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Compositional Analysis of *Helicobacter pylori* Rough-Form Lipopolysaccharides

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Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to analyze the macromolecular heterogeneity of lipopolysaccharides (LPS) from seven fresh clinical isolates and three culture collection strains of the human pathogen *Helicobacter pylori*. All the clinical isolates produced smooth-form LPS with O side chains of relatively homogeneous chain length, whereas the culture collection strains yielded rough-form LPS. A better yield of the latter LPS was obtained when combined protease pretreatment and hot phenol-water extraction were used than when the conventional phenol-water technique alone was used for extraction. The LPS of the three culture collection strains (S-24, C-5437, and NCTC 11637) were chemically characterized. Constituents common to all the LPS were fucose, D-mannose, D-glucose, D-galactose, D-*glycero*-D-*manno*-heptose, L-*glycero*-D-*manno*-heptose, and 3-deoxy-D-*manno*-2-octulosonic acid. The molar ratios of the hexoses differed between different strains, thereby reflecting structural differences. Phosphate, phosphorylethanolamine, and pyrophosphorylethanolamine were present also. Free lipid A contained D-glucosamine and fatty acids, with phosphate and a minor amount of ethanolamine. The major fatty acids were ester- and amide-bound 3-hydroxyoctadecanoic acid and ester-bound octadecanoic and 3-hydroxyhexadecanoic acids, with minor amounts of ester-bound tetradecanoic and hexadecanoic acids. In addition to the uncommonly long 3-hydroxy fatty acids, an unusual phosphorylation pattern was deduced to be present in the lipid A.

Helicobacter pylori (formerly *Campylobacter pylori*) is a microaerophilic bacterium which has been implicated as a causal agent of gastritis and has been associated with duodenal ulcers in humans (2, 19, 30). Despite its importance as a human pathogen, characterization of the surface structures of this bacterium is, to date, incomplete.

Lipopolysaccharides (LPS) are a characteristic cell wall constituent of gram-negative bacteria which are important in the structure and function of the outer membrane (25). Also, LPS can provide the basis for serological classifications, has value as a taxonomic marker, and is a potential toxin of the bacterium (3, 20, 21, 33, 48). Chemically, LPS are composed of a poly- or oligosaccharide and a lipid component, termed lipid A (3, 33). High-molecular-weight smooth (S)-form LPS consist of an O side chain, which is a polymer of repeating oligosaccharide units, a core oligosaccharide, and lipid A, whereas low-molecular-weight rough (R)-form LPS lack the O side chain (33). Mutants producing LPS lacking the O side chain, because of genetic defects in the biosynthesis of LPS, have proven useful for structural analysis of the core oligosaccharide and lipid A of those bacteria which normally produce S-form LPS, for example, members of the families *Enterobacteriaceae* and *Pseudomonadaceae* (3, 33).

Detailed compositional analysis is a prerequisite to structural analysis of the core oligosaccharide and lipid A, and this prompted the present investigation of *H. pylori* LPS. We undertook electrophoretic analysis of *H. pylori* culture collection strains and clinical isolates to determine the macromolecular heterogeneity of their LPS. The R-form LPS of three *H. pylori* strains were chemically characterized. The evidence presented suggests that structural differences in the oligosaccharide moiety of the LPS of the three strains exist and that *H. pylori* lipid A, in addition to containing uncommonly long 3-hydroxy fatty acids, possesses an unusual phosphorylation pattern compared with that of enterobacterial lipid A.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Three strains of *H. pylori* from culture collections were used. Strain NCTC 11637 was obtained from the Public Health Laboratory Service, London, England. Strains S-24 and C-5437 were provided by D. Danielsson, Medical Center Hospital, Örebro, Sweden, and H. Lior, Laboratory Center for Disease Control, Ottawa, Canada, respectively. In addition, *H. pylori* F-72/91, F-241/91, F-266/91, F-278/91, F-370/91, F-376/91, and F-384/91 were isolated from gastric biopsies. *Helicobacter* strains were grown on blood agar (Trypticase soy agar [Difco, Detroit, Mich.], 6% horse blood) supplemented with 10% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.) which was incubated in an atmosphere of 5% O_2, 10% CO_2, and 85% N_2 at 37°C for 48 h. *Salmonella enterica* serovar Typhimurium SH2183, Typhimurium mutant strain *his-515* (LPS chemotype Ra), and *S. enterica* serovar Minnesota mutant strain R595 (chemotype Re) were cultivated on L agar as described previously (40). 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.

Isolation of LPS. For analysis of the macromolecular heterogeneity of *H. pylori* LPS by gel electrophoresis, whole-cell lysates were prepared and treated with proteinase K (Sigma) by the procedure described by Hitchcock and Brown (14). Bulk extraction of LPS from bacteria (2 to 5 g

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[dry weight]) was performed with 45% aqueous phenol at 68°C for 30 min (50). Alternatively, bacteria (about 2 g [dry weight]) were pretreated with pronase (Calbiochem, Los Angeles, Calif.) (5) before LPS was extracted with 45% aqueous phenol. The LPS preparations were purified by treatment with RNase A (Sigma Chemical Co., St. Louis, Mo.), DNase II (Sigma), and proteinase K (Sigma) and by ultracentrifugation at 100,000 × *g* at 4°C for 18 h (22). LPS was extracted from *his-515* and R595 mutant strains by the phenol-chloroform-petroleum ether method (8).

SDS-PAGE and immunoblotting. Proteinase K-treated whole-cell lysates and LPS preparations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the discontinuous buffer system described by Laemmli (17). Electrophoresis was conducted with a constant current of 35 mA, a stacking gel of 5% acrylamide, and a separating gel of 15% acrylamide containing 3.2 M urea (ICN Biomedicals, Cleveland, Ohio). After SDS-PAGE, the gels were fixed and LPS was detected by silver staining, as described previously (46). Alternatively, LPS fractionated by SDS-PAGE was electrotransferred from gels to nitrocellulose membranes (pore size, 0.45 μm; Bio-Rad Laboratories, Richmond, Calif.) by using the buffer system described by Towbin et al. (44). Visualization of nitrocellulose blots was performed with rabbit antiserum as the first antibody and goat anti-rabbit immunoglobulin G–horseradish peroxidase conjugate (Bio-Rad) as the second antibody (28).

Preparative dephosphorylation of LPS. Preparative dephosphorylation was performed by treating LPS (20 mg [dry weight]) with 48% aqueous hydrofluoric acid (Merck, Darmstadt, Germany) at 4°C for 48 h (13). The resulting suspension was dialyzed against water. The retentate (dephosphorylated LPS) and, after neutralization, the dialysate were freeze-dried separately.

Preparation of free lipid A. LPS (20 mg) was treated with 1% CH_3COOH (5 ml) at 100°C for 1.5 h. Free lipid A was precipitated after centrifugation at 3,000 × *g* (4°C, 30 min), washed with water, freeze-dried, and subsequently solubilized by sonification and the addition of triethylamine to pH 8. The supernatant of the hydrolysate (degraded oligosaccharide) was concentrated and freeze-dried.

Neutral sugar analysis. Neutral sugars were liberated by acid hydrolysis (0.1 M HCl) at 100°C for 48 h and were determined quantitatively as their alditol acetate derivatives (36) by gas-liquid chromatography (GLC) with xylose as an internal standard. A Hewlett-Packard 5890 instrument (Avondale, Pa.) equipped with a chemically bonded NB-9C fused-silica capillary column (HNU-Nordion, Helsinki, Finland; 25 m by 0.32 mm) with helium as the carrier gas (0.05 MPa) was used. The column temperature program was 200°C for 3 min, increasing to 260°C at 5°C/min (program A); injector and detector temperatures were 260 and 280°C, respectively.

Characterization of the TBA-reactive constituent. For quantitation, thiobarbituric acid (TBA)-reactive material was liberated by using 0.1 M sodium acetate (pH 4.4) (100°C, 1 h) or 1 M HCl (100°C, 2 h) and estimated colorimetrically in terms of 3-deoxy-D-*manno*-2-octulosonic acid (KDO) equivalents (47). KDO was characterized as its partially methylated and acetylated 3-deoxyoctitol by GLC after treatment of LPS (4 mg) by the procedure of Tacken et al. (43) with permethylation with NaOH-dimethyl sulfoxide-CH_3I (6). KDO was also analyzed in the form of its methyl ketoside, which was obtained by acidic methanolysis of LPS (3 mg) and peracetylation according to the conditions described previously (23). GLC was performed on a fused-silica capillary column with chemically bonded HP-5 (Hewlett Packard; 25 m by 0.32 mm; column temperature, program B: 170°C, 3 min isothermally, increasing to 300°C at 3°C/min).

Ethanolamine and amino sugar analysis. Ethanolamine, amino sugars (except 4-amino-4-deoxyarabinose), and their phosphorylated derivatives were liberated with 4 M HCl at 100°C for 18 h. Ethanolamine and its phosphorylated derivatives were determined quantitatively in an amino acid analyzer (Alpha plus 4151; LKB Products, Bromma, Sweden). Amino sugars were analyzed in the amino acid analyzer or as their alditol acetate derivatives (36) by GLC on the HP-5 column (column temperature, program B). Analysis of 4-amino-4-deoxyarabinose was achieved after treatment of LPS with 4 M HCl at 65°C (15 h) and subsequent reduction and peracetylation (24).

Determination of the absolute configuration of sugars. Monosaccharides after liberation were treated with 2 M butanolic HCl [*S*(+)- or *R*(−)-2-butanol] at 86°C for 2 h and then peracetylated. The GLC retention times (NB-9C column; column temperature, program C: 150°C, 3 min isothermally, increasing to 250°C at 3°C/min. HP-5 column; column temperature, program B) of the peracetylated diastereomeric *S*(+)- and *R*(−)-2-butyl glycosides were compared with those of authentic reference compounds (D-mannose, D-glucose, D-galactose, and D-glucosamine) prepared in the same way.

Fatty acid determination. Fatty acids were liberated from LPS by acid hydrolysis (4 M HCl, 100°C, 5 h) and subsequent alkaline treatment (0.5 M NaOH, 100°C, 1 h). Free fatty acids were extracted with petroleum ether (fraction 40 to 60°C)-ethyl acetate (1:1 [vol/vol]) and subsequently carboxymethylated with diazomethane. Heptadecanoic acid (17:0) served as an internal standard. GLC of the methyl esters was performed on the HP-5 column (column temperature, program B). The absolute configuration of 3-hydroxy fatty acids was determined by GLC analysis (HP-5 column; column temperature, program C: 250°C, isothermally) of the diastereomeric D-phenylethylamide derivatives (31). Ester-bound fatty acids were selectively liberated from vacuum-dried LPS by alkaline transesterification with sodium methylate (0.25 M, 37°C, 15 h) and both qualitatively and quantitatively analyzed by GLC (HP-5 column; column temperature, program B) without and with additional diazomethane treatment as described previously (34).

Mass spectrometry. Combined GLC-mass spectrometry 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 carrier gas, and column temperature programs were the same as those for GLC. Electron impact mass spectra were recorded at 70 eV; chemical ionization mass spectra were achieved with ammonia as the reactant gas. The ion-source temperature was 185°C.

High-voltage paper electrophoresis. High-voltage paper electrophoresis (16) was performed with pyridine-formic acid-acetic acid-water buffer (2:3:20:180 [vol/vol/vol/vol], pH 2.8). Pherograms were stained with alkaline silver nitrate (45), TBA (4), ninhydrin, or molybdate reagent (10).

Miscellaneous colorimetric estimations. Covalently bound uronic acid was quantitated by the procedure of Bitter and Muir (1). Total hexosamine content was determined after acid hydrolysis (4 M HCl, 100°C, 18 h) by the Morgan-Elson reaction as modified by Strominger et al. (39). The direct Morgan-Elson reaction assay was performed without hydro-

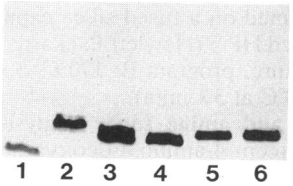

FIG. 1. Silver-stained SDS-PAGE gel of hot phenol-water-extracted LPS from *S. enterica* serovar Minnesota, *S. enterica* serovar Typhimurium, and *H. pylori* strains. Lanes: 1, Minnesota R595 (Re chemotype); 2, Typhimurium *his-515* (Ra chemotype); 3, *H. pylori* S-24; 4, *H. pylori* C-5437; 5, *H. pylori* NCTC 11637; and 6, *H. pylori* NCTC 11637 with pronase pretreatment. Samples of 0.2 μg were applied to the 15% acrylamide gel.

lysis. Organic and inorganic phosphates were determined by the method of Lowry et al. (18).

RESULTS

SDS-PAGE and immunoblotting. Silver-stained SDS-PAGE gels of hot phenol-water-extracted LPS from the *H. pylori* culture collection strains (S-24, C-5437, and NCTC 11637) exhibited R-form LPS (Fig. 1). Also, with visualization by silver staining or immunoblotting with homologous rabbit antisera, the migration patterns in SDS-PAGE of LPS from proteinase K-treated whole-cell lysates of these strains were identical to those of phenol-water-extracted LPS (data not shown). Although there were slight differences between strains in the mobility of their R-form LPS (Fig. 1, lanes 3 to 5), NCTC 11637 LPS extracted by phenol-water either without (Fig. 1, lane 5) or with (Fig. 1, lane 6) pronase pretreatment had the same mobility. *H. pylori* low-molecular-weight LPS migrated slower than Minnesota Re LPS (Fig. 1, lane 1) and slightly faster than Typhimurium Ra LPS (Fig. 1, lane 2). As Re LPS consists of lipid A linked to two KDO residues and has a molecular weight of 2,800, whereas Ra LPS, with a molecular weight of about 4,700, consists of lipid A plus a complete core oligosaccharide (46), it was estimated that the LPS of the culture collection strains had approximate molecular weights in the range of 4,000 to 4,500.

Proteinase K-treated whole-cell lysates of clinical isolates were examined by SDS-PAGE to compare their macromolecular heterogeneity with those of LPS from culture collection strains. In contrast to the latter strains, proteinase K-treated whole-cell lysates of all seven *H. pylori* clinical isolates, which had been passaged only once in vitro, showed profiles in silver-stained gels characteristic of high-molecular-weight LPS (Fig. 2). The absence of protein bands

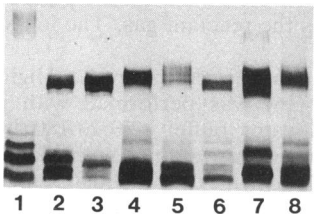

FIG. 2. Silver-stained SDS-PAGE gel of proteinase K-treated whole-cell lysates of *S. enterica* serovar Typhimurium SH2183 (lane 1) and *H. pylori* F-72/91 (lane 2), F-241/91 (lane 3), F-266/91 (lane 4), F-278/91 (lane 5), F-370/91 (lane 6), F-376/91 (lane 7), and F-384/91 (lane 8). Samples of 3 μl were applied to the 15% acrylamide gel.

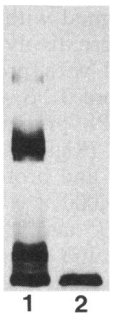

FIG. 3. Silver-stained SDS-PAGE gel of proteinase K-treated whole-cell lysates of *H. pylori* F-376/91 before (lane 1) and after (lane 2) numerous in vitro passages. Samples of 3 and 2 μl, respectively, were applied to the 15% acrylamide gel.

in the gels was confirmed by the absence of staining with Coomassie blue (data not shown). Unlike S-form LPS of *S. enterica* serovar Typhimurium with its orderly series of high-molecular-weight bands forming a ladderlike pattern (Fig. 2, lane 1), those of *H. pylori* had a profile characteristic of LPS with O side chains of relatively homogeneous length (Fig. 2, lanes 2 to 8). Strain-to-strain differences in the mobility, staining properties, and banding pattern of *H. pylori* S-form LPS were observed, reflecting possible structural differences in the LPS. In addition, the high-molecular-weight LPS of some strains could not be resolved into discrete bands even at low loading concentrations, suggesting the presence of homopolymers or additional charged groups in the O side chains. Upon numerous in vitro passages of the isolates, however, loss of O side chains occurred, resulting in the production of R-form LPS as determined by SDS-PAGE. The LPS profiles of F-370/91, shown in Fig. 3, are representative examples.

Yields of R-form LPS. The yields of LPS (percent bacterial dry weight) from the water phase of phenol-water extracts of the three culture collection strains were low (S-24, 0.4%; C-5437, 0.4%; and NCTC 11637, 0.3%). Pretreatment of bacteria with pronase before hot phenol-water extraction from one strain gave a 13-fold higher yield of LPS (NCTC 11637, 3.9%). The chemical compositions of the LPS obtained are shown in Table 1; these analyses accounted for 79 to 94% of the LPS (dry weight). The compositions of NCTC 11637 LPS prepared by the two methods were comparable. The LPS preparations were essentially free from proteins (<1%) and nucleic acids (<1%). Overall, a low ratio of sugar to fatty acid was observed.

Neutral sugars. All the R-form LPS contained D-mannose, D-glucose, and D-galactose, although the molar ratios of these hexoses differed between strains (Table 1). Also, two heptoses were common to the LPS. Upon electron impact mass spectrometry, the alditol acetate derivatives of both these compounds yielded primary fragments at m/z = 145, 217, 289, and 361 and secondary fragments at m/z = 103, 115, 127, 139, 157, 187, 199, 229, and 259. Chemical ionization with ammonia yielded the expected pseudomolecular ions at m/z = 507 $[M + H]^+$ and 524 $[M + NH_4]^+$. Upon GLC (NB-9C, program A) of their alditol acetate derivatives, one heptose comigrated with authentic D-*glycero*-D-*manno*-heptose, and the other comigrated with L-*glycero*-D-*manno*-heptose (relative retention times to xylitol pentaacetate, 1.57 and 1.67, respectively). An increased amount of L-*glycero*-D-*manno*-heptose was detectable after dephosphorylation of LPS with hydrofluoric acid, suggesting the presence of this

TABLE 1. Chemical composition of LPS of *H. pylori* strains extracted by hot phenol-water without or with pronase pretreatment

Constituent	Amt of constituent (nmol/mg) in LPS of *H. pylori*:			
	S-24	C-5437	NCTC 11637	
			Without pronase	With pronase[a]
Sugars				
Fucose	21	8	8	9
D-Mannose	64	129	49	49
D-Glucose	177	333	297	294
D-Galactose	273	499	353	354
D-*glycero*-D-*manno*-Heptose	282	341	241	232
L-*glycero*-D-*manno*-Heptose	385	479	356	342
L-*glycero*-D-*manno*-Heptose-phosphate[b]	58	72	54	52
3-Deoxy-D-*manno*-2-octulosonic acid[c]	259	291	255	266
D-Glucosamine	490	630	457	475
Ethanolamine	564	598	417	484
Ethanolamine-phosphate	Tr[d]	Tr	Tr	Tr
Ethanolamine-pyrophosphate	25	12	72	26
Fatty acids				
Tetradecanoic	20	29	19	21
Hexadecanoic	28	24	29	28
Octadecanoic	285	346	269	280
(*R*)-3-Hydroxyhexadecanoic	173	246	169	188
(*R*)-3-Hydroxyoctadecanoic	560	651	513	571
Phosphate				
Organic	591	611	481	497
Inorganic	Tr	Tr	Tr	Tr

[a] LPS was extracted with the hot phenol-water technique after pretreatment of bacteria with pronase.
[b] The increase in amount of heptose after LPS was dephosphorylated with hydrofluoric acid.
[c] Determined by the TBA assay after acid hydrolysis (1 M HCl, 100°C, 2 h).
[d] Tr, Trace (≤5 nmol/mg).

heptose-phosphate. The ratio of L-*glycero*-D-*manno*- to D-*glycero*-D-*manno*-heptose was about 2:1 for all the LPS (Table 1).

Another sugar whose alditol acetate had the same electron impact mass spectrum as 1,2,3,4,5-penta-*O*-acetyl-6-deoxyhexitol was present. Mass spectrometric fragmentation showed primary fragments at m/z = 145, 159, 217, 231, and 289. Secondary fragments were observed at m/z = 99, 113, 115, 117, 127, 141, 157, 187, and 201. Chemical ionization yielded peaks at m/z = 377 $[M + H]^+$ and 394 $[M + NH_4]^+$. Identity of the bacterial sugar with authentic fucose (6-deoxygalactose) was obtained upon GLC of the respective alditol acetate derivatives (NB-9C, program A; relative retention time to xylitol acetate, 0.70). As fucose was present in *H. pylori* LPS in nonstoichiometric amounts and only limited quantities of the LPS were available, it was not possible to determine the absolute configuration of fucose. Treatment of LPS with hydrofluoric acid and subsequent dialysis, in addition to dephosphorylating the LPS, liberated fucose, which was detectable after desalting of the dialysate by gel chromatography on a Sephadex G-10 column.

Uronic acid was not detectable in hydrolysates (4 M HCl, 1 h, or 0.1 M HCl, 100°C, 48 h) when analyzed by high-voltage paper electrophoresis or in a carbazole colorimetric assay (1).

KDO. After mild acid hydrolysis, a constituent from *H. pylori* LPS reacted with TBA and produced a chromophore with an absorption maximum at 549 nm. To identify the TBA-reactive constituent, LPS was subjected to acidic methanolysis (1 M HCL, 1 h) and the products were subsequently deuterium reduced (NaB_2H_4), permethylated, hydrolyzed with trifluoroacetic acid, reduced ($NaBH_4$), and peracetylated (43). A partially methylated and acetylated derivative of KDO (2,6-di-*O*-acetyl-3-deoxy-1,4,5,7,8-penta-*O*-methyl-[1,1-$^2H_2$]octitol), whose mass spectrum (electron impact) was identical to that of derivatized authentic compound (spectra not shown), was detected. The octitol derivative showed primary fragments at m/z = 45, 89, 161, 177, 205, 221, and 337, with secondary fragments at m/z = 101, 117, 129, 157, and 261, as described previously (43). Chemical ionization gave the expected m/z = 383 $[M + H]^+$ and 400 $[M + NH_4]^+$.

By acidic methanolysis (1 M HCl, 1 h) and peracetylation, pyranosidic KDO was detected as its peracetylated methyl ketoside methyl ester derivative. The KDO derivative was identical in all parameters to authentic 3-deoxy-D-*manno*-2-octulosonic acid derivatized in the same way, indicating the D-*manno* configuration of the bacterial sugar (42).

The quantity of KDO present was determined by the TBA assay after liberation with sodium acetate (pH 4.4) (0.1 M, 100°C, 1 h) or HCl (1 M, 100°C, 2 h). Sodium acetate treatment liberated low amounts of KDO (35 to 44 nmol), whereas stoichiometric amounts were liberated by HCl (255 to 291 nmol; Table 1). After dephosphorylation of LPS with hydrofluoric acid, no increase in KDO was observed, suggesting the absence of KDO-phosphate. Furthermore, KDO-phosphate was not detectable on paper electropherograms of acid hydrolysates (4 M HCl, 100°C, 1 h) of LPS or by GLC after acidic methanolysis of LPS (1 M HCl, 1 h) and either peracetylation or permethylation (13).

Amino sugars, phosphorylated amino sugars, ethanolamine, and ethanolamine-phosphate. D-Glucosamine was present in all the LPS and, after hydrolysis, was detected in the amino acid analyzer and as its alditol acetate by GLC. Neither

glucosamine-phosphate nor any other amino sugar was detected. The total hexosamine content determined by the Morgan-Elson reaction assay was comparable to that of D-glucosamine determined in the amino acid analyzer (Table 1). Stoichiometric amounts of ethanolamine and minor amounts of ethanolamine-pyrophosphate (12 to 72 nmol/mg) and trace amounts of ethanolamine-phosphate (≤5 nmol/mg) were also found in the LPS hydrolysates by using the amino acid analyzer (Table 1).

Fatty acids. The fatty acids present in LPS of all four strains were tetradecanoic (14:0), hexadecanoic (16:0), octadecanoic (18:0), 3-hydroxyhexadecanoic (3-OH-16:0), and 3-hydroxyoctadecanoic (3-OH-18:0). The approximate ratios of these fatty acids were 0.1:0.1 to 0.2:1.5:1:3 (Table 1).

The absolute configuration of the 3-hydroxy fatty acids present in *H. pylori* LPS was determined by GLC analysis of their 3-methoxy-D-phenylethylamide derivatives (31). The GLC retention times of the D-phenylethylamide derivatives of a homologous series of nonhydroxylated saturated fatty acids [dodecanoic (12:0), 14:0, 16:0, 17:0, and 18:0] when plotted semilogarithmically against the number of carbon atoms present in the fatty acid yielded a straight line (slope = 0.54, *y*-intercept = 1.46). The 3-methoxy-D-phenylethylamides derived from 3-OH-16:0 and 3-OH-18:0 of *Helicobacter* strains when plotted in the same manner yielded another straight line, parallel to the first and with the same slope (*y*-intercept = 1.81), on which also fell the D-phenylethylamide of authentic (*R*)- but not (*S*)-3-hydroxytetradecanoic acid (3-OH-14:0). Therefore, the 3-hydroxy fatty acids found in *H. pylori* LPS possess the (*R*) configuration.

Sodium methylate treatment released quantitatively 14:0, 16:0, and 18:0 in the form of their methyl esters. Proportions of 3-OH-16:0 and 3-OH-18:0 also were released (84 and 28%, respectively) as methyl esters. The remainder of 3-OH-16:0 was released as its 3-*O*-methylhexadecanoic acid [3-O-Me16:0] ester, which suggested the presence of glucosamine-bound 3-acyloxyacyl residues (34). The de-*O*-acylated LPS contained exclusively 18:0(3-OH), which, thus, is the only amide-bound fatty acid.

Lipid A and degraded oligosaccharide. Acid hydrolysis (1% CH_3COOH, 100°C, 1.5 h) of LPS of NCTC 11637 gave free lipid A and degraded oligosaccharide with yields of 41 and 48% of LPS (dry weight), respectively. The analyses, results of which are shown in Table 2, accounted for 80% of the lipid A and 70% of the oligosaccharide. Free lipid A contained all of the D-glucosamine and fatty acids of LPS, as well as some ethanolamine. Although phosphate was present in a significant amount, no glucosamine-phosphate was detected. The degraded oligosaccharide contained all of the neutral sugars and KDO. In addition, phosphate and ethanolamine were present. The presence of inorganic phosphate (41 nmol) showed that cleavage of some lateral and/or terminal phosphate had occurred during acid hydrolysis.

About 30% of the total hexosamine content of free lipid A reacted in the direct Morgan-Elson assay, whereas LPS did not react. These results indicated that loss of a glycosidically bound substituent from the reducing end sugar of lipid A had occurred during the acidic treatment used to liberate free lipid A from LPS. Since 52% of the total hexosamine content of dephosphorylated LPS reacted in the direct Morgan-Elson assay, it could be deduced that phosphate was the glycosidically bound group. In support of this deduction, after treatment of lipid A with 0.1 M HCl (100°C, 30 min), reactivity in the assay had increased to 48%, and liberated phosphate was detectable in the supernatant from the treatment, but organic phosphate was no longer detectable in the resulting precipitate (degraded lipid A).

TABLE 2. Chemical composition of degraded oligosaccharide and lipid A of *H. pylori* NCTC 11637[a]

Constituent	Amt of constituent (nmol/mg) in given LPS fraction	
	Degraded oligosaccharide	Lipid A
Sugars		
Fucose	5	ND[b]
D-Mannose	63	ND
D-Glucose	495	ND
D-Galactose	563	ND
D-*glycero*-D-*manno*-Heptose	541	ND
L-*glycero*-D-*manno*-Heptose	817	ND
L-*glycero*-D-*manno*-Heptose-phosphate[c]	123	ND
3-Deoxy-D-*manno*-2-octulosonic acid[d]	159	ND
D-Glucosamine	ND	822
Ethanolamine	1,025	6
Fatty acids		
Tetradecanoic	ND	35
Hexadecanoic	ND	58
Octadecanoic	ND	557
(*R*)-3-Hydroxyhexadecanoic	ND	363
(*R*)-3-Hydroxyoctadecanoic	ND	1,068
Phosphate		
Organic	662	269
Inorganic	41	10

[a] LPS was extracted with the hot phenol-water technique after pretreatment of bacteria with pronase. Separation of lipid A and oligosaccharide was achieved by acid hydrolysis (1% CH_3COOH, 100°C, 1.5 h).
[b] ND, Not detected.
[c] The increase in amount of heptose after LPS was dephosphorylated with hydrofluoric acid.
[d] Determined by the TBA assay after acid hydrolysis (1 M HCl, 100°C, 2 h).

DISCUSSION

The relative mobility of individual LPS molecules in SDS-PAGE depends on structural variation and the degree of polymerization of the O side chain (14, 26). All the LPS of *H. pylori* clinical isolates we examined by SDS-PAGE had O side chains which are remarkably homogeneous in length compared with that of enterobacterial LPS. Such LPS profiles have been encountered with other pathogenic bacteria that have proteinaceous S layers (e.g., *Aeromonas hydrophila* and *Aeromonas salmonicida*), with which the LPS has been shown to protrude through the S layer and suggested to act as an anchor for it (7, 37). Thus, the possible presence of an S layer on the surface of *H. pylori* strains should be evaluated in future studies.

A previous electrophoretic analysis of *H. pylori* antigens suggested that fresh clinical isolates produced either R- or S-form LPS, and the S-form LPS had a ladderlike pattern resembling that of enterobacterial LPS (27). In that study, however, isolates had been subjected to a number of in vitro passages, which could result in mutations leading to loss of the O side chain from LPS. Furthermore, heavy loading of gels with LPS can result in the visualization of less-predominant molecules, thus producing a ladderlike pattern. In fact, upon numerous in vitro passages of clinical isolates on a solid medium, we were able to show loss of O side chains.

Nevertheless, the observed differences in S-form LPS profiles between clinical isolates suggests the possibility that a typing system based on LPS could be developed for these bacteria once difficulties in their culturing without loss of O side chains were overcome.

In contrast to the fresh clinical isolates, LPS of all the *H. pylori* culture collection strains showed profiles, by SDS-PAGE, characteristic of R-form LPS, with differences in electrophoretic mobility between LPS reflecting possible structural differences. No loss of O side chains occurred during extraction of LPS since proteinase K-treated whole-cell lysates and hot phenol-water-extracted LPS had the same profiles. In addition, the presence of O side chains which do not stain with silver or which are present in a low concentration, as encountered in *Campylobacter jejuni* (28), was excluded by visualization of LPS in gels by immunoblotting as well as by silver staining. Chemical characterization of the LPS confirmed its low-molecular-weight nature, especially since a low ratio of sugar to fatty acid was observed. We suggest that the R-form LPS of these strains represent lipid A plus core oligosaccharide and have arisen because of spontaneous mutation with loss of O side chains.

The increased yield of R-form LPS obtained by combined pronase treatment and hot phenol-water extraction compared with phenol-water extraction alone reflects a close interaction between the LPS and protein. An increased yield of LPS is beneficial, as *H. pylori* is a fastidious microorganism and large quantities of biomass are difficult to obtain.

R-form LPS is more amenable for structural studies of the core oligosaccharide and lipid A components of LPS (3, 33); e.g., structural analysis of the core oligosaccharide is impeded by structures originating from the O side chain of S-form LPS, and lower yields of free lipid A are obtained from S-form than from R-form LPS. Since chemical characterization is a prerequisite before structural analysis the composition of *H. pylori* R-form LPS was studied in greater detail.

Structural variability within the core oligosaccharide mainly arises from differences in the outer core region, e.g., with members of the family *Enterobacteriaceae* (33). The common hexoses, D-mannose, D-glucose, and D-galactose, which normally occur in the outer core region, were present in *H. pylori* R-form LPS. The occurrence of these neutral sugars in differing ratios in LPS of different strains indicates structural and/or substitution differences in the core and are in agreement with the earlier electrophoretic observations. The presence of low nonstoichiometric amounts of fucose in all the LPS reflects incomplete substitution of the core oligosaccharide by this 6-deoxyhexose. Hydrofluoric acid treatment, as used in the present study for dephosphorylation of LPS (13), also liberates 6-deoxyhexoses (12). When they are chain linked in poly- or oligosaccharides, the lability of 6-deoxyhexoses in response to hydrofluoric acid can be taken advantage of to isolate smaller units from them. For instance, the chemical repeat unit of the O antigen 4,5,12 of *S. enterica* serovar Typhimurium, which contains L-rhamnose, can be isolated by hydrofluoric acid treatment (12). In the case of *H. pylori* LPS, fucose alone was released upon hydrofluoric acid treatment. Thus, it is apparent that fucose occurs in a terminal or lateral location in the LPS.

In the inner core region of all LPS characterized so far, KDO, or a derivative thereof, is present (3, 33). Similarly, *H. pylori* core oligosaccharide contained pyranosidic KDO as the only TBA-reactive material. The increase in the quantity of KDO liberated from LPS after hydrolysis with 1 M HCl compared with that liberated by sodium acetate (pH 4.4) may be attributed to the substitution and location of KDO in the molecule, i.e., whether it is laterally linked or chain linked. Heptose, another constituent common to the inner core region (3, 33), occurred in both the L-*glycero*-D-*manno* and D-*glycero*-D-*manno* configurations, with the former predominating. In the inner core of *H. pylori* LPS, phosphorylation was limited to heptose, namely, L-*glycero*-D-*manno*-heptose-phosphate, as suggested by the increase in this heptose after hydrofluoric acid treatment.

In LPS, exemplified by that of the family *Enterobacteriaceae*, phosphorylethanolamine and pyrophosphorylethanolamine are present in the core oligosaccharide, and ethanolamine, when present in lipid A, occurs as a substituting headgroup on phosphate or pyrophosphate of the lipid A backbone (3, 33). Likewise, ethanolamine and ethanolamine-pyrophosphate, with trace amounts of ethanolamine-phosphate, were present in the LPS, and upon hydrolysis, degraded oligosaccharide as well as free lipid A each contained ethanolamine.

The fatty acid composition of *H. pylori* LPS (lipid A), containing nonstoichiometric amounts of 14:0 and 16:0, but with 18:0, 3-OH-16:0, and 3-OH-18:0 in stoichiometric quantities, was relatively simple. Qualitatively, our results agree with those of a previous study on the composition of *H. pylori* lipids, which also found these fatty acids present in LPS (9). Like all 3-hydroxy fatty acids in LPS which have been characterized to date (31, 33), those in *H. pylori* had the *R* configuration. The predominant fatty acids, particularly the 3-hydroxy fatty acids, are relatively long. Furthermore, 18:0, 3-OH-16:0, and 3-OH-18:0 are not common constituents of LPS, although individually they have been reported present in LPS of diverse bacteria (e.g., *Brucella* spp. and *Thermus* spp. [21]; *Rhodopseudomonas* spp., *Bacteroides* spp., and *Coxiella burnetti* [20, 21, 49, 51]; *Rhodopila globiformis*, *Rhizobium trifolii*, and *Francisella tularensis* [20, 35, 41]). Like *H. pylori*, however, only *Brucella abortus* and *Legionella pneumophila* have collectively 18:0, 3-OH-16:0, and 3-OH-18:0 in their LPS (21, 38). Although our results show the presence of ester- and amide-bound 3-OH-18:0, with ester-linked 14:0, 16:0, 18:0, and 3-OH-16:0, and that 3-acyloxyacyl residues are present, more detailed studies are needed to determine the distribution of fatty acids on the lipid A backbone of *H. pylori*.

The backbone of lipid A, in a number of bacterial families including *Enterobacteriaceae*, is composed of a β(1→6)-linked D-glucosamine disaccharide with a glycosidically bound phosphate attached at position 1 and an ester-bound phosphate attached at position 4′ (11, 21, 33). Lipid A variants that contain 2,3-diamino-2,3-dideoxy-D-glucose or glucosaminuronic acid as their backbone sugars have been described (15, 20, 21, 24, 48). Since compositional analysis showed that D-glucosamine was the only amino sugar present in free lipid A and about 50% of its total was detectable in the direct Morgan-Elson reaction after dephosphorylation, it is likely that a glucosamine disaccharide forms the backbone of *H. pylori* lipid A. In addition, no glucosamine-phosphate was detected when strong acid hydrolysates of LPS and lipid A were tested in the amino acid analyzer, suggesting the absence of 4′-phosphate. Confirming this, treatment of free lipid A with mild acid, to liberate glycosidically bound phosphate, released all the phosphate from lipid A. During biosynthesis of enterobacterial lipid A, tetraacylglucosamine disaccharide-1-phosphate is an intermediate which is converted to tetraacylglucosamine disaccharide 1,4′-diphosphate by a 4′-kinase before attachment of KDO and final acylation (29). Assuming a

similar biosynthetic pathway in *H. pylori* would mean that the lack of 4′-phosphate in its lipid A is the result of a secondary process(es).

Although endotoxic activity is not dependent on one single constituent of lipid A, changes in lipid A structure can result in decreased endotoxic activity (32, 33). A phosphorylation pattern as observed with *H. pylori* lipid A, i.e., 1- but not 4′-phosphate is present, has been reported as one of the unusual structural features in *B. fragilis* lipid A, which has low endotoxic activity (49). As investigations of atypical lipid A's with differing architectures, such as that apparently possessed by *H. pylori*, can yield useful information on structure-bioactivity relationships, the lipid A of *H. pylori* merits further investigation.

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