JOURNAL OF BACTERIOLOGY, Oct. 1997, p. 6453–6463 Vol. 179, No. 20
0021-9193/97/$04.00+0


# Structural Characterization of the Lipid A Component of *Helicobacter pylori* Rough- and Smooth-Form Lipopolysaccharides

ANTHONY P. MORAN,[1]* BUKO LINDNER,[2] AND EVELYN J. WALSH[1]

*Department of Microbiology, University College, Galway, Ireland,*[1] *and Forschungszentrum Borstel, Zentrum für Medizin und Biowissenschaften, D-23845 Borstel, Germany*[2]

Received 18 April 1997/Accepted 15 August 1997

**The chemical structure of free lipid A isolated from rough- and smooth-form lipopolysaccharides (R-LPS and S-LPS, respectively) of the human gastroduodenal pathogen *Helicobacter pylori* was elucidated by compositional and degradative analysis, nuclear magnetic resonance spectroscopy, and mass spectrometry. The predominant molecular species in both lipid A components are identical and tetraacylated, but a second molecular species which is hexaacylated is also present in lipid A from S-LPS. Despite differences in substitution by acyl chains, the hydrophilic backbone of the molecules consisted of β(1,6)-linked D-glucosamine (GlcN) disaccharide 1-phosphate. Because of microheterogeneity, nonstoichiometric amounts of ethanolamine-phosphate were also linked to the glycosidic hydroxyl group. In S-LPS, but not in R-LPS, the hydroxyl group at position 4′ was partially substituted by another phosphate group. Considerable variation in the distribution of fatty acids on the lipid A backbone was revealed by laser desorption mass spectrometry. In tetraacyl lipid A, the amino group of the reducing GlcN carried (*R*)-3-hydroxyoctadecanoic acid (position 2), that of the nonreducing GlcN carried (*R*)-3-(octadecanoyloxy)octadecanoic acid (position 2′), and ester-bound (*R*)-3-hydroxyhexadecanoic acid was attached at position 3. Hexaacyl lipid A had a similar substitution by fatty acids, but in addition, ester-bound (*R*)-3-(dodecanoyloxy)hexadecanoic acid or (*R*)-3(tetradecanoyloxy)hexadecanoic acid was attached at position 3′. The predominant absence of ester-bound 4′-phosphate and the presence of tetraacyl lipid A with fatty acids of 16 to 18 carbons in length differentiate *H. pylori* lipid A from that of other bacterial species and help explain the low endotoxic and biological activities of *H. pylori* LPS.**

*Helicobacter pylori* is a prevalent pathogen of humans, and chronic infection of the gastric mucosa by the bacterium causes recurrent gastroduodenal inflammatory disease (5, 59). *H. pylori* is the primary cause of active chronic gastritis in humans and plays a pivotal role in the development of peptic ulcer disease (13, 31). Moreover, persistent infection with *H. pylori* is considered a risk factor for the development of adenocarcinoma and lymphoma of the stomach (11, 41).

Like the cell envelopes of other gram-negative bacteria, that of *H. pylori* contains lipopolysaccharides (LPSs). Chemically, LPSs are composed of a poly- or oligosaccharide covalently linked to a lipid component, lipid A (33, 51). Fresh clinical isolates of *H. pylori* produce high-molecular-weight smooth-form LPSs (S-LPSs), which consist of an O side chain, a core oligosaccharide, and lipid A (34, 36). However, strains of *H. pylori* that have been subcultured many times on conventional solid media may produce low-molecular-weight rough-form LPSs (R-LPSs) that lack the O side chain (36). Nevertheless, the phase shift from S- to R-LPS can be reversed when the strains are grown in liquid media, and thus expression of S-LPS can be stabilized in vitro (38).

The repeating units of the O side chains of LPSs of certain *H. pylori* strains have been shown to mimic Lewis$^x$ and Lewis$^y$ blood group antigens in structure (1, 2). Since these blood group antigens are expressed in the gastric mucosa of normal individuals (52), this mimicry may camouflage the bacterium and hence aid colonization (35). Furthermore, numerous investigations of the endotoxic and immunological properties of *H. pylori* LPSs have revealed significantly lower activities of these LPSs than of enterobacterial LPSs (34, 35). *H. pylori* LPS, by inducing a low immunological response, may prolong *H. pylori* infection longer than would occur with a more aggressive pathogen (34). Since lipid A represents the principal structural component responsible for the range of immunological activities of LPS (33, 45, 51, 64), it has been hypothesized that *H. pylori* LPS and, in particular, its lipid A component have evolved their present structure as a consequence of adaptation to chronic infection of the gastric mucosa (28). Preliminary investigations have indicated that the lipid A component of *H. pylori* LPS contains fatty acids longer than those encountered in enterobacterial lipid A and that the lipid A is underphosphorylated (28, 34, 37).

To clarify the molecular basis for the lower immunological activities of *H. pylori* LPS, we have investigated in detail the primary structure of its lipid A moiety. Investigations were initially undertaken with free lipid A liberated by acid hydrolysis from R-LPS, since on a weight basis, lipid A represents a larger proportion of R-LPS than S-LPS, and thus, greater quantities of free lipid A were obtained for analysis. Nevertheless, we also investigated the structure of lipid A from S-LPS, because this high-molecular-weight molecule occurs in vivo. The evidence presented shows that lipid A moieties from both R- and S-LPS are underphosphorylated and underacylated compared with those from enterobacterial LPS.

(A preliminary report of this research was presented at the Fourth Conference of the International Endotoxin Society, 22 to 25 October 1996, Nagoya, Japan.)

* Corresponding author. Mailing address: Department of Microbiology, University College, University Road, Galway, Ireland. Phone: 353-91-524411. Fax: 353-91-525700. E-mail: anthony.moran@ucg.ie.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *H. pylori* NCTC 11637 was obtained from the National Collection of Type Cultures (London, England). Stock cultures were maintained at −70°C in 15% (vol/vol) glycerol-Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). *H. pylori* was grown routinely on blood agar under microaerobic conditions at 37°C for 48 h (36). *H. pylori* NCTC 11637 grown under these conditions expressed R-LPS (36). Bacteria were harvested in sterile distilled water, centrifuged at 5,000 × *g* (4°C, 30 min), and washed twice, and the bacterial pellets were freeze-dried. To obtain *H. pylori* cells expressing S-LPS, bacteria were grown in a broth of brain heart infusion (Oxoid Ltd., London, England) containing 2% (vol/vol) fetal calf serum (Oxoid) as described by Moran and Walsh (38). Bacteria were harvested by centrifugation of broth cultures (5,000 × *g*, 4°C, 30 min) and washed twice, and the pellets were freeze-dried.

**Preparation of LPS.** After pretreatment of bacterial biomass with pronase (Calbiochem, Los Angeles, Calif.) (7), LPS was extracted by the hot phenol-water technique (61). The LPS preparations were purified by treatment with RNase (Sigma Chemical Co., St. Louis, Mo.), DNase II (Sigma), and proteinase K (Sigma) and by ultracentrifugation as described previously (36).

**Preparation of free lipid A.** Free lipid A was obtained by treatment of LPS with HCl (0.1 M, 100°C, 30 min) or with 0.1 M sodium acetate (pH 4.4) (100°C, 2 h) as described previously (36, 39). The former treatment yielded lipid $A_{HCl}$ from R- and S-LPS (R-lipid $A_{HCl}$ and S-lipid $A_{HCl}$, respectively) that lacked substitution at position 1 of the backbone disaccharide, whereas treatment of R-LPS and S-LPS with sodium acetate buffer yielded lipid $A_{NaAc}$ (R-lipid $A_{NaAc}$ and S-lipid $A_{NaAc}$, respectively) with partial substitution at position 1.

**Degradation and derivatization reactions.** Reduction with $NaBH_4$ or $NaB^2H_4$ was accomplished in water (monosaccharides) or in 0.02% aqueous triethylamine (lipid $A_{HCl}$ and derivatives) at 56°C for 16 h. Strong hydrazinolysis was performed essentially as described by Hase and Rietschel (14), but with modifications. The reaction was performed at 100°C for 48 h, and after cooling, the remaining hydrazine was removed by evaporation in a vacuum desiccator over concentrated $H_2SO_4$ (39). Unless otherwise stated, de-O-acylation was performed by mild hydrazinolysis at 37°C as described by Helander et al. (16). N-acetylation was carried out with acetic anhydride-NaOH (14), and O-acetylation was performed with acetic anhydride-pyridine (100°C, 30 min). Methylation was performed according to the procedure of Ciucanu and Kerek (8). Acetolysis was carried out as described by Stellner et al. (56), and acidic methanolysis was performed with methanolic HCl (1 M, 86°C, 1 h) in sealed glass tubes.

**Isolation of the lipid A backbone.** Preparation of the lipid A backbone was accomplished by the degradative procedure described previously (25, 39). Briefly, lipid $A_{HCl}$ was reduced and subjected to strong hydrazinolysis and N-acetylation, and subsequently the product was purified by gel permeation chromatography.

**Preparation of dephosphorylated lipid A derivatives.** Preparative dephosphorylation of LPS was performed with 48% aqueous hydrofluoric acid (Merck, Darmstadt, Germany) at 4°C for 48 h as described previously (16). Free dephosphorylated lipid A (lipid A-HF) was obtained by treatment of dephosphorylated LPS with mild acid (0.1 M HCl, 100°C, 1 h). De-O-acylation of dephosphorylated lipid A was achieved by alkaline methanolysis (0.2 M $NaOCH_3$, 4°C, 24 h) (39).

**Preparation of reduced and dephosphorylated derivatives of LPS and lipid A.** First, LPS was de-O-acylated and purified by gel permeation chromatography (39). The isolated product, which had increased solubility compared with LPS, was then dephosphorylated with hydrogen fluoride and reduced (with $NaBH_4$) to yield reduced-dephosphorylated LPS (25). Second, LPS was de-O-acylated and dephosphorylated, and the product was subjected to mild acid hydrolysis (0.1 M HCl, 100°C, 30 min) (39). Subsequently, this material was reduced (with $NaBH_4$) to produce reduced-dephosphorylated lipid A. Finally, reduced lipid A was produced by reduction of lipid $A_{HCl}$ (with $NaBH_4$).

**Methods of chemical analysis.** Phosphate content was determined according to the method of Lowry et al. (30). Amino components (glucosamine, D-glucosaminitol, and ethanolamine and their phosphorylated derivatives) were liberated by strong acid hydrolysis (4 M HCl, 100°C, 18 h) and quantified in an amino acid analyzer (Alpha Plus 4151; LKB, Bromma, Sweden). Hexosamine content was also determined by the Morgan-Elson assay as modified by Strominger et al. (57). The direct Morgan-Elson assay was performed without hydrolysis. Analysis of 4-amino-4-deoxyarabinose was achieved after treatment of LPS with 4 M HCl (65°C, 15 h) and subsequent reduction and per-O-acetylation (39).

Total fatty acids were liberated from LPS or lipid A preparations by combined acid- and base-catalyzed hydrolysis (39) and, with heptadecanoic acid (17:0) as an internal standard, were carboxymethylated with diazomethane. The resulting methyl esters were determined quantitatively by gas-liquid chromatography (GLC). Ester-bound fatty acids were selectively liberated from vacuum-dried lipid A by alkaline transesterification with sodium methylate (0.25 M, 37°C, 15 h) as described previously (50). Amide-bound acyloxyacyl residues were investigated according to the procedure of Wollenweber et al. (62).

**Chromatographic techniques.** High-voltage paper electrophoresis was performed at pH 2.8, and pherograms were stained with alkaline silver nitrate, thiobarbituric acid, ninhydrin, or molybdate as described previously (36, 39). Gel permeation chromatography was carried out on Sephadex G-25 (Pharmacia, Uppsala, Sweden; column, 50 by 2 cm) and on TSK-HW40S (Merck, Darmstadt, Germany; column, 24 by 1 cm) with water as an eluent. The effluent was monitored at 206 nm (Uvicord S spectrophotometer; LKB), and 2-ml fractions were collected. High-pressure liquid chromatography was performed as described previously (15) with an Aminex HPX-87H column (300 by 7.8 mm; Bio-Rad, Hercules, Calif.) at 40°C under isocratic conditions with 5 mM $H_2SO_4$ as the eluent at a flow rate of 0.75 ml/min.

GLC was performed with a 5890 gas chromatograph (Hewlett-Packard, Avondale, Pa.) equipped with flame-ionization detectors. Injector and detector temperatures were 260 and 280°C, respectively. The carrier gas was helium (0.05 MPa). The chromatograph was equipped with a fused-silica chemically bonded NB-9C capillary column (HNU-Nordion, Helsinki, Finland; 25 m by 0.32 mm) and a chemically bonded HP-5 column (Hewlett-Packard; 25 m by 0.32 mm). The following temperature program for the NB-9C column was used: 200°C for 3 min, increasing to 260°C at 5°C/min (analysis of neutral sugar and amino sugar alditol acetates). The temperature programs for the HP-5 column were 150°C for 3 min, increasing to 300°C at 3°C/min (fatty acid methyl esters and permethylated monosaccharide derivatives); 250°C for 3 min, increasing to 320°C at 5°C/min (permethylated lipid A backbone disaccharides); 150°C for 3 min, increasing to 300°C at 5°C/min (monosaccharide derivatives); and 200°C for 3 min, increasing to 320°C at 10°C/min (monosaccharides carrying amide-bound fatty acid groups).

**MS.** Combined GLC-mass spectrometry (MS) was performed on a Hewlett-Packard 5890 series II gas chromatograph equipped with a chemically bonded HP-5 fused-silica capillary column (25 m by 0.2 mm) and mass selective detector (model 5971A). Helium was used as the carrier gas, and the temperature programs were the same as those used in GLC. Electron-impact (EI) mass spectra were recorded at 70 eV; chemical ionization (CI) mass spectra were obtained with ammonia as the reactant gas. The ion-source temperature was 185°C.

Laser-desorption (LD)-MS of free dephosphorylated lipid A and dephosphorylated and de-O-acylated lipid A was performed with a laser microprobe mass analyzer (LAMMA 500; Leybold AG, Cologne, Germany) under the conditions described previously for the generation of spectra (29). Spectra were recorded after admixture of either sodium or cesium iodide.

**NMR spectroscopy.** Proton nuclear magnetic resonance ($^1$H-NMR) spectra were recorded on a Varian 500 Unity instrument at room temperature in 0.5 ml of $^2H_2O$ (99.96% $^2$H). Signals were referenced to acetonitrile (1.93 ppm) as an internal standard. Samples (5 to 10 mg) for phosphorous ($^{31}$P-NMR) spectroscopy were dissolved in $^2H_2O$ containing 2% (wt/vol) sodium deoxycholate and 5 mM EDTA and adjusted to pH 9.5 with triethylamine. $^1$H broad-band decoupled spectra were recorded over a spectral range of 7 kHz (15). Samples were referenced to an 80% (wt/vol) solution of phosphoric acid (0.00 ppm) as an external standard.

TABLE 1. Chemical composition of lipid A components obtained by differing acidic hydrolyses of *H. pylori* R-LPS and S-LPS

| Constituent | Amt of constituent in nmol/mg (mol/2 mol of GlcN) in: | | | |
|---|---|---|---|---|
| | R-lipid $A_{NaAc}$ | R-lipid $A_{HCl}$ | S-lipid $A_{NaAc}$ | S-lipid $A_{HCl}$ |
| GlcN | 1,033 (2.0) | 1,150 (2.0) | 921 (2.0) | 1,014 (2.0) |
| Phosphate | 520 (1.0) | 0 (−)[a] | 665 (1.4) | 217 (0.4) |
| Ethanolamine | 8 (Tr)[b] | 0 (−) | 10 (Tr) | 0 (−) |
| Fatty acids | | | | |
| 12:0 | 10 (Tr) | 6 (Tr) | 226 (0.5) | 254 (0.5) |
| 14:0 | 67 (0.1) | 69 (0.1) | 189 (0.4) | 203 (0.4) |
| 16:0 | 41 (0.1) | 52 (0.1) | 46 (0.1) | 51 (0.1) |
| 18:0 | 610 (1.2) | 679 (1.2) | 438 (1.0) | 507 (1.0) |
| 16:0(3-OH) | 382 (0.7) | 431 (0.8) | 602 (1.3) | 659 (1.3) |
| 18:0(3-OH) | 1,160 (2.3) | 1,300 (2.3) | 922 (2.0) | 1,014 (2.0) |

[a] −, none detected.
[b] Tr, trace (<0.05 mol).

## RESULTS

**Chemical composition of lipid A.** Free lipid A preparations were obtained from R-LPS and S-LPS of *H. pylori* NCTC 11637 by mild acid hydrolysis with either 0.1 M sodium acetate buffer, pH 4.4, or 0.1 M HCl, and their compositions are shown in Table 1. Besides fatty acids, D-glucosamine (GlcN), phosphate, and ethanolamine were present in R-lipid $A_{NaAc}$ and S-lipid $A_{NaAc}$. However, phosphate and ethanolamine were absent from R-lipid $A_{HCl}$. Although ethanolamine was absent

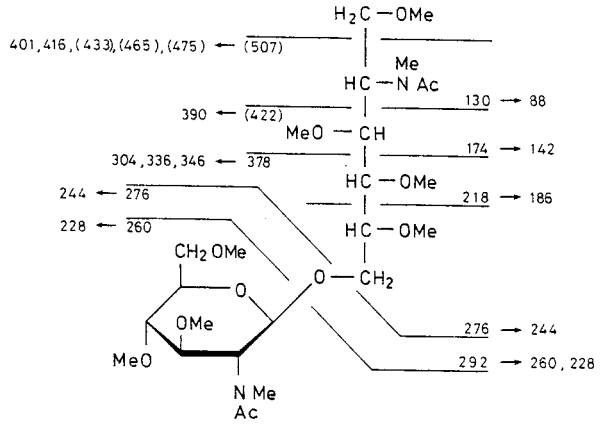
from S-lipid $A_{HCl}$, phosphate was present in a lower molar ratio (0.4 mol) than in S-lipid $A_{NaAc}$ (1.4 mol). Unlike lipid A of some other bacterial species, in which 4-amino-4-deoxyarabinose is present as a polar head group (33, 64), this sugar was not detected in *H. pylori* lipid A. The major fatty acids present in all of the lipid A preparations were hexadecanoic (16:0),
octadecanoic (18:0), 3-hydroxyhexadecanoic [16:0(3-OH)], and 3-hydroxyoctadecanoic [18:0(3-OH)] acids, with minor amounts of dodecanoic (12:0) and tetradecanoic (14:0) acids. The constituents accounted for 89 to 94% (dry weight) of the preparations. The free lipid A preparations were essentially free from proteins (<0.1%) and nucleic acids (<0.1%).

**Structural analysis of the lipid A backbone.** The pathway of chemical degradation developed earlier for lipid A backbone analysis was used (25, 39). Approximately 50% of the total GlcN content of R-lipid $A_{HCl}$ and S-lipid $A_{HCl}$ (Table 1) was detectable in the direct Morgan-Elson reaction, indicating that one-half of the GlcN is in the reducing form and that both lipid A's are likely to contain a GlcN disaccharide as the backbone.

R-lipid $A_{HCl}$ (96 mg) was reduced with $NaBH_4$ to yield a product (82.5 mg) in which the molar ratio of GlcN (667 nmol/mg) and D-glucosaminitol was 1.0:0.9, as determined with an amino acid analyzer. Phosphate was absent, and the product was negative in the direct Morgan-Elson reaction. Subsequently, this material was subjected to strong hydrazinolysis, and high-voltage paper electrophoresis of an aliquot (about 50 μg) of the deacylated and dephosphorylated material (28.9 mg) revealed a major component with an $M_{GlcN}$ of 1.15, which readily reacted with ninhydrin. The remainder of the hydrazinolysate was N-acetylated, the product (26 mg) was subjected to gel chromatography, and a purified disaccharide was obtained (5.1 mg). Similarly, S-lipid $A_{HCl}$ (45 mg) was reduced to yield a product (37.4 mg) in which the molar ratio of GlcN (542 nmol/mg), D-glucosaminitol, and phosphate was 1:0.9:0.4. This material was negative in the direct Morgan-Elson reaction. Subsequent degradation and gel chromatography yielded a disaccharide product (2.0 mg).

The disaccharides isolated from R-lipid $A_{HCl}$ and S-lipid $A_{HCl}$ had a retention time on high-pressure liquid chromatography analysis (13.1 min) identical to that of authentic 2-acetamido-2-deoxy-6-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-D-glucitol (GlcNAcβ1-6GlcNAc-ol). In the $^1$H-NMR spectra of the bacterial disaccharides, signals were assigned to H-1′ = 4.43 ppm with $J_{1',2'}$ of 8.6 Hz, values identical to those obtained with authentic GlcNAcβ1-6GlcNAc-ol, thus indicating that the nonreducing GlcN in the backbone is present as a β anomer. In addition, the N-acetylated backbone disaccharides were permethylated and analyzed by GLC and GLC-MS. The samples contained a single compound which comigrated (retention time, 14.9 min) and exhibited identical EI mass spectra, including the characteristic fragment at $m/z$ = 218 (Fig. 1), and CI mass spectra (pseudomolecular ion, [M + H] = 553) as authentic permethylated GlcNAcβ1-6GlcNAc-ol.

Finally, the permethylated disaccharides were acetolyzed, reduced ($NaB^2H_4$), and per-O-acetylated. Subsequent analysis by GLC and GLC-MS identified two compounds. These were 1,6-di-*O*-acetyl-2-deoxy-3,4,5-tri-*O*-methyl-2-(*N*-methylacetamido)-glucitol (obtained via C-1 de-O-methylation during acetolysis and acetylation [17]) from the reducing GlcN residue and 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-[1-$^2$H]glucitol from the nonreducing GlcN of the lipid A backbone, which shows that the disaccharides are (1,6)-linked. The occurrence of an *O*-acetyl group at C-5 in the $^2$H-incorporated compound indicates that the nonreducing GlcN of the disaccharide is in the pyranose form. Collectively,



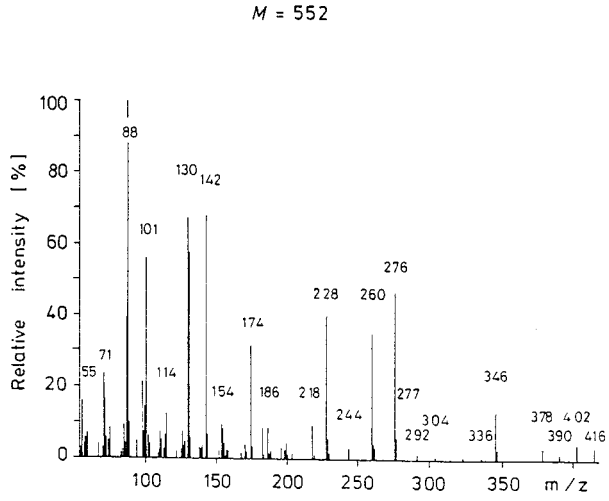




FIG. 1. EI mass spectrum of the permethylated and N-acetylated disaccharide derived from free lipid A of *H. pylori* R-LPS.

these data show that the backbone of both R-lipid A and S-lipid A of *H. pylori* is a β(1,6)-linked GlcN disaccharide.

**Attachment site of the core oligosaccharide to lipid A.** To determine the linkage site of the core oligosaccharide, partially degraded LPS and free lipid A preparations were subjected to methylation analysis. Three products each, (i) reduced-dephosphorylated LPS (LPS-$HF_{red}$), (ii) reduced-dephosphorylated lipid A (lipid A-$HF_{red}$), and (iii) reduced lipid A (lipid $A_{red}$), were derived from R- and S-LPS and were subsequently subjected to permethylation, acetolysis, reduction ($NaB^2H_4$) and per-O-acetylation. The resultant partially methylated alditol acetates were analyzed by GLC and GLC-MS. As shown in Table 2, all six products yielded 1,6-di-*O*-acetyl-2-deoxy-3,4,5-tri-*O*-methyl-2-(*N*-methylacetamido)-glucitol (3,4,5-$Me_3$GlcNMeAc-ol). LPS-$HF_{red}$ derived from R-LPS and LPS-$HF_{red}$ from S-LPS yielded the same additional methylation product, 1,5,6-tri-*O*-acetyl-2-deoxy-3,4-di-*O*-methyl-2-(*N*-methylacetamido)-[1-$^2$H]glucitol (3,4-$Me_2$*GlcNMeAc-ol). Likewise, lipid A-$HF_{red}$ from R-LPS and S-LPS yielded another product, 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-[1-$^2$H]glucitol (3,4,6-$Me_3$*GlcNMeAc-ol), which was also a product of methylation analysis of lipid $A_{red}$ from R-LPS and S-LPS. However, lipid $A_{red}$ derived from S-LPS gave an additional product, 1,4,5-tri-*O*-acetyl-2-deoxy-

TABLE 2. Identity of methylated sugars obtained after methylation analysis of selected preparations derived from *H. pylori* R-LPS and S-LPS

| Preparation | Methylated sugar in derivative of: | |
|---|---|---|
| | R-LPS | S-LPS |
| HF-$LPS_{red}$ | 3,4,5-$Me_3$GlcNMeAc-ol[a]<br>3,4-$Me_2$*GlcNMeAc-ol[b] | 3,4,5-$Me_3$GlcNMeAc-ol<br>3,4-$Me_2$*GlcNMeAc-ol |
| Lipid $A_{red}$ | 3,4,5-$Me_3$GlcNMeAc-ol<br>3,4,6-$Me_3$*GlcNMeAc-ol | 3,4,5-$Me_3$GlcNMeAc-ol<br>3,6-$Me_2$*GlcNMeAc-ol<br>3,4,6-$Me_3$*GlcNMeAc-ol |
| HF-lipid $A_{red}$ | 3,4,5 $Me_3$GlcNMeAc-ol<br>3,4,6-$Me_3$*GlcNMeAc-ol | 3,4,5-$Me_3$GlcNMeAc-ol<br>3,4,6-$Me_3$*GlcNMeAc-ol |

[a] Obtained via C-1 de-O-methylation during acetolysis and acetylation (17).
[b] Asterisk denotes a $^2H$-incorporated compound.

3,6-di-*O*-methyl-2-(*N*-methylacetamido)-[1-$^2$H]glucitol (3,6-$Me_2$*GlcNMeAc-ol).

Comparing the derivatives from this methylation analysis, a 6-*O*-methyl group was present in the $^2H$-incorporated compound obtained from lipid $A_{red}$ and lipid A-$HF_{red}$, but absent in such a compound obtained from LPS-$HF_{red}$. This shows that in free R- and S-lipid A, position 6′ of the backbone disaccharide is free, but it is occupied in LPS. Thus, the core oligosaccharide is attached to position 6′ of the nonreducing GlcN residue of the backbone of R-lipid A and S-lipid A.

**Location of phosphoryl groups and their substitution.** About 4% (40 nmol/mg) of the total hexosamine content of R-lipid $A_{NaAc}$ reacted in the direct Morgan-Elson reaction, indicating that the glycosidic hydroxyl group was only partially free. In R-lipid $A_{HCl}$, the amount of GlcN determined by the direct Morgan-Elson reaction was about one-half (500 nmol/mg) of the total hexosamine content. Compared with R-lipid $A_{NaAc}$, preparation of R-lipid $A_{HCl}$ resulted in loss of phosphorylation (Table 1), and thus it could tentatively be deduced that phosphate was the glycosidically bound group in R-lipid $A_{NaAc}$. In a similar manner, only 2% (20 nmol/mg) of the total hexosamine content of S-lipid $A_{NaAc}$, but 50% (501 nmol/mg) of the GlcN content of S-lipid $A_{HCl}$, reacted in the direct Morgan-Elson reaction. However, S-lipid $A_{NaAc}$ contained 1.4 mol of phosphate, compared with 0.4 mol in S-lipid $A_{HCl}$ (Table 1), suggesting that in addition to a glycosidically linked phosphate, nonstoichiometric amounts of a second acid-stable phosphate group were present in the latter lipid A.

In order to confirm the substitution of the glycosidic position of the GlcN disaccharide by phosphate, S-lipid $A_{NaAc}$ (2 mg) in 0.1% sodium dodecyl sulfate (wt/vol) was treated with 0.1 M HCl at 100°C. Samples were taken at different times and analyzed for total phosphate, inorganic phosphate, and reactivity in the direct Morgan-Elson assay. The results, depicted in Fig. 2, show that after 30 min of incubation, reducing GlcN, detected in the direct Morgan-Elson assay, reached a maximum value (approximately 1 mol/2 mol of GlcN) which did not change during further acidic treatment (up to 120 min). The appearance of reducing GlcN was paralleled by the release of inorganic phosphate (maximum, 0.95 mol), whereas total phosphate remained constant (1.4 mol). The concomitant release of phosphate with the appearance of reducing GlcN, as well as the identical kinetics of its release compared with that of α-glycosidically bound phosphate (6, 16), indicates that this group is α-glycosidically bound to GlcN I of the lipid A backbone.

Methylation analysis of partially degraded LPS and free lipid A preparations derived from R-LPS and S-LPS indicated heterogeneity in substitution by phosphate of the nonreducing GlcN of the backbone (Table 2). The occurrence of a 4-*O*-methyl group in 3,4,6-$Me_3$*GlcNMeAc-ol obtained from LPS-$HF_{red}$, lipid A-$HF_{red}$, and lipid $A_{red}$ indicates that position 4′ of the nonreducing GlcN is unsubstituted in lipid A of R-LPS and S-LPS. However, lipid $A_{red}$, but not lipid A-$HF_{red}$, derived from S-LPS also yielded 3,6-$Me_2$*GlcNMeAc-ol, which shows that position 4′ in this lipid A is partially substituted by a hydrogen fluoride-labile substituent, probably phosphate.

To investigate this potential heterogeneity in phosphate substitution further, R-lipid $A_{HCl}$ and S-lipid $A_{HCl}$ (2.5 mg, respectively) were reduced ($NaBH_4$) and subjected to acidic methanolysis (1 h), and the methanolysate was permethylated. Analysis by GLC and GLC-MS of the permethylated products of S-lipid $A_{HCl}$ revealed three peaks. One peak, derived from the reducing GlcN of the lipid A backbone, exhibited an EI mass spectrum (primary fragments at $m/z$ = 89, 133, 177, 384, 428, 472, and 516; secondary fragments at $m/z$ = 57, 101, 145, 352, 396, 440, and 484) and CI mass spectrum (pseudomolecular ion, $[M + H]^+$ = 562) corresponding to permethylated glucosaminitol N-acylated by 3-methoxyoctadecanoic acid [18:0(3-OMe)] (Fig. 3A). The two remaining compounds (peaks 2 and 3), derived from the nonreducing GlcN of the backbone, had relative peak intensities of 2:1 and corresponded to permethylated pyranosidic GlcN N-acylated by 18:0(3-OMe) (EI-MS, characteristic fragments = 117 and 396; CI-MS, $[M + H]^+$ = 546) and the 4-*O*-phosphorylated derivative of the latter compound (EI-MS, characteristic fragments = 117 and 490; CI-MS, $[M + H]^+$ = 640) (Fig. 3B and C). On the other hand, analysis of the permethylated products of R-lipid $A_{HCl}$ yielded only two compounds corresponding to peaks 1 and 2 above, indicating lack of phosphorylation of the nonreducing GlcN residue in this lipid A preparation. Collectively, these results provided evidence that the nonglycosidic phosphate group is linked in nonstoichiometric amounts to position 4′ of the back-

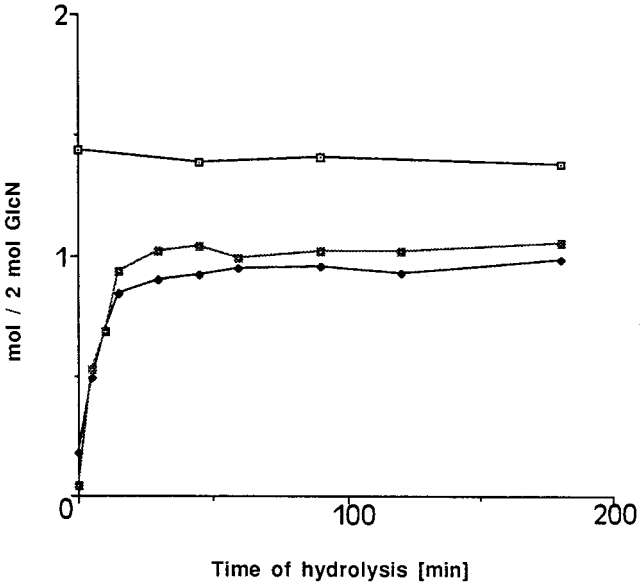



FIG. 2. Time dependence of phosphate release and appearance of reducing glucosamine on treatment of *H. pylori* S-lipid $A_{NaAc}$ with 0.1 M HCl at 100°C. The mixture was assayed at different times for total phosphate (⊡), inorganic phosphate (◆), and reducing glucosamine reactivity in the direct Morgan-Elson assay without prior hydrolysis (■).

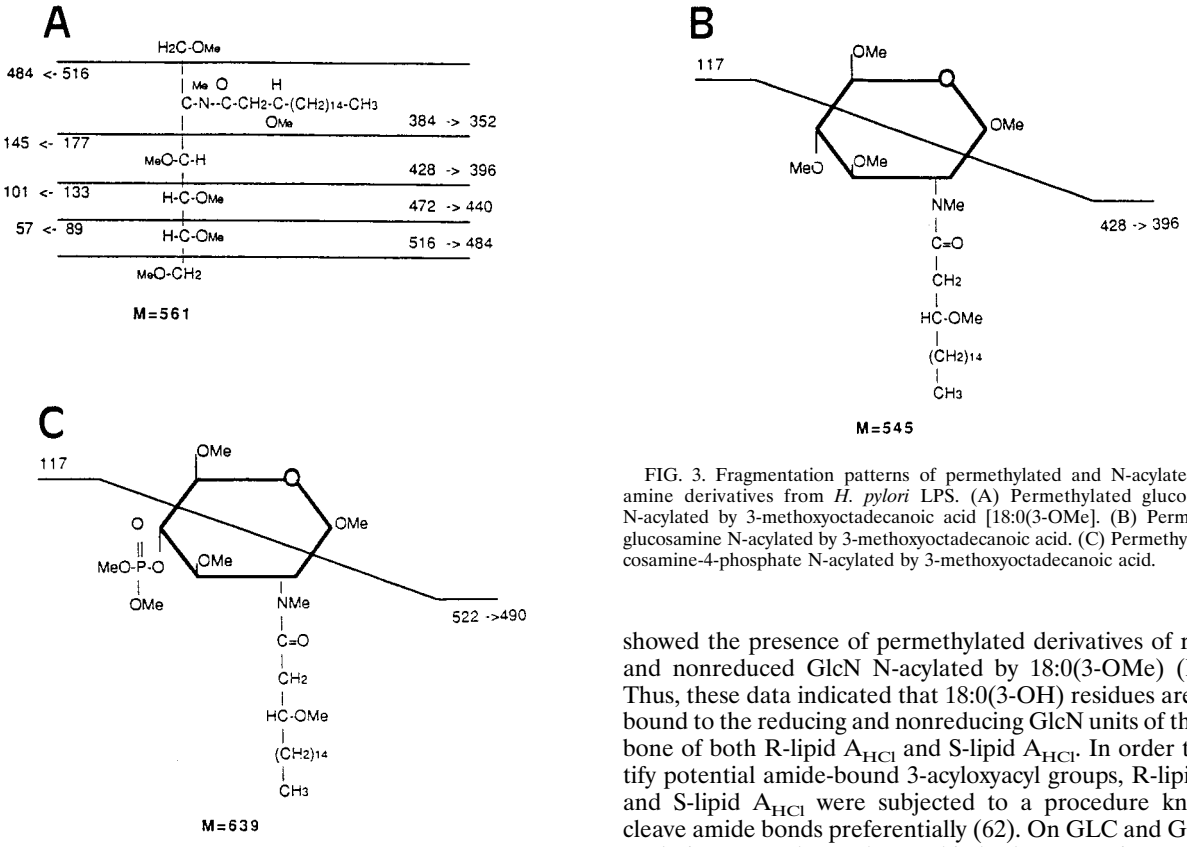



FIG. 3. Fragmentation patterns of permethylated and N-acylated glucosamine derivatives from *H. pylori* LPS. (A) Permethylated glucosaminitol N-acylated by 3-methoxyoctadecanoic acid [18:0(3-OMe]. (B) Permethylated glucosamine N-acylated by 3-methoxyoctadecanoic acid. (C) Permethylated glucosamine-4-phosphate N-acylated by 3-methoxyoctadecanoic acid.

bone of S-lipid $A_{HCl}$ and that this position was not phosphorylated in R-lipid $A_{HCl}$.

Analysis by $^{31}$P-NMR confirmed the above conclusions (Fig. 4). The analysis revealed a signal (2.96 ppm) for a glycosidic phosphomonoester and a second for a phosphodiester (1.32 ppm) in R-lipid $A_{NaAc}$. Since acidic treatment (0.1 M HCl, 100°C, 30 min) of R-lipid $A_{NaAc}$ led to the loss of both signals and the liberation of phosphate and ethanolamine-phosphate, the signals were deduced to correspond to glycosidic phosphate and ethanolamine-phosphate, respectively. In addition to these signals, analysis of S-lipid $A_{NaAc}$ revealed a further signal which corresponded to an ester-bound phosphomonoester (4.52 ppm) which was not affected by acidic treatment and hence was attributed to 4′-phosphate.

**Nature, quantity, and type of linkage of fatty acids.** All of the lipid A preparations derived from *H. pylori* R- and S-LPS contained 16:0, 18:0, 16:0(3-OH), and 18:0(3-OH) as the major fatty acids, with minor amounts of 12:0 and 14:0 (Table 1). As determined previously (36), the 3-hydroxy fatty acids possessed the (*R*)-configuration. The molar ratios of fatty acids in the lipid A preparations derived from R-LPS (R-lipid $A_{NaAc}$ and R-lipid $A_{HCl}$) differed from those of lipid A derived from S-LPS (S-lipid $A_{NaAc}$ and S-lipid $A_{HCl}$) (Table 1). However, in our previous investigation (42), we showed that quantitatively similar fatty acid profiles were present in *H. pylori* LPS and the respective lipid A preparations, showing that differences do not result from the hydrolysis conditions used to liberate free lipid A. Nevertheless, the observed differences in molar ratios suggested greater substitution by certain fatty acids in lipid A of S-LPS than in R-LPS (Table 1).

Analysis of the products of R-lipid $A_{HCl}$ and S-lipid $A_{HCl}$ after methanolysis and permethylation (described above) showed the presence of permethylated derivatives of reduced and nonreduced GlcN N-acylated by 18:0(3-OMe) (Fig. 3). Thus, these data indicated that 18:0(3-OH) residues are amide bound to the reducing and nonreducing GlcN units of the backbone of both R-lipid $A_{HCl}$ and S-lipid $A_{HCl}$. In order to identify potential amide-bound 3-acyloxyacyl groups, R-lipid $A_{HCl}$ and S-lipid $A_{HCl}$ were subjected to a procedure known to cleave amide bonds preferentially (62). On GLC and GLC-MS analysis, one peak was detected in both preparations, which by comparison with available mass spectrometric data (54, 63) was identified as 3-(octadecanoyloxy)octadecanoic acid [3-(18:0-*O*)-18:0]. Quantitatively, 0.8 mol (811 nmol/mg) and 0.9 mol (1,035 nmol/mg) of the product were detected in S-lipid $A_{HCl}$ and R-lipid $A_{HCl}$, respectively. These results show that in lipid A of *H. pylori*, approximately 1 mol of amide-linked 18:0(3-OH) can be substituted by 18:0.

Ester-linked fatty acids were investigated by treatment of R-lipid $A_{HCl}$ and S-lipid $A_{HCl}$ with sodium methylate (Table 3). This treatment of R-lipid $A_{HCl}$ released quantitatively 18:0 (1.0 mol) and 16:0(3-OH) (1.1 mol) as their methyl esters, showing that they had been exclusively ester linked. In the alkaline methanolysate, only trace amounts of 16:0(3-OMe) were detectable, indicating the predominant absence of ester-bound 3-acyloxyacyl groups in this lipid A. Sodium methylate treatment of S-lipid $A_{HCl}$ released quantitatively 18:0 (1.1 mol) and 16:0(3-OH) (1.0 mol), and in addition, a nonstoichiometric amount of 16:0 (0.1 mol), as their methyl esters. In contrast to R-lipid $A_{HCl}$, 16:0(3-OMe) (0.6 mol) was present in the methanolysate of S-lipid $A_{HCl}$, and methyl esters of 12:0 (0.6 mol) and 14:0 (0.5 mol) appeared after diazomethane treatment of the methanolysate (i.e., carboxymethylation). Hence, based on previous experience of the behavior of variously linked fatty acids in transmethylation and β-elimination reactions (50), it was deduced that ester-bound 3-acyloxyacyl groups consisting of 3-(dodecanoyloxy)hexadecanoyl [3-(12:0-*O*)-16:0] and 3-(tetradecanoyloxy)hexadecanoyl [3-(14:0-*O*)-16:0] residues were present in this lipid A. In addition, the 18:0 (and the nonstoichiometric amounts of 16:0) released as methyl ester was likely to have been the ester-bound substituent of amide-bound 3-acyloxyacyl residues (described above).

**Distribution of fatty acids on the lipid A backbone.** Free dephosphorylated and de-O-acylated lipid A preparations de-

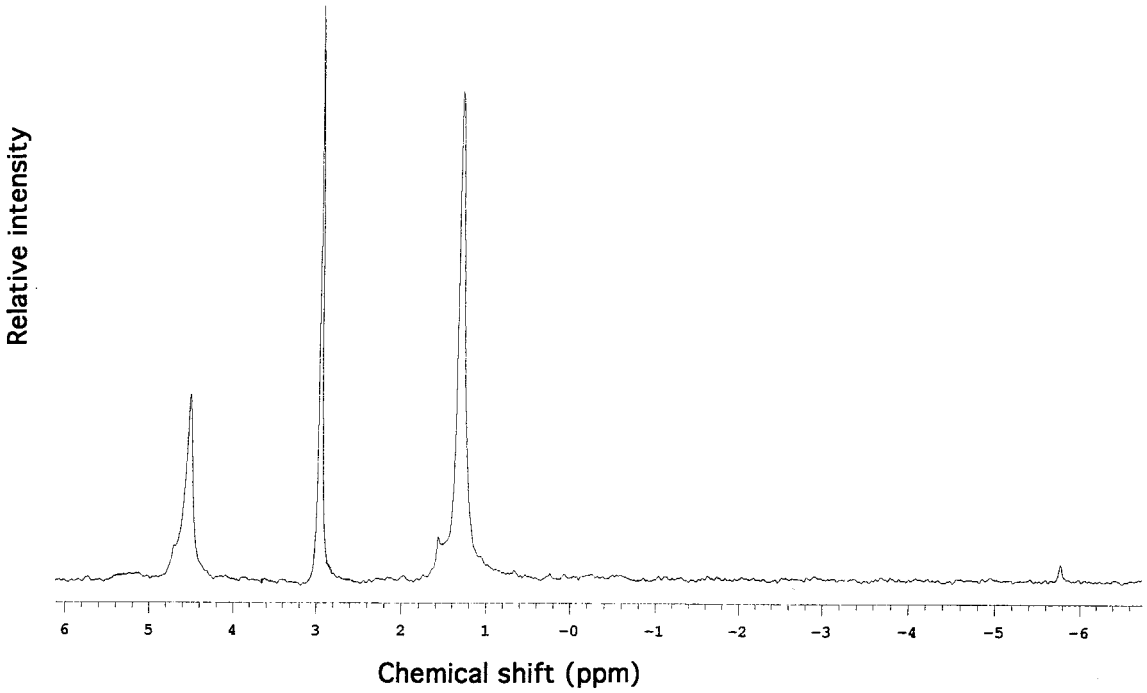



FIG. 4. $^{31}$P-NMR spectrum of *H. pylori* free lipid A (S-lipid $A_{NaAc}$).

rived from R- and S-LPSs of *H. pylori* were subjected to LD-MS, after cationization by the NaI admixture, and were analyzed in the positive-ion mode. The LD mass spectra of both of these materials yielded identical spectra (not shown) with a quasimolecular ion ($[M + Na]^+$) at $m/z$ = 928 corresponding to a GlcN disaccharide carrying two amide-bound 18:0(3-OH) residues.

Subsequently, free dephosphorylated lipid A preparations derived from R- and S-LPSs of *H. pylori* (dephosphorylated R-lipid A and S-lipid A, respectively) were analyzed in a similar manner. These two lipid A preparations yielded differing spectra, as shown in Fig. 5. The assignments of ions for dephosphorylated R-lipid A and S-lipid A are shown in Tables 4 and 5, respectively, and the fragmentation scheme and processes compatible with these assignments are shown in Fig. 6. The depicted $m/z$ values represent quasimolecular ions derived from the acylated GlcN disaccharide (M) and fragment ions including those derived from the acylated reducing and nonreducing GlcN units ($M_I$ and $M_{II}$, respectively). Also, analysis of dephosphorylated S-lipid A by LD-MS after admixture of CsI gave a similar fragmentation pattern, and in addition showed the presence of nonstoichiometric amounts of amide-bound 3-(16:0-*O*)-18:0 replacing 3-(18:0-*O*)-18:0 on the nonreducing GlcN unit of the lipid A backbone (data not shown).

Collectively, these data and the results of chemical studies show that the predominant lipid A molecular species in *H. pylori* R-LPS is tetraacyl, whereby the reducing GlcN of the backbone carries amide-bound 18:0(3-OH) and ester-linked 16:0(3-OH), and the nonreducing GlcN carries only amide-bound 3-(18:0-*O*)-18:0. Likewise, tetraacyl lipid A may be present in S-LPS, but hexaacyl lipid A is also present, whereby the nonreducing GlcN has ester-bound 3-(12:0-*O*)-16:0 or 3-(14:0-*O*)-16:0. To resolve this issue of heterogeneity, which had been observed in our previous analysis of free lipid by thin-layer chromatography (42), free dephosphorylated lipid A preparations derived from R- and S-LPSs of *H. pylori* were subjected to silica-gel chromatography as described previously (39). Upon fractionation of the dephosphorylated material, one predominant fraction was obtained from R-LPS, and one major and a second minor fraction were obtained from S-LPS. Analysis of the major fractions from R- and S-LPSs by LD-MS confirmed the presence of tetraacyl lipid A, whereas analysis of the minor fraction derived from S-LPS showed the hexaacyl distribution of fatty acids as described above.

## Discussion

In the present study, the lipid A components of *H. pylori* R- and S-LPS were isolated and chemically characterized. The proposed structures of the molecular species in both of these

TABLE 3. Nature and amount of fatty acid methyl esters released from free lipid A of *H. pylori* by sodium methylate

| Fatty acid methyl ester | Amt of methyl ester in nmol/mg (mol/2 mol of GlcN)[a] detectable before and after $CH_2N_2$ treatment[b] | | | |
|---|---|---|---|---|
| | R-lipid $A_{HCl}$ | | S-lipid $A_{HCl}$ | |
| | Before | After | Before | After |
| 12:0 | 0 (−)[c] | 7 (Tr)[d] | 0 (−) | 328 (0.6) |
| 14:0 | 0 (−) | 24 (Tr) | 0 (−) | 262 (0.5) |
| 16:0 | 18 (Tr) | 20 (Tr) | 48 (0.1) | 50 (0.1) |
| 18:0 | 558 (1.0) | 561 (1.0) | 537 (1.1) | 553 (1.1) |
| 16:0(3-OH) | 632 (1.1) | 626 (1.1) | 482 (1.0) | 500 (1.0) |
| 16:0(3-OMe) | 0 (−) | 14 (Tr) | 279 (0.6) | 298 (0.6) |
| 18:0(3-OH) | 0 (−) | 0 (−) | 0 (−) | 0 (−) |

[a] The GlcN values of 1,150 and 1,014 nmol were the basis of the calculation of molar ratios in R-lipid $A_{HCl}$ and S-lipid $A_{HCl}$, respectively.

[b] Diazomethane treatment results in carboxymethylation of free acids liberated by β-elimination and hence their detection as methyl esters.

[c] −, none detected.

[d] Tr, trace (<0.05 mol).

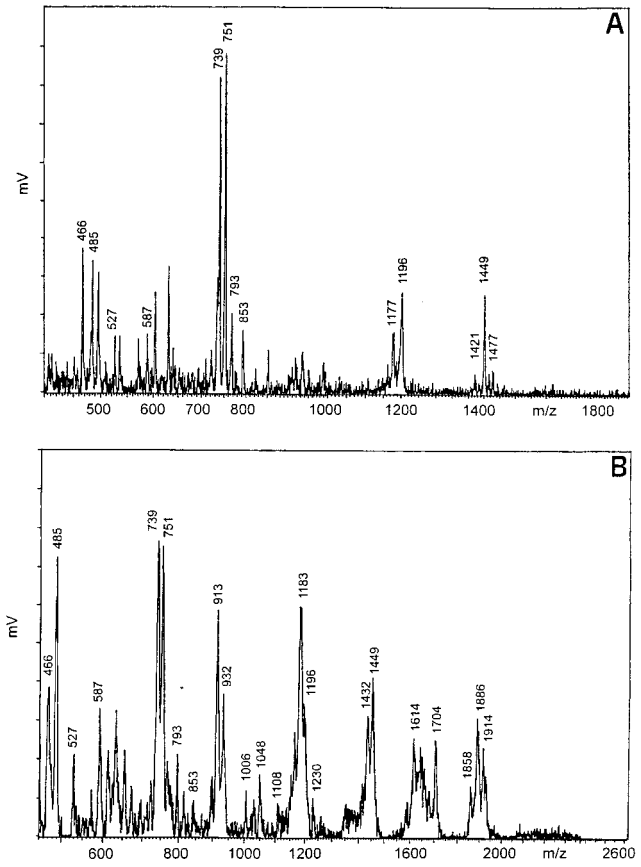



FIG. 5. Positive-ion LD mass spectra of free dephosphorylated lipid A preparations derived from R-LPS (A) and S-LPS (B) of *H. pylori* after admixture of NaI.

lipid A's from *H. pylori* R- and S-LPSs are shown in Fig. 7. Despite variations in acylation and phosphorylation patterns, the structure of *H. pylori* lipid A possesses the common architectural and structural principles encountered in many distinct bacterial groups (33, 44, 48, 51, 64). The predominant molecular species in both lipid A components are identical and are tetraacylated in an asymmetric manner (Fig. 7A). In addition, a second molecular species which is hexaacylated, also in an asymmetric manner, is present in lipid A from S-LPS (Fig. 7B). Despite differences in substitution, the backbone of the molecules consists of a β(1,6)-linked GlcN disaccharide with a glycosidically linked phosphate at position 1. Because of microheterogeneity, nonstoichiometric amounts of ethanolaminephosphate may also be linked to the glycosidic hydroxyl group.

A significant finding of the present investigation is the absence of an ester-bound phosphate group at position 4′ of the nonreducing GlcN unit of the backbone disaccharide of lipid A from R-LPS (Fig. 7A). Although the hydroxyl group at position 4′ on the backbone disaccharide of lipid A from S-LPS was predominantly free and unsubstituted, phosphorylation at position 4′ was present in this lipid A in nonstoichiometric amounts. It was not possible in the present study to determine whether 4′-phosphate was present on the backbone of the tetraacyl or hexaacyl molecular species in lipid A of S-LPS. However, because of the nonstoichiometric nature of this phosphate substitution and by analogy with lipid A of R-LPS, it is more likely that the minor hexaacyl species in S-LPS, rather than the predominant tetraacyl species, is phosphorylated at position 4′ (Fig. 7B). An ester-bound phosphate group bound to the hydroxyl group at position 4′ of the backbone disaccharide is a common constituent of lipid A of other bacterial species, particularly enterobacterial lipid A's (33, 44, 48, 64). However, this phosphate group is absent from lipid A's of *Bacteroides fragilis*, *Bacteroides intermedius*, and *Rhodomicrobium vannielii* (17, 19, 60). In *R. vannielii* lipid A, the 4-*O*-phosphoryl group is positionally replaced by D-mannose (17). Similar to lipid A from *H. pylori* S-LPS, the predominant mo-

TABLE 4. Assignment of peaks in the LD mass spectrum of free dephosphorylated lipid A derived from *H. pylori* R-LPS[a]

| Peak (*m/z*) | Cleavage process | Structure[b] |
|---|---|---|
| 1,477 | | $[M - 16:OH(3-OH) + 18:OH(3-OH) + Na]^+$ |
| 1,449 | | $[M + Na]^+$ |
| 1,421 | | $[M - 18:OH(3-OH) + 16:0 + Na]^+$ |
| 1,177 | B | $[M - 16:OH(3-OH) + H + Na]^+$ |
| 853 | | $[M_{II} + 102 + Na]^+$ |
| 793 | | $[M_{II} + 42 + Na]^+$ |
| 751 | | $[M_{II} + Na]^+$ |
| 739 | | $[M_I + Na]^+$ |
| 587 | H | $[M_{II} + 102 - 18:0 + H + Na]^+$ |
| 527 | G | $[M_{II} + 42 - 18:0 + H + Na]^+$ |
| 485 | F | $[M_{II} - 18:0 + H + Na]^+$ |
| 466 | B | $[M_I - 16:0(3-OH) + H + Na]^+$ |

[a] The relevant cleavage process and the resultant structure are shown in Fig. 6.
[b] M, quasimolecular ion derived from the acylated GlcN disaccharide; $M_{II}$, fragment ion derived from the acylated nonreducing GlcN unit of the backbone; $M_I$, fragment ion derived from the acylated reducing GlcN unit of the backbone.

TABLE 5. Assignment of peaks in the LD mass spectra of free dephosphorylated lipid A derived from *H. pylori* S-LPS[a]

| *m/z* | Cleavage process | Structure[b] |
|---|---|---|
| 1,914 | | $[M - 12:0 + 14:0 + Na]^+$ |
| 1,886 | | $[M + Na]^+$ |
| 1,858 | | $[M - 18:0 + 16:0 + Na]^+$ |
| 1,704 | A | $[M - 12:0 + H + Na]^+$ |
| 1,614 | B | $[M - 16:0(3-OH) + H + Na]^+$ |
| 1,449[c] | C | $[M - 12:0 - 16:0(3-OH) + H + Na]^+$ |
| 1,432 | D | $[M - 12:0 - 16:0(3-OH) + H_2O + Na]^+$ |
| 1,230 | G | $[M_{II} + 42 + Na]^+$ |
| 1,196[c] | C, E | $[M - 16:0(3-OH) + H - 12:0 - 16:0(3-OH) + H + Na]^+$ |
| 1,188 | | $[M_{II} + Na]^+$ |
| 1,183[c] | C, F | $[M - 18:0 + H - 12:0 - 16:0(3-OH) + H + Na]^+$ |
| 1,108 | A, H | $[M_{II} + 102 - 12:0 + H + Na]^+$ |
| 1,048 | A, G | $[M_{II} + 42 - 12:0 + H + Na]^+$ |
| 1,006 | A | $[M_{II} - 12:0 + H + Na]^+$ |
| 853[c] | C, H | $[M_{II} + 102 - 12:0 - 16:0(3-OH) + H + Na]^+$ |
| 793[c] | C, G | $[M_{II} + 42 - 12:0 - 16:0(3-OH) + H + Na]^+$ |
| 751[c] | C | $[M_{II} - 12:0 - 16:0(3-OH) + H + Na]^+$ |
| 739 | | $[M_I + Na]^+$ |
| 587[c] | C, F, H | $[M_{II} + 102 - 18:0 + H - 12:0 - 16:0(3-OH) + H + Na]^+$ |
| 527[c] | C, F, G | $[M_{II} + 42 - 18:0 + H + 12:0 - 16:0(3-OH) + H + Na]^+$ |
| 485[c] | C, F | $[M_{II} - 18:0 + H + 12:0 - 16:0(3-OH) + H + Na]^+$ |
| 466 | B | $[M_I - 16:0(3-OH) + H + Na]^+$ |

[a] The relevant cleavage process and the resultant structure are shown in Fig. 6.
[b] M, quasimolecular ion derived from the acylated GlcN disaccharide; $M_{II}$, fragment ion derived from the acylated nonreducing GlcN unit of the backbone; $M_I$, fragment ion derived from the acylated reducing GlcN unit of the backbone.
[c] Peak that can be derived from tetraacyl lipid A as well as from cleavage of acyl chains from hexaacyl lipid A. Compare with Table 4.

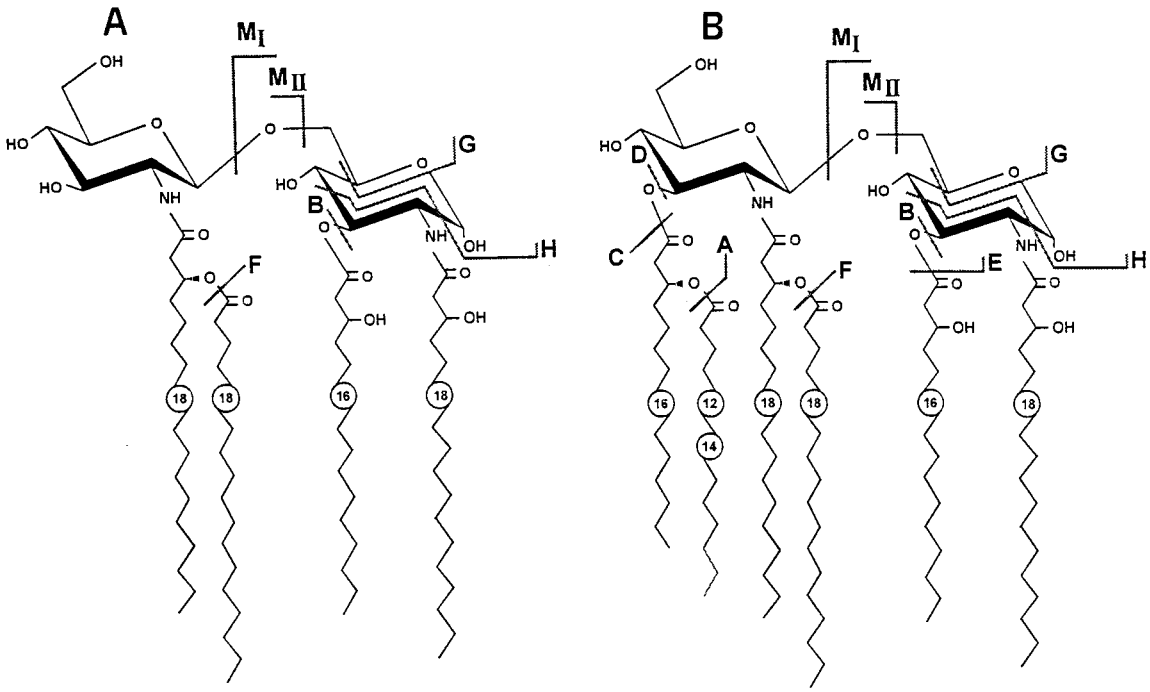



FIG. 6. Interpretations of the fragmentation patterns of free dephosphorylated lipid A derived from R-LPS (A) and S-LPS (B) of *H. pylori*. Numbers in circles refer to the number of carbon atoms in acyl chains, and letters indicate the designated cleavage process. Refer to Tables 4 and 5 for details of the formation of positive ions.

lecular species in lipid A of the oral pathogen *Porphyromonas gingivalis* lacks a phosphate group at position 4′ of the backbone disaccharide, but a minor constituent is phosphorylated at this position (26).

Another significant finding of the present investigation is that although hexaacyl lipid A is present in S-LPS, tetraacyl lipid A predominates in both R- and S-LPSs of *H. pylori*. Although heterogeneity was observed in the lipid A preparations, it is unlikely to derive from conditions used to liberate lipid A or from other treatments used during analysis, since

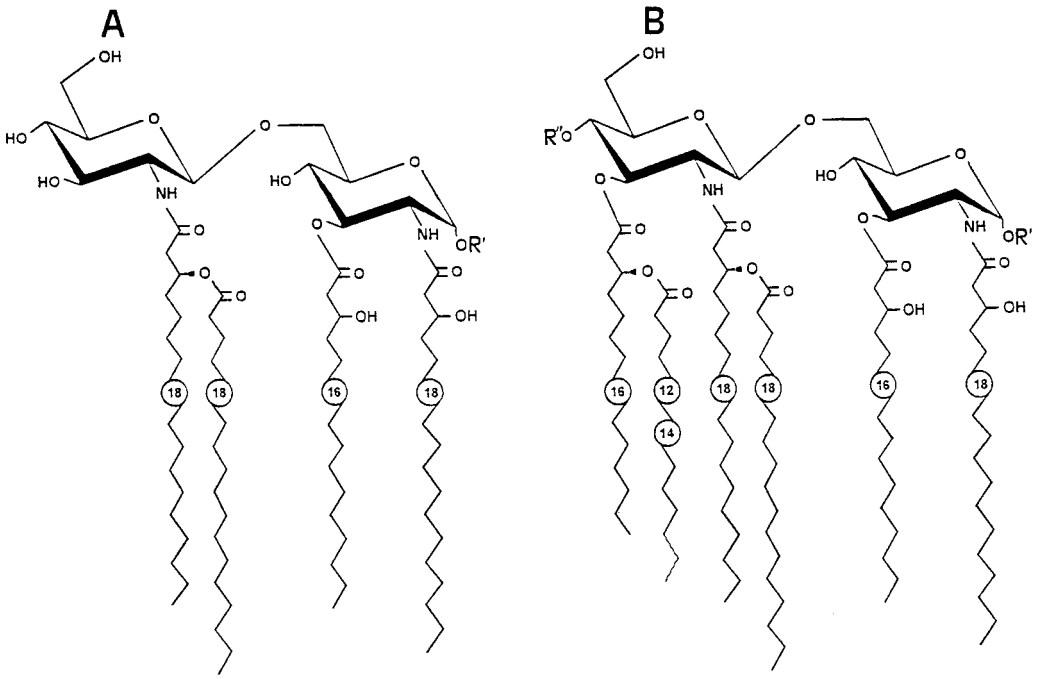



FIG. 7. Proposed chemical structures of the predominant lipid A molecular species found in both R-LPS and S-LPS (A) and a minor lipid A molecular species found in S-LPS (B) of *H. pylori*. Numbers in circles refer to the number of carbon atoms in acyl chains, and shaded lines indicate partial substitution due to microheterogeneity. Polar headgroups are indicated by R′ (phosphate or phosphorylethanolamine) and R″ (H or phosphate).

treatment conditions had been optimized and the natural heterogeneity in lipid A had been verified in our previous study (42). The asymmetric acylation pattern in *H. pylori* hexaacyl lipid A is similar to that found in a number of bacterial species of diverse origins, e.g., *Escherichia coli*, *Salmonella minnesota*, *Campylobacter jejuni*, *Haemophilus influenzae*, *Pectinatus* spp., and *Proteus mirabilis* (15, 16, 33, 39, 54, 64). However, the predominant fatty acids, particularly the 3-hydroxy fatty acids, are relatively long and are not common constituents of LPS. Like *H. pylori*, however, *Brucella abortus* and *Legionella pneumophila* have collectively 18:0, 16:0(3-OH), and 18:0(3-OH) in their LPSs and also possess low endotoxic activity (32, 55). Although nontoxic *Rhodobacter capsulatus* contains predominantly pentaacyl lipid A (24), a tetraacyl lipid A has been found in this organism, but with acyl chains of 10 to 14 carbons in length only (48). Thus, the predominance of a tetraacyl lipid A with long acyl chains of 16 to 18 carbons in length is an unusual property of *H. pylori* lipid A.

The relationships between the chemical structure of lipid A and the endotoxic and immunological activities of lipid A and LPS have been examined by many investigators using both natural and chemically synthesized lipid A analogs. In particular, studies of synthetic *S. minnesota*- and *E. coli*-like lipid A partial structures and analogs have shown that phosphorylation patterns (12, 18, 20, 22, 23), fatty acid composition (21, 27), and the presence of acyloxyacyl groups (49, 51) are important for the full expression of a range of biological activities. Based on these findings, it has been deduced that certain structural prerequisites in lipid A are needed for full expression of endotoxicity and biological activities by LPS. These include a molecule with a β(1,6)-linked D-hexosamine disaccharide backbone which is bisphosphorylated and carries six fatty acids in a defined arrangement as it is present in *E. coli* lipid A (33, 51, 64). Because synthetic lipid A partial structures that are monophosphorylated and lipid A analogs that carry acyl chains of increased chain length have been shown to exhibit decreased biological activity (18, 23, 27), it is likely that the underphosphorylation and underacylation patterns in *H. pylori* lipid A would produce an LPS with low endotoxic and biological activities.

Consistent with this, the lower endotoxic and immunological activities of *H. pylori* LPS have been well documented (34, 35). Compared with *Salmonella typhimurium* LPS, the pyrogenicity and mitogenicity of *H. pylori* LPS are 1,000-fold lower, and the lethal toxicity is 500-fold lower (40). *H. pylori* LPS exhibits a 100- to 500-fold lower activity when tested in the *Limulus* amebocyte lysate assay (42). The induction of cytokines, nitric oxide, and prostaglandin $E_2$ by *H. pylori* LPS is significantly lower than that by enterobacterial LPS (4, 9, 42, 43). Also, the ability of *H. pylori* LPS to induce the production of procoagulant activity is 1,000-fold lower than that of *S. typhimurium* LPS (53). Unlike enterobacterial LPS, *H. pylori* LPS does not abolish the expression of suppressor T-cell activity (3) or induce E-selectin expression or significant natural killer cell activity (10, 58).

With some analogy to the tetraacyl lipid A of *H. pylori*, a tetraacyl lipid A partial structure which constitutes a precursor in lipid A biosynthesis is produced by an *S. typhimurium* mutant strain and has been termed "precursor Ia," or, alternatively, lipid $IV_a$ (46, 64). Synthetic precursor Ia, which is bisphosphorylated and contains four fatty acids but no acyloxyacyl groups (45, 47), exhibits high toxicity but low pyrogenicity and mitogenicity compared with intact lipid A (12, 18, 20, 22, 23).

Comparisons have been made between *B. fragilis*, which is a long-lived commensal of the human gut, and the ability of *H. pylori* to cause chronic infection in the stomach. In particular, it has been suggested that the lipid A and LPS of *H. pylori* may have evolved to avoid or minimize the host defense (28). Supporting this, like the LPS of *H. pylori* that of *B. fragilis* also expresses low pyrogenicity and toxicity and has a lipid A with a β(1,6)-linked GlcN disaccharide backbone phosphorylated at position 1 only, with long 3-hydroxy fatty acids whose chain length is 15 to 17 carbons (61). In addition, the repeating units of the O side chains of *H. pylori* LPS may mimic Lewis blood group antigens that are found in the gastric mucosa (1, 2, 52), thereby potentially camouflaging the organism and minimizing the host defence (37). Therefore, these findings support the view that the primary role of *H. pylori* LPS and lipid A should not be considered endotoxicity, but that it is to provide a functional macromolecular matrix through which the bacterium interacts with its environment.

## ACKNOWLEDGMENTS

This study was supported by grants from the Irish *Helicobacter pylori* Study Group and the Irish Health Research Board to A.P.M.

We thank J. J. McGovern for expert technical assistance.

## REFERENCES

1. **Aspinall, G. O., M. A. Monteiro, H. Pang, E. J. Walsh, and A. P. Moran.** 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.
2. **Aspinall, G. O., and M. A. Monteiro.** 1996. Lipopolysaccharides of *Helicobacter pylori* strains P466 and MO19: structures of the O antigen and core oligosaccharide regions. Biochemistry **35:**2498–2504.
3. **Baker, P. J., T. Hraba, C. E. Taylor, P. W. Stashak, M. B. Fauntleroy, U. Zähringer, K. Takayama, T. R. Sievert, X. Hronowski, R. J. Cotter, and G. Perez-Perez.** 1994. Molecular structures that influence the immunomodulatory properties of the lipid A and inner core region oligosaccharides of bacterial lipopolysaccharides. Infect. Immun. **62:**2257–2269.
4. **Birkholz, S., U. Knipp, C. Nietzki, R. J. Adamek, and W. Opferkuch.** 1993. Immunological activity of lipopolysaccharide of *Helicobacter pylori* on human peripheral mononuclear blood cells in comparison to lipopolysaccharides of other intestinal bacteria. FEMS Immunol. Med. Microbiol. **6:**317–324.
5. **Blaser, M. J.** 1993. *Helicobacter pylori*: microbiology of a 'slow' bacterial infection. Trends Microbiol. **1:**255–260.
6. **Caroff, M., A. Tacken, and L. Szabo.** 1988. Detergent-accelerated hydrolysis of bacterial endotoxins and determination of the anomeric configuration of the glycosyl phosphate present in the "isolated lipid A" fragment of the *Bordetella pertussis* endotoxin. Carbohydr. Res. **175:**273–282.
7. **Chester, I. R., and R. G. E. Murray.** 1975. Analysis of the cell wall and lipopolysaccharide of *Spirillum serpens*. J. Bacteriol. **124:**1168–1176.
8. **Ciucanu, I., and F. Kerek.** 1984. A simple and rapid method for the permethylation of carbohydrates. Carbohydr. Res. **131:**209–217.
9. **Crabtree, J. E., S. Perry, A. Moran, P. Peichl, D. S. Tompkins, and I. J. D. Lindley.** 1994. Neutrophil IL-8 secretion induced by *Helicobacter pylori*. Am. J. Gastroenterol. **89:**1337.
10. **Darveau, R. P., M. D. Cunningham, T. Bailey, C. Seachord, K. Ratcliffe, B. Bainbridge, M. Dietsch, R. C. Page, and A. Aruffo.** 1995. Ability of bacteria associated with chronic inflammatory disease to stimulate E-selectin expression and promote neutrophil adhesion. Infect. Immun. **63:** 1311–1317.
11. **Forman, D., D. G. Newell, F. Fullerton, J. W. Yarnell, A. R. Stacey, N. Wald, and F. Sitas.** 1991. Association between infection with *Helicobacter pylori* and risk of gastric cancer: evidence from a prospective investigation. Br. Med. J. **302:**1302–1305.
12. **Galanos, C., V. Lehmann, O. Lüderitz, E. T. Rietschel, O. Westphal, H. Brade, L. Brade, M. A. Freudenberg, T. Hansen-Hagge, T. Lüderitz, G. McKenzie, U. Schade, W. Strittmatter, K.-I. Tanamoto, U. Zähringer, M. Imoto, H. Yoshimura, M. Yamamoto, T. Shimamoto, S. Kusumoto, and T. Shiba.** 1984. Endotoxic properties of chemically synthesized lipid A part structures. Comparison of synthetic lipid A precursor and synthetic analogues with biosynthetic lipid A precursor and lipid A. Eur. J. Biochem. **140:**221–227.
13. **Graham, D. Y., G. M. Lew, P. D. Klein, D. G. Evans, D. J. Evans, Z. A. Saeed, and H. M. Malaty.** 1992. Effect of treatment of *Helicobacter pylori* infection on the long-term recurrence of gastric or duodenal ulcer. Ann. Intern. Med. **116:**705–708.
14. **Hase, S., and E. T. Rietschel.** 1976. Isolation and analysis of the lipid A

backbone. Lipid A structure of lipopolysaccharides from various bacterial groups. Eur. J. Biochem. **63:**101–107.
15. **Helander, I. M., I. Kilpeläinen, M. Vaara, A. P. Moran, B. Lindner, and U. Seydel.** 1994. Chemical structure of the lipid A component of lipopolysaccharides of the genus *Pectinatus*. Eur. J. Biochem. **224:**63–70.
16. **Helander, I. M., B. Lindner, H. Brade, K. Altmann, A. A. Lindberg, E. T. Rietschel, and U. Zähringer.** 1988. Chemical structure of the lipopolysaccharide of *Haemophilus influenzae* strain I-69 $Rd^{-}/b^{+}$. Description of a novel deep-rough chemotype. Eur. J. Chem. **177:**483–492.
17. **Holst, O., D. Borowiak, J. Weckesser, and H. Mayer.** 1983. Structural studies on the phosphate-free lipid A of *Rhodomicrobium vannielii* ATCC 17100. Eur. J. Biochem. **137:**325–332.
18. **Homma, J. Y., M. Matsuura, S. Kanegasaki, Y. Kawakubo, Y. Kojima, N. Shibukawa, A. Yamamoto, K.-I. Tanamoto, T. Yasuda, M. Imoto, H. Yoshimura, S. Kusumoto, and T. Shiba.** 1985. Structural requirements of lipid A responsible for the functions: a study of chemically synthesized lipid A and its analogues. J. Biochem. (Tokyo) **98:**395–406.
19. **Johne, B., I. Olsen, and K. Bryn.** 1988. Fatty acids and sugars in lipopolysaccharides from *Bacteroides intermedius*, *Bacteroides gingivalis* and *Bacteroides loescheii*. Oral Microbiol. Immunol. **3:**22–27.
20. **Kanegasaki, S., Y. Kojima, M. Matsuura, J. Y. Homma, A. Yamamoto, Y. Kumazawa, K.-I. Tanamoto, T. Yasuda, T. Tsumita, M. Imoto, H. Yoshimura, M. Yamamoto, T. Shimamoto, S. Kusumoto, and T. Shiba.** 1984. Biological activities of analogues of lipid A based chemically on the revised structural model. Comparison of mediator-inducing, immunomodulating and endotoxic activities. Eur. J. Biochem. **143:**237–242.
21. **Kanegasaki, S., K.-I. Tanamoto, T. Yasuda, J. Y. Homma, M. Matsuura, M. Nakatsuka, Y. Kumazawa, A. Yamamoto, T. Shiba, S. Kusumoto, M. Imoto, H. Yoshimura, and T. Shimamoto.** 1986. Structure-activity relationship of lipid A: comparison of biological activities of natural and synthetic lipid A's with different fatty acid compositions. J. Biochem. (Tokyo) **99:**1203–1210.
22. **Kotani, S., H. Takada, M. Tsujimoto, T. Ogawa, K. Harada, Y. Mori, A. Kawasaki, A. Tanaka, S. Nagao, S. Tanaka, T. Shiba, S. Kusumoto, M. Imoto, H. Yoshimura, M. Yamamoto, and T. Shimamoto.** 1984. Immunobiologically active lipid A analogs synthesized according to a revised structural model of natural lipid A. Infect. Immun. **45:**293–296.
23. **Kotani, S., H. Takada, M. Tsujimoto, T. Ogawa, I. Takahashi, T. Ikeda, K. Otsuka, H. Shimauchi, N. Kasai, J. Mashimo, S. Nagao, A. Tanaka, S. Tanaka, K. Harada, K. Nagaki, H. Kitamura, T. Shiba, S. Kusumoto, M. Imoto, and H. Yoshimura.** 1985. Synthetic lipid A with endotoxic and related biological activities comparable to those of a natural lipid A from an *Escherichia coli* Re-mutant. Infect. Immun. **49:**225–237.
24. **Krauss, J. H., U. Seydel, J. Weckesser, and H. Mayer.** 1989. Structural analysis of the nontoxic lipid A of *Rhodobacter capsulatus* 37b4. Eur. J. Biochem. **180:**519–526.
25. **Kulshin, V. A., U. Zähringer, B. Lindner, K. E. Jäger, B. A. Dmitriev, and E. T. Rietschel.** 1991. Structural characterization of the lipid A component of *Pseudomonas aeruginosa* wild-type and rough mutant lipopolysaccharides. Eur. J. Biochem. **198:**697–704.
26. **Kumada, H., Y. Haishima, T. Umemoto, and K.-I. Tanamoto.** 1995. Structural study on the free lipid A isolated from lipopolysaccharide of *Porphyromonas gingivalis*. J. Bacteriol. **177:**2098–2106.
27. **Kumazawa, Y., N. Nakatsuka, H. Takimoto, T. Furuya, T. Nagumo, A. Yamamoto, J. Y. Homma, K. Inada, M. Yoshida, M. Kiso, and A. Hasegawa.** 1988. Importance of fatty acid substituents of chemically synthesized lipid A-subunit analogs in the expression of immunopharmacological activity. Infect. Immun. **56:**149–155.
28. **Lee, A., and A. P. Moran.** 1994. Lipopolysaccharide (LPS)-related damage by *H. pylori*, p. 169–179. *In* R. H. Hunt and G. N. J. Tytgat (ed.), *Helicobacter pylori*: basic mechanisms to clinical cure. Kluwer Academic Publishers, Dordrecht, The Netherlands.
29. **Lindner, B., U. Zähringer, E. T. Rietschel, and U. Seydel.** 1990. Structural elucidation of lipopolysaccharides and their lipid A component: application of soft ionization mass spectrometry, p. 149–161. *In* A. Fox, S. L. Morgan, L. Larsson, and G. Odham (ed.), Analytical microbiology methods. Plenum Press, New York, N.Y.
30. **Lowry, O. H., N. R. Roberts, K. Y. Leiner, M. L. Wu, and A. L. Farr.** 1954. The quantitative histochemistry of brain. I. Chemical methods. J. Biol. Chem. **207:**1–17.
31. **Marshall, B. J., and J. R. Warren.** 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet **i:**1311–1315.
32. **Mayer, H., and J. Weckesser.** 1984. 'Unusual' lipid A's: structures, taxonomical relevance and potential value for endotoxin research, p. 221–247. *In* E. T. Rietschel (ed.), Handbook of endotoxin, vol. 1. Chemistry of endotoxin. Elsevier Science Publishers B.V., Amsterdam, The Netherlands.
33. **Moran, A. P.** 1995. Structure-bioactivity relationships of bacterial endotoxins. J. Toxicol. Toxin Rev. **14:**47–83.
34. **Moran, A. P.** 1995. Cell surface characteristics of *Helicobacter pylori*. FEMS Immunol. Med. Microbiol. **10:**271–280.
35. **Moran, A. P.** 1996. The role of lipopolysaccharide in *Helicobacter pylori* pathogenesis. Aliment. Pharmacol. Ther. **10**(Suppl. 1)**:**39–50.
36. **Moran, A. P., I. M. Helander, and T. U. Kosunen.** 1992. Compositional analysis of *Helicobacter pylori* rough-form lipopolysaccharides. J. Bacteriol. **174:**1370–1377.
37. **Moran, A. P., J. J. McGovern, B. Lindner, and E. J. Walsh.** 1995. Preliminary structural analysis of *Helicobacter pylori* lipid A. Gut **37**(Suppl. 1)**:**A16.
38. **Moran, A. P., and E. J. Walsh.** 1993. *In vitro* production of endotoxin by the gastroduodenal pathogen *Helicobacter pylori*—relevance to serotyping. Ir. J. Med. Sci. **162:**384.
39. **Moran, A. P., U. Zähringer, U. Seydel, D. Scholz, P. Stütz, and E. T. Rietschel.** 1991. Structural analysis of the lipid A component of *Campylobacter jejuni* CCUG 10936 (serotype O:2) lipopolysaccharide. Description of a lipid A containing a hybrid backbone of 2-amino-2-deoxy-D-glucose and 2,3-diamino-2,3-dideoxy-D-glucose. Eur. J. Biochem. **198:**459–469.
40. **Muotiala, A., I. M. Helander, L. Pyhälä, T. U. Kosunen, and A. P. Moran.** 1992. Low biological activity of *Helicobacter pylori* lipopolysaccharide. Infect. Immun. **60:**1714–1716.
41. **Parsonnet, J., S. Hansen, L. Rodriguez, A. B. Gelb, R. A. Warnke, E. Jelum, N. Orentreich, J. H. Vogelman, and G. D. Friedman.** 1994. *Helicobacter pylori* infection and gastric lymphoma. N. Engl. J. Med. **330:**1267–1271.
42. **Pece, S., D. Fumarola, G. Giuliani, E. Jirillo, and A. P. Moran.** 1995. Activity in the *Limulus* amebocyte lysate assay and induction of tumor necrosis factor-α by diverse *Helicobacter pylori* lipopolysaccharide preparations. J. Endotoxin Res. **2:**455–462.
43. **Perez-Perez, G. I., V. L. Shepherd, J. D. Morrow, and M. J. Blaser.** 1995. Activation of human THP-1 cells and rat bone marrow-derived macrophages by *Helicobacter pylori* lipopolysaccharide. Infect. Immun. **63:**1183–1187.
44. **Raetz, C. R. H.** 1990. Biochemistry of endotoxins. Annu. Rev. Biochem. **59:**129–170.
45. **Raetz, C. R. H.** 1993. Bacterial endotoxins: extraordinary lipids that activate eucaryotic signal transduction. J. Bacteriol. **175:**5745–5753.
46. **Raetz, C. R. H., S. Purcell, M. V. Meyer, N. Qureshi, and K. Takayama.** 1985. Isolation and characterization of eight lipid A precursors from a 3-deoxy-D-*manno*-octulosonic acid-deficient mutant of *Salmonella typhimurium*. J. Biol. Chem. **260:**16080–16088.
47. **Ray, B. L., and C. R. H. Raetz.** 1987. The biosynthesis of gram-negative endotoxin: a novel kinase in *Escherichia coli* membranes that incorporates the 4′ phosphate of lipid A. J. Biol. Chem. **262:**1122–1128.
48. **Rietschel, E. T., L. Brade, B. Lindner, and U. Zähringer.** 1992. Biochemistry of lipopolysaccharides, p. 3–41. *In* D. C. Morrison and J. L. Ryan (ed.), Bacterial endotoxic lipopolysaccharides, vol I. Molecular biochemistry and cellular biology. CRC Press, Boca Raton, Fla.
49. **Rietschel, E. T., L. Brade, U. Schade, C. Galanos, M. Freudenberg, O. Lüderitz, S. Kusumoto, and T. Shiba.** 1987. Endotoxic properties of synthetic pentaacyl lipid A Ib and a structural isomer. Eur. J. Biochem. **169:**27–31.
50. **Rietschel, E. T., H. Gottert, O. Lüderitz, and O. Westphal.** 1972. Nature and linkages of fatty acids present in the lipid A component of *Salmonella* lipopolysaccharides. Eur. J. Biochem. **28:**166–173.
51. **Rietschel, E. T., T. Kirikae, F. U. Schade, U. Mamat, G. Schmidt, H. Loppnow, A. J. Ulmer, U. Zähringer, U. Seydel, F. Di Padova, M. Schreier, and H. Brade.** 1994. Bacterial endotoxin: molecular relationships of structure to activity and function. FASEB J. **8:**217–225.
52. **Sakamoto, J., T. Watanabe, T. Tokumaru, H. Takagi, H. Nakazato, and K. O. Lloyd.** 1989. Expression of $Lewis^{a}$, $Lewis^{b}$, $Lewis^{x}$, $Lewis^{y}$, sialyl-$Lewis^{a}$, and sialyl-$Lewis^{x}$ blood group antigens in human gastric carcinoma and in normal gastric tissue. Cancer Res. **49:**745–752.
53. **Semeraro, N., P. Montemurro, C. Piccolo, V. Muolo, M. Colucci, G. Giuliani, D. Fumarola, S. Pece, and A. P. Moran.** 1996. Effect of *Helicobacter pylori* lipopolysaccharide (LPS) and LPS derivatives on the production of tissue factor and plasminogen activator inhibitor type 2 by human blood mononuclear cells. J. Infect. Dis. **174:**1255–1260.
54. **Sidorczyk, Z., U. Zähringer, and E. T. Rietschel.** 1983. Chemical structure of the lipid A component of the lipopolysaccharide from a *Proteus mirabilis* Re-mutant. Eur. J. Biochem. **137:**15–22.
55. **Sonesson, A., E. Jantzen, K. Bryn, L. Larsson, and J. Eng.** 1989. Chemical composition of a lipopolysaccharide from *Legionella pneumophila*. Arch. Microbiol. **153:**72–78.
56. **Stellner, K., H. Saito, and S. Hakomori.** 1973. Determination of aminosugar linkages in glycolipids by methylation of aminosugar linkages of ceramide pentasaccharides of rabbit erythrocytes and of Forssman antigen. Arch. Biochem. Biophys. **155:**464–472.
57. **Strominger, J. L., J. T. Park, and R. E. Thompson.** 1959. Composition of the cell wall of *Staphylococcus aureus*: its relation to the action of penicillin. J. Biol. Chem. **234:**3263–3268.
58. **Tarkkanen, J., T. U. Kosunen, and E. Saksela.** 1993. Contact of lymphocytes with *Helicobacter pylori* augments natural killer cell activity and induces production of gamma interferon. Infect. Immun. **61:**3012–3016.
59. **Taylor, D. N., and M. J. Blaser.** 1991. The epidemiology of *Helicobacter*

*pylori* infection. Epidemiol. Rev. **13:**42–59.
60. **Weintraub, A., U. Zähringer, H.-W. Wollenweber, U. Seydel, and E. T. Rietschel.** 1989. Structural characterization of the lipid component of *Bacteroides fragilis* strain NCTC 9343 lipopolysaccharide. Eur. J. Biochem. **183:** 425–431.
61. **Westphal, O., O. Lüderitz, and F. Bister.** 1952. Über die Extraktion von Bakterien mit Phenol/Wasser. Z. Naturforsch. Teil B **7:**148–155.
62. **Wollenweber, H.-W., K. W. Broady, O. Lüderitz, and E. T. Rietschel.** 1982. The chemical structure of lipid A. Demonstration of amide-linked 3-acyloxyacyl residues in *Salmonella minnesota* Re lipopolysaccharide. Eur. J. Biochem. **124:**191–198.
63. **Wollenweber, H.-W., U. Seydel, B. Lindner, O. Lüderitz, and E. T. Rietschel.** 1984. Nature and location of amide-bound (*R*)-3-acyloxyacyl groups in lipid A of lipopolysaccharides from various gram-negative bacteria. Eur. J. Biochem. **145:**265–272.
64. **Zähringer, U., B. Lindner, and E. T. Rietschel.** 1994. Molecular structure of lipid A, the endotoxic center of bacterial lipopolysaccharides. Adv. Carbohydr. Chem. **50:**211–276.