Hep constituents were confirmed by the chiral glycoside procedure (Leontein et al., 1978). However, we were unable to detect more than traces of the previously reported Man as a constituent (Moran et al., 1992), and we now regard this sugar as an artifact for which no independent evidence was obtained in other experiments. Control experiments have shown that the addition of sodium borohydride before complete removal of acid after hydrolysis may, with formation of sodium borate, produce sufficiently basic conditions for enolization and epimerization at C-2 to occur as a competing reaction, before reduction is complete, with generation of mannitol from glucose in amounts up to 25%. The anomeric configurations of the dominant sugar residues were defined by ^1H 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], β-D-galactose ($\delta_{H\text{-}1}$ 4.69, $J_{1,2}$ 7.2 Hz), and *N*-acetyl-β-D-glucosamine ($\delta_{H\text{-}1}$ 4.46, $J_{1,2}$ 7.7 Hz). The presence of these main constituents was confirmed in a $^1H-^{13}C$ HMQC spectrum with the assigment of anomeric carbon resonances at δ_C 103 ($J_{C,H}$ 162 Hz) for β-Gal, δ_C 102.6 and 102.4 (each $J_{C,H}$ 163 Hz) for β-GlcNAc, and δ_C 99.6 ($J_{C,H}$ 169 Hz) for α-Fuc residues (the resonance for the minor component was too weak for detection) and additional resonances for GlcNAc residues at δ_C 175.1 (**C**O), 55.7 (**C**-2), and 22.6 (**C**H_3). The ^{31}P NMR spectrum for **PS-1** showed a single resonance at δ 3.79 at pD 6 assignable to a phosphoric monoester.

Linkage analysis performed on the permethylated LPS showed the presence of terminal Fuc, 3-linked Gal, and 4-linked and 3,4-linked GlcNAc residues in the approximate molar ratio of 5:9:4:4, together with the sugar residues (approximately 1 mol equiv each of variously substituted

Table 1: Methylation Linkage Analysis of LPS and Derived Oligosaccharides **OS-1**, **OS-2**, **OS-3**, and **OS-4** from *H. pylori* NCTC 11637

structural units from methylationanalysis	approximate molar ratios[a]				
	LPS	**OS-1**	**OS-2**	**OS-3**	**OS-4**
O antigen chain					
Fuc	5	3	1		
Gal	1	1	0.5		
→3)Gal	9	4			
GlcNAc					1
→3)GlcNAc			0.5		
→4)GlcNAc	4	1			
→3/4)GlcNAc	4	3	0.5		
intervening and core regions					
Glc	1	1	2	1	1
→3)Glc	1	1	1		1
→6)Glc		1			
→4)Gal	1	1	1	1	1
DD-Hep				tr	
→7)DD-Hep	1	1	0.5	1	1
→2)DD-Hep					tr
→2/7)DD-Hep	1	1	1.5		1
→2)LD-Hep	1	1	1	1	1
→3)LD-Hep →3)-LDHep(P)	1	1	1	1	1
→5)Kdo	nd	nd	nd	1	nd

[a] For comment on the assessment of data, see Methylations in Experimental Procedures.

Glc, DD-Hep, and LD-Hep units) arising from the inner region of the LPS (Table 1). The major constituents were consistent with the presence of an *N*-acetyllactosaminoglycan with 3-linked Gal and 4-linked GlcNAc residues with approximately half of the latter carrying at O-3 terminal Fuc residues. An additional feature, not listed in Table 1, was the formation of a small proportion (<1 M proportion) of nonterminal 3-linked Fuc residues. In our preliminary report (Aspinall et al., 1994c), this constituent was postulated to arise from fucobiose units and this possibility seemed to be confirmed with the detection of periodate-resistant residues in the product from Smith degradation (*vide infra*). However, further studies failed to provide independent evidence for this postulate through the isolation of oligosaccharides containing the fucobiose unit, and we now consider that these observations arose respectively from incomplete methylation and inter-residue hemiacetal protection from complete periodate oxidation during the Smith degradation.

More detailed information for the distribution of linkages in the branched glycan was obtained from two series of degradations. The Smith degradation sequence [periodate oxidation, reduction ($NaBD_4$), and mild acid hydrolysis of acyclic acetals] yielded an essentially linear *N*-acetyllactosaminoglycan (**PS-2**) arising from the O antigen region (**PS-1**) of the LPS (Figure 2). Linkage analysis showed 3-linked Gal and uniformly 4-linked GlcNAc residues as the two major components, and FAB/MS of the permethylated derivative showed a series of glycosyloxonium fragment ions at *m*/*z* 260, 709, 1158, 1607, 2056, and 2505 from preferential cleavage at GlcNAc residues that indicated a regular alternation of GlcNAc and Gal residues. No additional units were detectable at the reducing terminus of **PS-2** that might have pointed to the connection of the O antigen chain to the core OS region of the LPS. A small proportion of Fuc residues may be regarded as having escaped oxidation but, in the absence of unambiguous supporting evidence, probably does not arise from genuine nonterminal Fuc residues. A

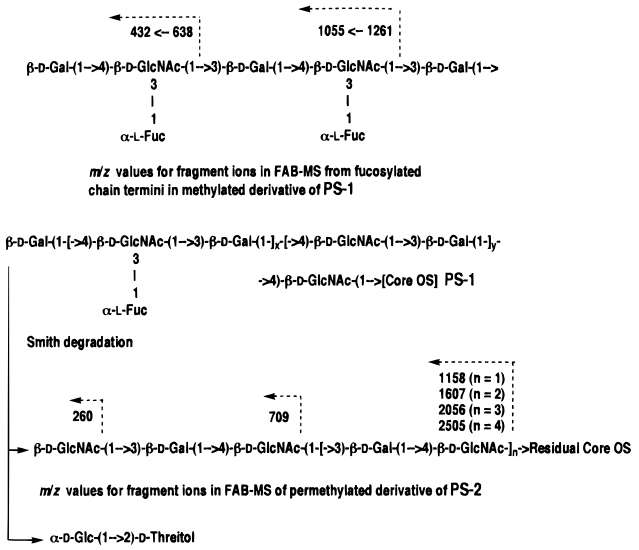

FIGURE 2: Analysis of positive ion FAB/MS data for the permethylated O antigen chain **PS-1** of *H. pylori* LPS and of the permethylated derivative of the product **PS-2** from Smith degradation.

minor component of low M_r was obtained when the material from the Smith degradation was fractionated by GPC on Bio-Gel P-6. Hydrolysis of this component gave Glc and threitol in equimolar amounts, and the compound was characterized by ^{1}H NMR ($\delta_{H\text{-}1}$ 4.90, $J_{1,2}$ 3.8 Hz), methylation analysis (2,3,4,6-tetra-*O*-methylglucose and 1,2,4-tri-*O*-methylthreitol), and GC/MS of the intact permethylated derivative as 2-*O*-α-D-glucopyranosyl-D-threitol-1-*d* (Figure 2) [$(M + H)^+$ at *m*/*z* 382, fragment ions at *m*/*z* 219 (A_1) and 146 (J_2)] (Aspinall, 1982). The significance of this degradation product within the LPS structure did not become apparent until after the inner regions of the R-LPS had been examined.

The second degradative approach to the determination of the fine structure of the O antigen chain in the S-LPS was by the action of *B. fragilis endo-β*-D-galactosidase. The specificity of the enzyme in the depolymerization of incompletely fucosylated lactosaminoglycans, whereby cleavage takes place at locations other than terminal galactosyl linkages to nonfucosylated GlcNAc residues (Kannagi et al., 1982), led to the formation of two categories of oligosaccharides (Figure 3): (a) those containing a nonreducing terminal *β*-D-Gal from a single glycosyl cleavage and (b) those containing a nonreducing *β*-D-GlcNAc end group resulting from internal regions of the chain by cleavage at linkages to two unbranched *β*-D-GlcNAc residues. The products of enzyme action were fractionated by GPC on Bio-Gel P-2 and furnished a degraded polysaccharide, higher oligosaccharides in a mixture that was too complex for unambiguous structural assignments, and three oligosaccharide fractions A, B, and C. Fraction C contained a single component **4**, but despite incomplete preparative separation of oligosaccharides **8** and **9** in fraction A and of oligosaccharides **5**−**7** in fraction B, the structures of oligosaccharides **4**−**9** were assigned from the following experiments. ^{1}H NMR spectroscopy showed the characteristic anomeric resonances of the known sugar residues in glycosidic linkages and for each component, after treatment with $NaBD_4$, the disappearance of the dominant anomeric resonance ($\delta_{H\text{-}1}$ 5.13, $J_{1,2}$ 3.9 Hz) of the reducing α-D-Gal terminus. Pseudomolecular ions $[M + H]^+$ from FAB/MS of the intact permethylated glycosylalditols defined the compositions of

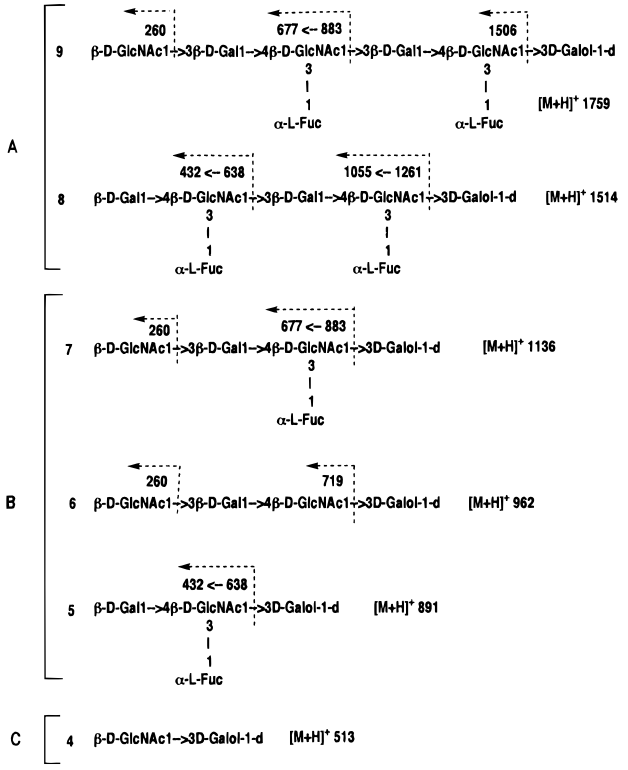

FIGURE 3: Analysis of positive ion FAB/MS data of permethylated oligoglycosylalditols derived from oligosaccharides **4**−**9** liberated from the LPS O antigen chain by the action of *endo*-β-D-galactosidase.

oligosaccharides, and determination of sequences of sugar units was aided by the formation of fragment ions by preferential cleavage at GlcNAc residues. The ready loss by β-elimination of Fuc units (206 amu) attached at O-3 of GlcNAc glycosyloxocarbenium ions served to identify those residues bearing side chains. Subsequent methylation linkage analysis using standard GC/EIMS confirmed the presence of 3-O-substituted galactitol termini and the expected glycose residues in the *N*-acetyllactosaminoglycan backbone with variable degrees of fucosylation.

The results of these experiments are summarized in Table 2, and the structures of oligosaccharides **4**−**9**, even when in admixture, were unambiguously assigned. Disaccharide **4** and tetrasaccharide **6** arose from unbranched internal GlcNAc-Gal segments without Fuc side chains, and Fuc-containing pentasaccharide **7** and octasaccharide **9** derive from branched internal regions. Tetrasaccharide **5** and heptasaccharide **8** were assigned to nonreducing terminal sequences which incorporate monomeric and dimeric Lewis^x (Le^x) determinants. The presence of these terminal sequences was also shown in the FAB/MS of the permethylated LPS (Figure 2) with the detection of the same glycosyloxocarbenium fragment ions at *m*/*z* 638 and 1261, each of these showing ready loss of 206 amu by β-elimination of Fuc residues attached at O-3 of GlcNAc units. No evidence was obtained for Gal-terminated chains in which the outermost GlcNAc residue did not carry a fucosyl side chain. Confirmation of the compositions of oligosaccharides in fraction B was obtained after HPLC separation of the permethylated glycosylalditols and examination by electrospray mass (ES) spectrometry. Thus, one subfraction showed pseudomolecular ions $[M + NH_4]^+$ at *m*/*z* 908 and $[M + H]^+$ at *m*/*z* 891 for **5** (Figure 4, panel **a**) with a prominent fragment ion at *m*/*z* 638. Another subfraction showed **7** as the dominant component with pseudomolecular ions for $[M + NH_4]^+$ at *m*/*z* 1153 and $[M + H]^+$ at *m*/*z* 1136, and a prominent fragment ion at *m*/*z* 883, and **6** as a minor component with $[M + NH_4]^+$ at 962 (Figure 4, panel **b**). No oligosaccharides containing fucobiose units could be detected.

The most striking conclusion from the results from this part of the investigation was the recognition of bacterial components closely resembling in structure the extended oligomeric Le^x chains in fucosylated *N*-acetyllactosaminoglycans (Spooncer et al., 1984) and glycolipids (Fukuda et al., 1985) and in many adenocarcinomas (Hakomori et al., 1984) with fucosylated type 2 chains. Evidence for the connection of the repetitive structure in the O antigen chains to the LPS core emerged from studies on an R-LPS of *H. pylori* strain NCTC 11637.

Examination of Core Oligosaccharides Liberated from R-LPS. Since R-LPS of *H. pylori* NCTC 11637 isolated from phenol−water extraction was largely water-insoluble, detailed studies of core OS structure were carried out on material obtained after treatment with 1% aqueous acetic acid under standard conditions. Compositional analysis of the liberated OS preparation showed the presence of significant amounts of sugar constituents from O antigen chains in addition to those arising from previously reported R-LPS (Moran et al., 1992). Separation of the OS preparation by GPC on Bio-Gel P-2 afforded in succession three fractions, of which that of lowest M_r, **OS-3**, had a composition showing an absence of O chain constituents. The first two fractions **OS-1** and **OS-2** were less completely resolved but were sufficiently different in extent of O chain sugar constituents to justify separate examination. Parallel studies of the fractions involved ^{1}H and ^{31}P NMR of the parent OS, FAB/MS of permethylated derivatives, and accompanying linkage analyses (Table 1).

*Characterization of Core **OS-3.*** **OS-3** had the approximate composition of D-Glc, D-Gal, DD-Hep, and LD-Hep in the molar ratio of 1:1:1:2 with additional single units of phosphate as phosphoric acid monoester (from ^{31}P NMR) and Kdo (from FAB/MS and linkage analysis of the permethylated derivative); Fuc and GlcNAc as typical O chain constituents were absent. The ^{1}H NMR spectrum showed anomeric resonances for α-D-Glc (δ 4.85), and β-D-Gal (δ 4.48) and unresolved doublets at δ 5.39 and 5.02 (double intensity) typical of Hep residues with the α-*manno* configuration. Confirmation of the ring configurations of the two Hex and three Hep residues was obtained from ^{1}H−^{1}H COSY and TOCSY experiments showing expected values for coupling constants $J_{2,3}$ and $J_{3,4}$ for the *gluco*, *galacto*, and *manno* configurations. Methylation linkage analysis gave derivatives of the major components (Table 1) from a linear hexasaccharide. Derivatives of terminal Gal and DD-Hep residues were formed as minor components, and only small amounts of a 3,7-di-O-substituted LD-Hep were liberated from a phosphorylated Hep residue. A similar linkage analysis of **OS-3a** after dephosphorylation of **OS-3** yielded a derivative of 3-linked LD-Hep residue as an additional major component. Analysis by the procedure of Tacken et al. (1986) showed the presence of the 5-linked Kdo reducing terminus. FAB/MS of the permethylated **OS-3a** (Figure 5) gave pseudomolecular ions, $[(M + Na) - 46]^+$, at *m*/*z* 1451 for the major component corresponding to a composition of Hex_2Hep_3Kdo and minor components at *m*/*z* 1247 and 1043

Table 2: Methylation Linkage Analysis of Oligosaccharides **4**−**9** Formed by the Action of *endo*-β-D-Galactosidase on Smooth-Form LPS

		structural units from methylation analysis of oligoglycosylalditols-1-*d*[a]							
fraction	oligosaccharide	Fuc1→	GlcNAc1→	4GlcNAc1→	3/4GlcNAc1→	Gal1→	f3Gal1→	3Galol-1-d	[M + H]
A	**9**	2	1		2		2	1	1759
A	**8**	2			2	1	1	1	1514
B	**7**	1	1		1		1	1	1136
B	**6**		1	1			1	1	962
B	**5**	1			1	1		1	891
C	**4**		1					1	513

[a] The identities of the constituent sugar residues were confirmed by GC/MS, and their relative proportions in individual oligosaccharides present in mixtures were calculated on the basis of the pseudomolecular ions $[M + H]^+$ and the fragment ions shown in Figure 3.

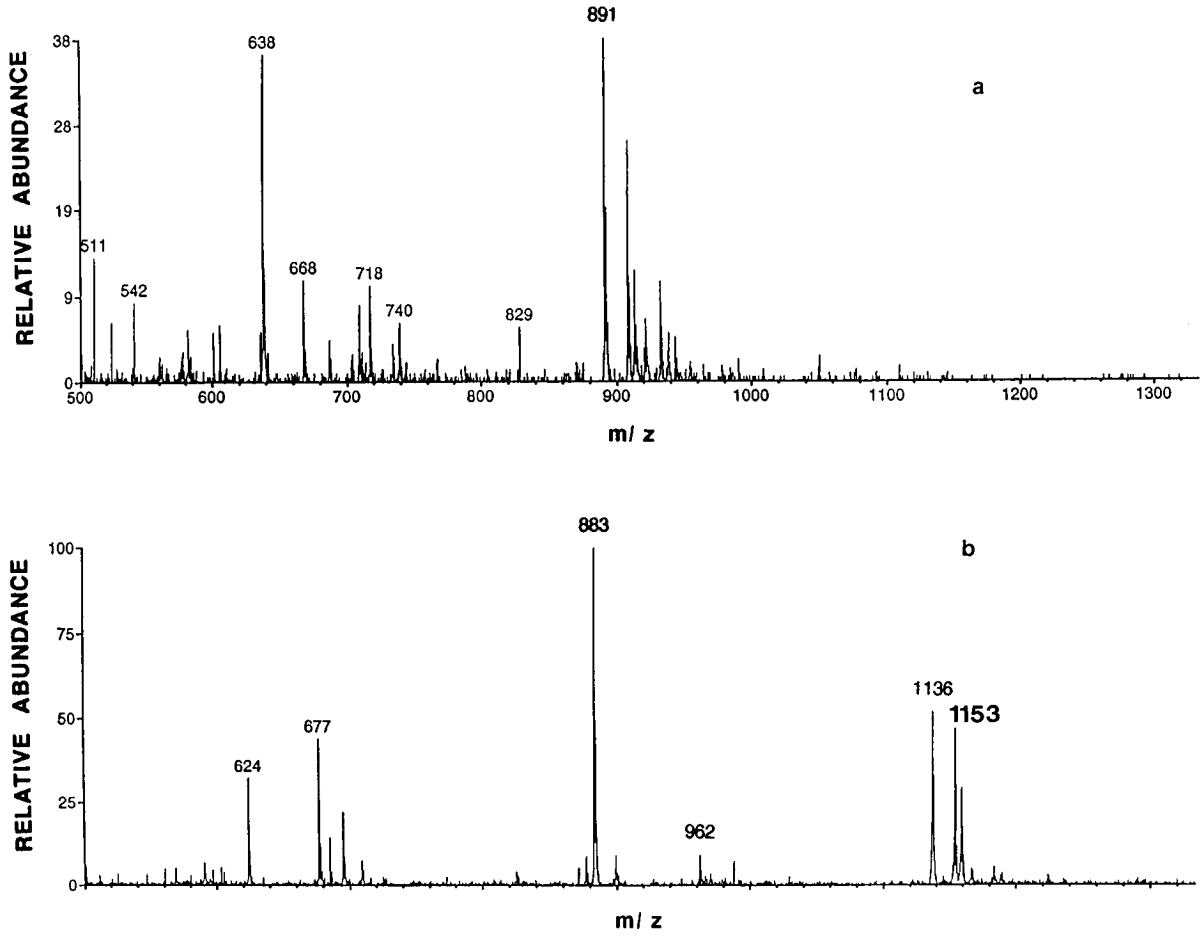

FIGURE 4: Electrospray/mass spectra of selected permethylated oligoglycosylalditols in fraction B: (a) subfraction containing permethylated **5** and (b) subfraction containing permethylated **7**.

with compositions of $HexHep_3Kdo$ and Hep_3Kdo, respectively. Glycosyloxonium ions, interpreted in Figure 5 as arising from the penta- and tetrasaccharide species in **OS-3a** in addition to those from the major component, defined the sequence of sugar residues in the complete hexasaccharide. The detection of a small amount of terminal DD-Hep residues served to place that sugar unit as distal to the two units of LD-Hep. FAB/MS of the permethylated derivative of the phosphorylated **OS-3** showed pseudomolecular ions, $[M + H]^+$, at *m*/*z* 1592 for the major component and *m*/*z* 1184 for a minor tetrasaccharide component with composition Hep_3PKdo. The presence, in low abundance, of fragment ions at *m*/*z* 511 (Hep_2) and 853 (Hep_3 P) arising from the attenuated tetrasaccharide and at *m*/*z* 919 (Hex_2Hep_2) arising from the major hexasaccharide served to establish the attachment of phosphate to the inner Hep residue (Figure 5).

*Characterization of Core **OS-2.*** Compositional analysis of **OS-2** showed the presence of the following constituents in addition to those in **OS-3**, Fuc, GlcNAc, a second DD-Hep, on average less than one extra Gal, and about two further Glc residues per chain, these hexose residues being distributed between four locations. Lack of uniformity in the placing of the additional Gal and Glc residues was indicated by the less than stoichiometric proportions of methylated derivatives in linkage analysis (Table 1) which showed the introduction of the new structural units and the creation of new branch points. The locations of the chain extensions were assessed from variations in the fragment ions shown by FAB/MS, and Figure 6 shows a proposed structure for **OS-2** with an interpretation of the origins of newly observed fragment ions in the FAB/MS of the permethylated derivative. Ions that could be assigned to preferential cleavage at HexNAc residues were observed at *m*/*z* 434 (Fuc,

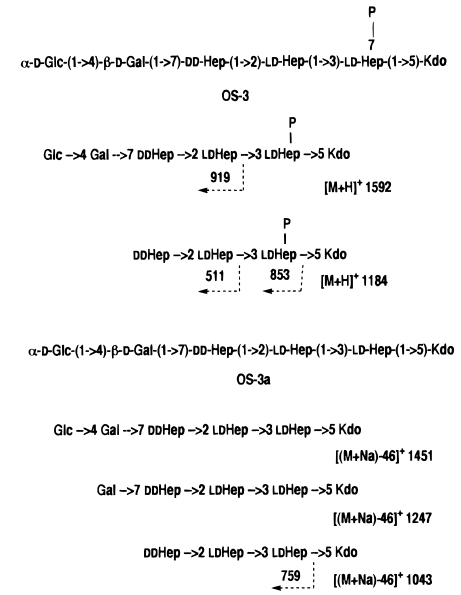

FIGURE 5: Analysis of positive ion FAB/MS data of permethylated **OS-3** and permethylated **OS-3a** (from dephosphorylation of **OS-3**).

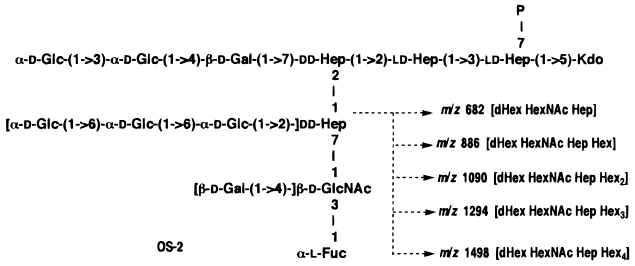

FIGURE 6: Interpretation of fragment ion data in the FAB/MS of permethylated **OS-2**.

GlcNAc) and 638 (Fuc, Gal, GlcNAc). Two ions of unexpectedly high relative abundance were observed at *m*/*z* 682 (Fuc, GlcNAc, Hep) and 886 (Fuc, GlcNAc, Hep, Hex), and progressively less abundant ions at *m*/*z* 1090 (Fuc, GlcNAc, Hep, Hex_2), 1294 (Fuc, GlcNAc, Hep, Hex_3), and 1498 (Fuc, GlcNAc, Hep, Hex_4) had compositions which could be unambiguously assigned from the incremental chain extensions of new Hex residues. With the known structural units of **OS-2**, the ion at *m*/*z* 682 was of unambiguous structure, Fuc(1→3)GlcNAc(1→7)DD-Hep, and the other rather conspicuous glycosyloxonium ions could only have originated from cleavage at Hep residues. We suggest that the ready formation and presumed relative stability of these ions may be a consequence of the anomeric effect of sugar residues having the α-D-*manno* configuration. In agreement with the detection of fragment ions at *m*/*z* 682 and 886, linkage analysis showed the addition of single Gal end groups to some of the GlcNAc residues with the formation of an Le^x terminus. Linkage analysis of **OS-2** showed that both 3- and 6-linked Glc residues were present, and 2D COSY and 2D TOCSY experiments (not shown) indicated the presence of five anomeric signals (Table 3) with the α-D-Glc configuration, but of unequal intensity.

In light of the earlier observation that 2-*O*-α-D-glucopyranosyl-D-threitol was isolated from Smith degradation of S-LPS, it is proposed that these differently linked α-D-Glc residues may be assigned to specific locations. Only one type of Glc residue would be resistant to periodate oxidation,

Table 3: ^{1}H NMR Chemical Shift Data (ppm) from 2D COSY and 2D TOCSY for Assignments of Anomeric[a] and Ring[b] Configurations of Residues in **OS-2**

residue[c]	H-1 ($J_{1,2}$, Hz)	H-2	H-3	H-4	H-5	H-6
α-Hep	5.41 (ud)	4.04	3.99	3.95		
α-Hep	5.39 (ud)	4.04	3.98	3.94		
α-Hep†	5.32 (ud)	4.05				
α-Fuc	5.09 (3.8)	4.01	3.82			
α-Hep	5.08 (ud)	4.11				
α-Hep	4.99 (ud)	4.13				
α-Glc	4.98 (3.9)	3.79	3.89	3.93		
α-Glc†	4.97 (3.8)	3.72	3.70			
α-Glc†	4.95 (3.8)	3.75		3.68		
α-Glc	4.90 (3.9)	3.52	3.71			
α-Glc	4.88 (3.9)	3.49	3.39			
β-Gal†	4.62 (7.7)	3.54	3.74	3.99		
β-GlcNAc	4.54 (7.9)	3.78	3.61	3.56	4.19	3.41
β-Gal	4.49 (7.8)	3.53	3.72	4.01		

[a] Anomeric configurations were assigned on the basis of $J_{1,2}$ values in the table. [b] Ring configurations were assigned on the basis of coupling constants as follows: $J_{2,3} \sim 8-10$ Hz and $J_{3,4} \sim 8-10$ Hz for α-*gluco*; $J_{2,3} \sim 8-10$ Hz and $J_{3,4} \sim 8-10$ Hz for β-*gluco*; $J_{2,3} = 8.8$ Hz and $J_{3,4}$, 3.8 Hz for α-*fuco*; $J_{2,3} \sim 8-10$ Hz and $J_{3,4} \sim 3-4$ Hz for β-*galacto*; and $J_{2,3} \sim 3$ Hz and $J_{3,4} \sim 8-10$ Hz for α-*manno* (in Hep). [c] Residues with resonances of lower intensity are marked with a dagger.

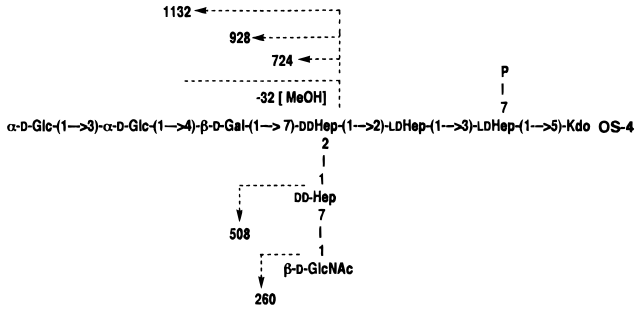

FIGURE 7: Analysis of positive ion FAB/MS data of permethylated **OS-4**.

and the formation of threitol could only arise from a 4-linked Gal residue in the core OS after chain extension with an extra α-D-Glc residue attached by a 1→3 linkage. It is, therefore, implied that O-2 of approximately 50% of the outer DD-Hep residues is the site of attachment of an α-D-Glc residue and on some chains up to two further α-D-Glc residues in 1→6 linkages.

Notwithstanding variability in hexose content and distribution, FAB/MS data showed two main sets of pseudomolecular ions. The first set of ions included $[M + H]^+$ at *m*/*z* 2348, $[(M + H) - 46]^+$ at *m*/*z* 2302, and $[(M + H) - 116]^+$ at *m*/*z* 2232, corresponding to those of permethylated nonphosphorylated oligosaccharides with composition of dHex, Hex_3, HexNAc, Hep_4, Kdo; ions for phosphorylated derivatives were observed at $[M + H]^+$ at *m*/*z* 2442 and $[(M + H) - 46]^+$ at *m*/*z* 2396. The second set of ions included $[M + H]^+$ at *m*/*z* 2552 and $[(M + H) - 46]^+$ at *m*/*z* 2506 for a composition dHex, Hex_4, HexNAc, Hep_4, Kdo. The Hep_4 composition confirmed the presence of a second DD-Hep residue. Since, on average, more than one of the two DD-Hep residues was 2,7-di-O-substituted with the remaining portion singly substituted at O-7, it may be inferred that the 7-linked DD-Hep residues in the core OS backbone of **OS-3** are extended at O-2 and that some of the DD-Hep residues in the side chain are also branched and are the probable site of attachment of the 6-linked α-D-Glc residues. ES/MS of permethylated **OS-2** showed a series of pseudomolecular ions

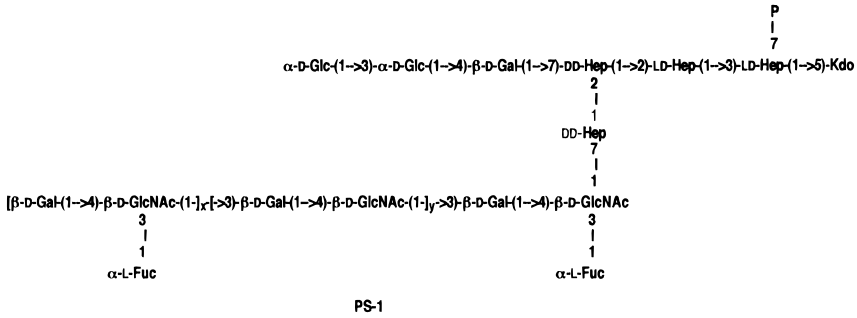

FIGURE 8: O antigen chain, intervening, and inner core regions of smooth-form LPS of *H. pylori* type strain NCTC 11637.

corresponding to the above-mentioned oligosaccharides together with those of compositions of dHex, Hex_5, HexNAc, Hep_4, Kdo and dHex, Hex_6, HexNAc, Hep_4, Kdo in a further demonstration of the size heterogeneity of **OS-2**.

*Characterization of Core **OS-1**.* Linkage analysis data for **OS-1** (Table 1) indicated the presence a more extended O chain than that present in **OS-2**, and hence resembling intact S-LPS. FAB/MS of the permethylated **OS-1** showed fragment ions derived from terminal sequences of dimeric Le^x units (as shown for permethylated LPS in Figure 2). A noteworthy feature was the absence of 6-linked α-D-Glc residues as shown by linkage analysis and by decreases in the number of anomeric resonances for α-D-Glc residues in the ^1H NMR spectrum (not shown).

*Degradation of **PS-3** from S-LPS and Characterization of **OS-4**.* The preceding experiments performed on the oligosaccharides liberated from R-LPS provided essential evidence for the connection of the extended O antigen chain to the inner core segment. Confirmation of this aspect of structure was sought by degradation of the S-LPS. A more extended treatment of LPS with 5% aqueous acetic acid at 100 °C for 2 h resulted in complete defucosylation, in addition to cleavage of the ketosidic linkage of Kdo to lipid A, and afforded **PS-3**. Complete depolymerization of the unbranched lactosaminoglycan chains in **PS-3** was achieved upon treatment with *endo-β*-galactosidase to give the previously characterized disaccharide **4** and **OS-4**. Linkage analysis of **OS-4** (Table 1) showed the presence of a single residual GlcNAc residue as a nonreducing terminus of the side chain, together with most of the residues from the inner region of the S-LPS which were present in **OS-2**. However, a notable difference was the absence of the 6-linked α-D-Glc residues attached to the outer of the two DD-Hep units. FAB/MS of permethylated **OS-4** (for an interpretation, see Figure 7) showed fragment ions at *m*/*z* 260 (HexNAc) and 508 (HexNAc, Hep) and a series of secondary fragment ions at *m*/*z* 724, 928, and 1132. The ion at *m*/*z* 724 may be assigned to that of an elimination ion from an unbranched $HexNAcHep_2$ ion at *m*/*z* 754 with loss of methanol, since no 3-*O*-glycosylheptose residues had been detected. Taken together with the detection of small amounts of 2-linked DD-Hep as a constituent of chains lacking the outer Hex residues in the core component of **OS-4**, it is reasonable to assign the residue undergoing elimination as that of the DD-Hep in the core OS region. The formation of fragment ions from permethylated **OS-4** at *m*/*z* 928 and 1132 possessing incremental differences in hexose content (HexNAc, Hep_2, Hex_n-MeOH) could then be assigned (Figure 7) to similar fragmentations from the more prominent species retaining the outer hexose residues of the core OS chain in the microheterogeneous population of molecules.

DISCUSSION

The results of this investigation on the structure of **PS-1** of *H. pylori* strain NCTC 11637, comprising the O antigen chain and the intervening and core OS regions, may be considered in terms of the structural proposal shown in Figure 8. The O antigen chains of the LPS were shown to consist of partially fucosylated *N*-acetyllactosaminoglycan chains of type 2 with 4-linked GlcNAc residues in the backbone, to which approximately half of the α-L-Fuc residues were attached at O-3. From our FAB/MS experiments, there was evidence for monomeric and dimeric Le^x units at the chain terminus, and from FAB/MS of the permethylated polysaccharide, fragment ions were observed for trimeric Le^x units.

The inner regions of the polysaccharide structure were delineated by the characterization of oligosaccharide fractions liberated from the water-insoluble presumed R-LPS. In place of a single core OS fraction representing the region to which the repetitive chains of O antigen might be directly linked, a mixture of oligosaccharides was isolated. Chromatographic separation gave three fractions; each displayed natural microheterogeneity, but they differed sufficiently in overall structure to provide evidence for the progression of glycosylations leading to the assembly of the mature LPS. Thus, the hexasaccharide, **OS-3**, defined the inner core OS region; the next higher **OS-2** fraction with a microheterogeneous collection of molecules pointed to three types of chain extension: an extension of the core OS by attachment of a second α-D-Glc residue in a 1→3 linkage, the introduction of branching from the DD-Hep residue in the inner core through a second such residue to which the first Le^x unit was to be developed, and in some chains yet further branching in a sequence of up to three α-D-Glc residues in 1→6 linkages. The **OS-1** fraction of highest M_r showed the development of the O chain structure with up to five repeating units. However, in **OS-1**, as in the complete LPS, the 6-linked α-D-Glc residues were no longer present, and their role in the LPS assembly is not known. Complementary evidence from controlled degradation of the S-LPS to give **OS-4** confirmed the proposed connection of the single remaining GlcNAc residue in O antigen chain via an intervening DD-Hep residue to the inner core. In the complete structure (Figure 8) for O antigen chain, and intervening and core OS regions of the polysaccharide portion, **PS-1**, of S-LPS, present estimates suggest that there are eight to nine repeating units in the O antigen chain. The detailed distribution of Fuc residues is not known.

The most significant conclusion from these investigations concerns the elaboration by *H. pylori* of LPS with O antigen chains with oligomeric Le^x epitopes in mimicry of structures expressed mainly on glycolipids in certain human cell surface glycoconjugates. Although found on normal cells, e.g. on granulocytes (Fukuda et al., 1984), the abundant expression of these tumour-associated carbohydrate antigens is limited to malignant cells (Hakomori, 1989). Nevertheless, the $Lewis^x$ is expressed in normal gastric tissue (Sakamoto et al., 1989) which thus poses the question as to whether mimicry of this blood group antigen by *H. pylori* in the gastric mucosa may camouflage the bacterium from the host and thus aid survival of *H. pylori* in that environment. Conversely, the presence of antibodies against *H. pylori* in patients has been found to strongly correlate with the presence of autoantibodies against human antral gastric mucosa (Negrini et al., 1991). Since antibodies cross-reacting with the gastric mucosa have been demonstrated in mice immunized with *H. pylori* (Negrini et al., 1991), the expression of $Lewis^x$-like epitopes on the surface of the bacterium may play a role in the development of an autoimmune reaction contributing to disease. The further implications of these surface structures in interaction between the bacterium and the human host will require biological experiments with chemically defined LPS from different strains of the organism. Furthermore, the structure described here will provide a reference point in the examination of antigenic variants. The accompanying paper reports evidence for differences in the structures of LPS from two other *H. pylori* strains (Aspinall & Monteiro, 1996).

ACKNOWLEDGMENT

We thank Mary Cheung for recording some of the mass spectra.

REFERENCES

Aspinall, G. O., Ed. (1982) *The Polysaccharides*, Vol. 1, pp 73−81, Academic Press, New York.

Aspinall, G. O., McDonald, A. G., Pang, H., Kurjanczyk, L. A., & Penner, J. L. (1993) *J. Biol. Chem. 268*, 18321−18329.

Aspinall, G. O., McDonald, A. G., Pang, H., Kurjanczyk, L. A., & Penner, J. L. (1994a) *Biochemistry 33*, 241−249.

Aspinall, G. O., McDonald, A. G., & Pang, H. (1994b) *Biochemistry 33*, 250−255.

Aspinall, G. O., Monteiro, M. A., Pang, H., Walsh, E. J., & Moran, A. P. (1994c) *Carbohydr. Lett. 1*, 151−156.

Aspinall, G. O., & Monteiro, M. A. (1996) *Biochemistry* 35, 2498−2504.

Ciucanu, I., & Kerek, F. (1984) *Carbohydr. Res. 131*, 209−217.

Cover, T. L., & Blaser, M. J. (1995) *ASM News 61*, 21−26.

Dell, A., Azadi, P., Thomas-Oates, J. E., Jennings, H. J., Beurret, M., & Michon, F. (1990) *Carbohydr. Res. 200*, 59−76.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350−356.

Fukuda, M. N., Dell, A., Oates, J. E., Wu, P., Klock, J. C., & Fukuda, M. (1985) *J. Biol. Chem. 260*, 1067−1082.

Hakomori, S.-i. (1989) *Adv. Cancer Res. 52*, 257−331.

Hakomori, S.-i., Nudelman, E., Levery, S. B., & Kannagi, R. (1984) *J. Biol. Chem. 259*, 4672−4680.

Kannagi, E., Nudelman, E., Levery, S. B., & Hakomori, S. (1982) *J. Biol. Chem. 257*, 14865−14874.

Leontein, K., Lindberg, B., & Lönngren, J. (1978) *Carbohydr. Res. 62*, 359−362.

Mills, S. D., Kurjanczyk, L. A., & Penner, J. L. (1992) *J. Clin. Microbiol. 30*, 3175−3180.

Moran, A. P. (1995) *FEMS Immunol. Med. Microbiol. 10*, 271−280.

Moran, A. P., & Walsh, E. J. (1993a) *Acta Gastro-Enterol. Belg. 56* (Suppl.), 98.

Moran, A. P., & Walsh, E. J. (1993b) *Ir. J. Med. Sci. 162*, 384.

Moran, A. P., Helander, I. M., & Kosunen, T. U. (1992) *J. Bacteriol. 174*, 1370−1377.

Negrini, R., Lisato, L., Zanella, I., Cavazzini, L., Gullini, S., Villianacci, V., Poiesi, C., Albertini, A., & Ghielmi, S. (1991) *Gastroenterology 101*, 437−445.

Piotrowski, J., Slomiany, A., & Slomiany, B. L. (1993) *Biochem. Mol. Biol. Int. 31*, 1051−1058.

Pritchard, D. G., Rener, B. P., Huang, D. H., & Krishna, N. R. (1988) *Carbohydr. Res. 173*, 255−262.

Sakamoto, J., Watanabe, T., Tokumaru, T., Takagi, H., Nakazato, H., & Lloyd, K. O. (1989) *Cancer Res. 49*, 745−752.

Sawardeker, J. H., Sloneker, J. H., & Jeanes, A. (1967) *Anal. Chem. 39*, 1602−1604.

Spooncer, E., Fukuda, M., Klock, J. C., Oates, J. E., & Dell, A. (1984) *J. Biol. Chem. 259*, 4792−4801.

Tacken, A., Rietschel, E. T., & Brade, H. (1986) *Carbohydr. Res. 149*, 279−291.

Valkonen, K. H., Wadström, & Moran, A. P. (1994) *Infect. Immun. 62*, 3640−3648.

Yamasaki, R., Griffiss, J. M., Quinn, K. P., & Mandrell, R. E. (1993) *J. Bacteriol. 175*, 4565−4568.

York, W. S., Darvill, A. G., McNeil, M., Stevenson, T. T., & Albersheim, P. (1986) *Methods Enzymol. 118*, 3−40.

Young, G. O., Stemmet, N., Lastovica, A., Van der Merwe, E. L., Louw, J. A., Modlin, I. M., & Marks, I. N. (1992) *Aliment. Pharmacol. Ther. 6*, 169−177.

BI951852S