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Lipid A in *Helicobacter pylori*

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Free lipid A of *Helicobacter pylori* was characterized with regard to chemical composition, reactivity with anti-lipid A antibodies, and activity in a *Limulus* lysate assay. The predominant fatty acids of *H. pylori* lipid A were 3-OH-18:0, 18:0, 3-OH-16:0, 16:0, and 14:0. Hexosamine was present in amounts similar to those in *Campylobacter jejuni* or *Salmonella typhimurium* lipid A. The lipopolysaccharide of *H. pylori* contained 2-keto-3-deoxyoctonic acid, a common constituent of enterobacterial and *C. jejuni* lipopolysaccharides. In the enzyme-linked immunosorbent assay, the doses of lipid A required to inhibit anti-lipid A by 50% (EI_{50} values) by absorption of the immune (rabbit) serum were 7.9, 1.2, and 1.4 μg of O-deacylated lipid A's from *H. pylori*, *C. jejuni*, and *S. typhimurium* per ml, respectively. The lower reactivity of *H. pylori* lipid A compared with those of the other two lipid A preparations (as shown by the higher EI_{50} value) was underscored by the use of a murine monoclonal anti-lipid A antibody in the inhibition assay. An EI_{50} value was not obtained at the concentrations tested for *H. pylori* lipid A; the corresponding figures for *C. jejuni* and *S. typhimurium* lipid A's were 13 and 14 μg/ml, respectively. No inhibition was obtained with *H. pylori* lipopolysaccharide, which showed a low-molecular-weight profile on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The activity of *H. pylori* lipid A in the *Limulus* assay was approximately 71 and 650 times lower than those of *C. jejuni* and *S. typhimurium* lipid A's, respectively. These findings suggest that lipid A is an integral part of the outer cell wall of *H. pylori*. The lower reactivity of *H. pylori* lipid A with anti-lipid A antibodies and in the *Limulus* assay compared with that of *C. jejuni* or *S. typhimurium* lipid A may be explained by a different composition of the fatty acids, especially the 3-hydroxy fatty acids, and a possible deviating phosphorylation pattern.

Lipid A is the moiety of gram-negative bacterial lipopolysaccharide (LPS) that is responsible for the toxic properties of LPS or endotoxin. These toxic properties include pyrogenicity, depression of blood pressure, intravascular coagulation, and shock (6). The polysaccharide of the LPS is linked through 2-keto-3-deoxyoctonic acid (KDO) to lipid A, which is anchored in the cell membrane. Long-chain nonhydroxylated and hydroxylated fatty acids are major components of lipid A. The backbone structure of lipid A is highly conserved, and in nearly all gram-negative bacteria the other main constituents of lipid A are diglucosamine and phosphate (6). Among the bacterial cellular fatty acids, the hydroxy fatty acids occur almost exclusively in the lipid A portion; and among nearly all species of the family *Enterobacteriaceae* and *Campylobacter* spp. the major hydroxy fatty acid is 3-hydroxy-tetradecanoic acid (3-OH-14:0) (24, 32).

Helicobacter pylori is now the correct name (8) for the spiral bacterium first isolated from the gastric mucosa of patients with gastritis and peptic ulcer in Western Australia and originally termed *Campylobacter pyloridis* (18). Goodwin et al. (10) reported that *H. pylori* has 3-hydroxyhexadecanoic acid (3-OH-16:0) but no 3-OH-14:0. Recently, Lambert et al. reported that *H. pylori* uniquely has 3-hydroxyoctadecanoic acid (3-OH-18:0), whereas *Campylobacter jejuni* has 3-OH-14:0 (16, 24).

The lipid A structure of *H. pylori* is not fully known. The hydroxy fatty acid composition of *H. pylori* lipid A is unique and may have consequences for its biological activity (22). This study was undertaken to investigate whether lipid A of *H. pylori* deviated from enterobacterial lipid A with regard to chemical markers, reactivity with anti-lipid A antibodies, and activity in the *Limulus* lysate assay (*Limulus* activity). Free *H. pylori* lipid A was compared with free *C. jejuni* and *Salmonella typhimurium* lipid A's.

MATERIALS AND METHODS

Bacterial strains. The *H. pylori* strain used was NCTC 11637 of the National Collection of Type Cultures, London. *C. jejuni* CCUG 8754 was obtained from the Culture Collection of Gothenburg University. The *H. pylori* strain was cultured on plates of brain heart infusion agar (Oxoid) with 7% horse blood and 1% IsoVitaleX (BBL Microbiology Systems) in a microaerobic atmosphere (9). *C. jejuni* was cultured on nutrient agar (Lab-lemco powder L29, peptone L34; Oxoid) plates under microaerobic conditions at 42°C and harvested after 48 h of incubation. Sufficient material for extraction was more readily obtained from *C. jejuni* than from *H. pylori*; the latter was cultured at 37°C and harvested after 72 h of incubation, washed once, and then freeze-dried. Cultures of *H. pylori* on 250 agar plates yielded only 1.5 g of freeze-dried bacteria. *S. typhimurium* 395 MR 10 (chemotype Rd) (17) and *Escherichia coli* EH 100 (chemotype R2) were cultured on nutrient agar for 24 h at 37°C.

Preparation of LPS and lipid A. For *H. pylori* and *C. jejuni*, the dried bacteria were first extracted with a chloroform-methanol mixture to remove the phospholipids. LPS was extracted by the phenol-water method of Westphal and Jann

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(31). The LPS of the rough mutant *S. typhimurium* 395 R10 was extracted by the phenol-chloroform-petroleum ether method (7). Thus, three LPS preparations were used in this study. Lipid A was prepared by 1% acetic acid hydrolysis of LPS at 100°C for 2 h. Precipitates were washed with distilled water and then lyophilized. The solubility of the *Campylobacter* and *Helicobacter* lipid A's was low; therefore, all lipid A preparations, including that of *S. typhimurium*, were O deacylated with alkali (0.25 M NaOH, 56°C, 1 h) to obtain soluble lipid A (only amide-linked fatty acids present). After alkali treatment, the solutions were neutralized and dialyzed against distilled water. The dialyzed solutions were tested in an inhibition enzyme-linked immunosorbent assay (ELISA) as described below.

Chemical analysis. The protein contents of the preparations were analyzed with a Bio-Rad protein assay (Bio-Rad Laboratories). KDO was determined by the method of Weissbach and Hurwitz (30). Glucosamine was determined by the hexosamine assay (2).

For studying fatty acid composition, the dried lipid A preparations were heated in 4 M methanolic HCl (1 ml) at 100°C overnight, and after water (1 ml) was added, the lipid A was extracted with *n*-hexane (1.5 ml). The organic phase was evaporated and made up to 100 μl with *n*-hexane; 1-μl samples were used for gas chromatography-mass spectrometry (GC-MS) analysis. A VG Trio-1 S GC-MS system (VG Instruments, Manchester, United Kingdom) was used. The GC was a Hewlett-Packard model 5890 GC (Hewlett-Packard, Avondale, Pa.) equipped with a fused-silica capillary column (25 m long, 0.25-mm inner diameter) containing cross-linked OV-1 as the stationary phase. Injections were made with a Hewlett-Packard model 7673 autosampler in the splitless mode; the split valve was opened 1 min after the injection. Helium was used as the carrier gas at an inlet pressure of 7 lb/in^2, and the temperature of the column was programmed from 120 to 260°C/min. Both the injector and the interface (between the GC and the MS) were kept at 260°C. The samples were analyzed in the electron impact mode, at an ion source temperature of 220°C. Ionization was performed at 70 eV.

Hydroxy fatty acids were also analyzed as heptafluorobutyryl methyl ester derivatives by a modified version of the method of Parker et al. (25). To all samples, including reference samples with known amounts of β-OH-14:0, an internal standard of β-OH pentadecanoic acid (3 μg) was added. The samples were hydrolyzed with concentrated HCl (12 M) for 1 h at 100°C. After extraction with hexane (3 ml) and then centrifugation, the hexane phase was evaporated. Fatty acids were methylated with borontrifluoride in methanol (14%) by the procedure of Dees and Moss (4), and water (0.5 ml) was added to the samples. The fatty acid methyl esters were extracted with hexane. After evaporation of the organic solvent phase, 0.5 ml of isooctane and 10 μl of heptafluorobutyric anhydride were added to the samples. The reaction mixture was heated to 85°C for 15 min. One milliliter of phosphate buffer (pH 6, 1 M) was added, and the mixture was shaken slowly for 30 min. After centrifugation, the organic solvent phase was taken to dryness. The dried material was dissolved in isooctane (0.2 ml), and 2 μl was injected into a Sigma 3B GC (Perkin Elmer Corp., Norwalk, Conn.) equipped with a ^{631}Ni (10 mCi) electron capture detector and an SGE (Victoria, Australia) split injector. A fused silica column (0.22 mm by 25 m) coated with nonpolar phase (BP-I, SGE) was used. A linear gradient of 10°C/min from 180 to 250°C was used for running the injected sample. The amount of β-OH-14:0 in a sample was determined by the ratio of β-OH-14:0 to the internal standard β-OH-15:0 and compared with known ratios.

SDS-PAGE. LPS preparations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (15) on a Miniprotean II slab cell (Bio-Rad Laboratories, Richmond, Calif.). The stacking gel contained 4% acrylamide, and the separating gel contained 12% acrylamide. Electrophoresis was performed with a constant voltage of 200 V. After the electrophoresis, the gel was fixed and stained with a silver stain (Bio-Rad).

Chromogenic *Limulus* lysate assay. The lipid A preparations were analyzed by a quantitative chromogenic *Limulus* lysate assay (Coatest endotoxin; Chromogenix, Mölndal, Sweden). *E. coli* O111:B4 LPS (1 ng, corresponding to 12 endotoxin units of Food and Drug Administration standard EC-5/USP lot F) was used as a standard. Samples of 50 μl were diluted (10 pg/ml to 10 ng/ml) and added to microtiter plates. Five standard points (12.5 to 100 pg/ml) and a blank were also included. *Limulus* lysate (50 μl) was added to the wells and incubated for 12 min at 37°C in a microtiter plate incubator. After 100 μl of the chromogenic substrate pentapeptid-*p*-nitroaniline (2 mmol/liter) was added, the plate was incubated for another 3 min at 37°C. The reaction was stopped by adding 20% acetic acid (100 μl). The plate was read in a spectrophotometer, and the absorbance of the samples was read against a standard curve.

Production of anti-lipid A rabbit antiserum and mouse monoclonal antibody to lipid A. Rabbits were immunized with *E. coli* EH 100 lipid A coated onto erythrocytes, and antiserum was obtained as described previously (21). The monoclonal antibodies were produced by the method of De St. Groth and Scheidegger (5). BALB/c mice were immunized with heat-killed freeze-dried *S. typhimurium* 395 MR10. Four immunization doses (0.25 mg in the first one and 0.5 mg thereafter) were given to the mice over a period of 6 months; the last dose was given 1 week before the fusion of spleen cells with SP2-0 myeloma cells. The first three injections were given subcutaneously, and the last dose was given intraperitoneally. Freund's complete adjuvant was included in the first dose. The two following doses contained Freund's incomplete adjuvant in addition to the immunogen. The last booster dose contained only the bacterial cells diluted in phosphate-buffered saline. Hybridomas were screened for antibody against lipid A of *S. typhimurium* 395 MR10 by an ELISA (21) in microtiter plates. For coating of the microtiter plate, lipid A diluted in sterile phosphate-buffered saline (pH 7.2) (5 μg/ml) was incubated overnight at room temperature. Specific-antibody-producing cells were cloned twice. To obtain large quantities of the monoclonal antibodies, the hybridomas were cultured in dialysis tubing as described by Sjögren-Jansson and Jeansson (28). One hybridoma (no. 895) produced antibodies of the immunoglobulin G3 subclass that were reactive against lipid A of *S. typhimurium* 395 MR10, *Salmonella minnesota* R595, *E. coli* O111, and *E. coli* EH100. It also showed antibody activity against the LPS of *S. typhimurium* MR10 (chemotype Rd). The antibody activity against the Rd LPS could be completely inhibited by absorption with lipid A of *S. typhimurium* 395 MR10 or *S. minnesota* R595. No antibody activity was recorded against LPS from any of the following bacterial strains: *S. typhimurium* 395 MS, *Salmonella enteritidis*, *Salmonella abortus-equi*, *S. minnesota*, *Salmonella typhi*, *Haemophilus influenzae*, *Pseudomonas pseudoalcaligenes*, *Agrobacterim tumefaciens*, *E. coli* EH100 (chemotype Ra), or *E. coli* O4.

Inhibition ELISA. Rabbit immune serum or mouse mono-

TABLE 1. Comparison of LPSs and lipid A's from *H. pylori* with those from *C. jejuni* and *S. typhimurium*

Source of LPS	% (wt/wt) in LPS			% (wt/wt) in Lipid A			
	Yield from bacteria[a]	KDO	Protein	Yield from LPS	Hexosamine	3-OH-14:0	Protein
H. pylori	1.8	1.6	1.0	35.4	4.9	0	1.8
C. jejuni	7.0	0.6	5.2	40.1	7.1	30.2	3.5
S. typhimurium		4.0	2.0	56.0	8.3	29.2	2.5

[a] Chloroform-methanol-extracted bacteria.

clonal antibody was diluted to an antibody activity in the ELISA corresponding to an A_{405} of approximately 1. Lipid A from *S. typhimurium* 395 MR10 was used as the solid-phase antigen. Various amounts of lipid A or LPS were added to the diluted antibody solution and incubated for 30 min at 37°C and then overnight at 4°C. The remaining anti-lipid A activity was compared with that of the unabsorbed antibody solution in the ELISA. The rabbit antiserum was used at a dilution of 1:20,000, and the monoclonal antibody was diluted 1:200. The method was identical to that used previously in this laboratory (3). The quantity of LPS or lipid A required to produce 50% inhibition of the lipid A-anti-lipid A reaction in ELISA by absorption of the anti-lipid A antibodies before the analysis (EI_{50}) was taken as the endpoint.

RESULTS

Yields and chemical markers of LPS and lipid A. The percentage of LPS and lipid A obtained from dried *H. pylori* bacterial cells was almost four times lower than that obtained from *C. jejuni* (Table 1). The content of KDO was higher in *H. pylori* than in *C. jejuni* but less than half the amount found in *S. typhimurium*. Similar amounts of lipid A were obtained from LPS of *H. pylori* and *C. jejuni*. Analysis of the *H. pylori* LPS by SDS-PAGE revealed one low-molecular-mass band on the gel that was similar in electrophoretic mobility to the band of the *S. typhimurium* LPS (<14.4 kDa). *C. jejuni* LPS showed four bands (<25 kDa).

3-OH-14:0, which is a chemical marker of enterobacterial and *C. jejuni* lipid A (Table 1), was found by GC-MS also in *H. pylori* lipid A (Table 2). Almost identical amounts were seen in the other two lipid A preparations (Table 1). Contaminating protein, ranging from 1 to 5%, was seen in all preparations.

The nonhydroxylated fatty acids found in *H. pylori* lipid A were 18:0, 14:0, 16:0, and 12:0, the corresponding 3-hydroxylated acids were 3-OH-18:0, 3-OH-16:0, and 3-OH-14:0 (Table 2).

Reactivity of deacylated lipid A preparations and LPS with anti-lipid A antibodies. To overcome the poor solubility of *H. pylori* and *C. jejuni* lipid A's, all preparations were deacylated (indicated as lipid A_{ac}). The EI_{50}s of lipid A_{ac} and LPS are shown in Fig. 1. With the rabbit antiserum, the EI_{50} of *H. pylori* lipid A_{ac} was 7.9 μg/ml. The corresponding EI_{50}s of *C. jejuni* and *S. typhimurium* lipid A_{ac} were approximately 6 times lower (1.2 and 1.4 μg/ml, respectively). None of the three LPS preparations inhibited the antibody activity to 50% at concentrations up to 200 μg/ml.

Since a monoclonal antibody has only one specificity, a small structural difference in lipid A of *H. pylori* compared with the other lipid A's may be more pronounced by such an anti-lipid A system. Indeed, the difference between *H. pylori* lipid A_{ac} and the other two was underscored by using the monoclonal antibody. Not even at 500 μg/ml was a 50% inhibition obtained with *H. pylori* lipid A_{ac}, whereas *C. jejuni* and *S. typhimurium* lipid A_{ac}s had very similar EI_{50}s (13 and 14 μg/ml, respectively). In this system, the LPS of *S. typhimurium* showed the same inhibition capacity as its corresponding lipid A preparation (EI_{50}, 11 μg/ml). *H. pylori* and *C. jejuni* LPS showed no inhibitory capacity.

***Limulus* activity of lipid A.** The *Limulus* activity of the *H. pylori* lipid A was significantly lower than those of *C. jejuni* and *S. typhimurium* lipid A's, differing by factors of 71 and 650, respectively (Table 3). This difference increased by a factor of 10 between *H. pylori* and *C. jejuni*, when lipid A_{ac}s were compared.

TABLE 2. Relative amounts of fatty acids in lipid A's of *H. pylori* and *C. jejuni* measured by GC-MS (averages of three injections)

Source of lipid A	% of fatty acids				% of 3-OH fatty acids		
	12:0	14:0	16:0	18:0	14:0	16:0	18:0
H. pylori	2.2	18.0	13.4	66.4	0.9	31.6	67.5
C. jejuni		10.0	71.2	18.8	100		

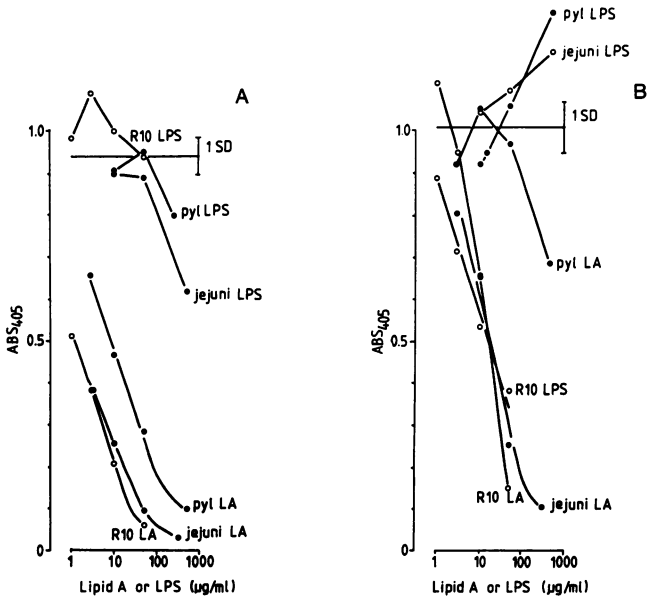

FIG. 1. Inhibition of the lipid A–anti-lipid A reaction in the ELISA by absorption with various amounts of lipid A_{ac} (LA) or LPS from *H. pylori* (pyl), *C. jejuni* (jejuni), or *S. typhimurium* (R10). (A) Rabbit anti-lipid A serum; (B) mouse monoclonal antibody.

TABLE 3. *Limulus* activities of lipid A's and lipid A_{ac}s

Source of lipid A	*Limulus* activity (EU/mg)	
	Lipid A	Lipid A_{ac}
H. pylori	3.4×10^4	4.0×10^3
C. jejuni	2.4×10^6	2.8×10^6
S. typhimurium	2.2×10^7	ND[a]

[a] ND, not determined.

DISCUSSION

A major problem with *H. pylori* is the slow and scanty growth of the organism; our yield of LPS from strain NCTC 11637 was only 1.8%, compared with 7.0% from *C. jejuni*. We have shown that LPS is an integral part of the cell wall of *H. pylori* containing KDO, which constitutes the link between the lipid A part and the polysaccharide chain in most LPSs. The low yield of LPS from *H. pylori* may be due to the presence of fatty acids with long carbon chains as well as a short-chain LPS that is less effectively extracted by the phenol-water method. Our short-chain LPS profile of the *H. pylori* strain on SDS-PAGE is commonly found among *H. pylori* strains, especially culture collection strains (22, 26). Longer LPS chains are found more frequently among clinical isolates (22).

Lipid A was readily cleaved from the LPS of *H. pylori* by hydrolysis. We have confirmed that the lipid A of *H. pylori* contains the hydroxy fatty acids 3-OH-18:0 and 3-OH-16:0 (22); in addition, small amounts of 3-OH-14:0 were found. Analysis in whole organisms of the cellular fatty acids has shown that 3-OH-16:0 is not found in enterobacteria, but this acid has been found in *Campylobacter fetus* subsp. *fetus*, "*C. cinaedi*," and "*C. fennelliae*" (9, 16), whereas 3-OH-18:0 was found only in *H. pylori* (9). The main nonhydroxy-fatty acid in *H. pylori* lipid A was 18:0; this is also a very rare feature for gram-negative bacteria (6).

The GC and GC-MS analyses of *H. pylori* lipid A gave chromatograms showing free aldehydes of 14- and 16-carbon atoms; these aldehydes were identified by GC-MS. Corresponding chromatograms of *C. jejuni* and *S. typhimurium* lipid A showed an aldehyde of 12 carbon atoms. It was recently shown that aldehydes can be readily formed in the sample introduction part of the gas chromatograph upon injection of methyl esters of 3-hydroxylated fatty acids with an underivatized hydroxyl group (12). The risk of aldehyde formation through degradation of 3-hydroxylated fatty acids should be taken into account, particularly if GC-MS is not available. It is likely that derivatization of the hydroxyl group, e.g., by silylation, could be used to avoid this problem.

We compared the abilities of *H. pylori* LPS and lipid A with those of *C. jejuni* and *S. typhimurium* LPSs and lipid A's to inhibit the lipid A–anti-lipid A reaction as measured by ELISA. With this technique we reported previously that the lipid A from *Bacteroides fragilis* and other *Bacteroides* species deviated from enterobacterial lipid A (3). Although a microheterogeneity is found in all lipid A's because of variations in the composition of the fatty acids and in phosphate groups (1, 20), the major fraction of *B. fragilis* lipid A was shown to contain 13-*methyl*-14:0, 3-OH-15:0, 3-OH-16:0, 15-*methyl*-(3-OH)-16:0, and 3-OH-17:0 fatty acids (29). In addition, the major species of *B. fragilis* lipid A contained monophosphorylated diglucosamine and not the diphosphorylated diglucosamine found in many enterobacterial lipid A's. With both rabbit antiserum and monoclonal antibody, we found that the lipid A of *H. pylori* was less efficient than the lipid A of *C. jejuni* or *S. typhimurium* at absorbing anti-lipid A antibody. We conclude from the inhibition studies that the lipid A of *H. pylori* is partly different in its immunoreactivity from the lipid A of *C. jejuni* or *S. typhimurium*. This difference in immunoreactivity could be explained by the presence of other fatty acids, such as 3-OH-16:0 and 3-OH-18:0, in *H. pylori* lipid A. Other structural differences, however, such as in the number of phosphate groups, are probably also involved (22). The absence of one phosphate group (at position 1) in the disaccharide backbone of *H. pylori* lipid A, as suggested by Moran et al., is supported by our results on the reactivity with anti-lipid A antibodies (22). The phosphorylation pattern of the lipid A molecule has been shown to play a decisive role in the immunoreactivity of certain lipid A antibodies (27). The partial similarity in immunoreactivity and the presence of similar amounts of hexosamine in the lipid A's of *H. pylori*, *C. jejuni*, and *S. typhimurium* would suggest that the diglucosamine backbone in *H. pylori* lipid A is similar to that in enterobacterial lipid A. Recently D-glucosamine was shown to be present in *H. pylori* lipid A (22). Rabbit polyclonal antibodies have been used to find at least five different epitopes on the lipid A molecule (27). A monoclonal antibody is specific for one epitope and may explain the differences in the reactivities of the rabbit antibodies and the mouse monoclonal antibody to the different lipid A preparations.

S. typhimurium LPS, but not the other two LPSs, inhibited the monoclonal antibody activity in the ELISA. This result indicated that *C. jejuni* LPS had a polysaccharide chain that was somewhat longer than that of the Rd chemotype LPS, which contained only heptose and KDO in addition to the lipid A part. A complete core oligosaccharide like *E. coli* EH100 LPS has been shown to be long enough to mask the lipid A antigen in the LPS molecule (19). The lack of reactivity of the *H. pylori* LPS is related to the differences in the lipid A or to an LPS chain longer than the Rd chemotype or to both. Both *H. pylori* and *C. jejuni* LPSs showed low-molecular-weight profiles in the gel analysis.

The *Limulus* activity of *H. pylori* lipid A was approximately 70 and 700 times lower than those of *C. jejuni* and *S. typhimurium* lipid A's, respectively. After deacylation, the difference in activity even increased. The use of synthetic lipid A analogs has shown that the composition of fatty acids is important for the *Limulus* activity (13). Also the phosphate groups have been shown to influence the *Limulus* activity, since lipid A molecules lacking both or one of the two phosphate groups was less active than the biphosphorylated lipid A (11, 14). A difference similar to that between the *Limulus* activities of *H. pylori* and enterobacterial lipid A's has been observed also for the LPS preparations with regard to pyrogenicity and mitogenicity (23).

Further studies are needed to evaluate whether the difference in fatty acids affects the reactivity with anti-lipid A antibodies and *Limulus* activity directly or indirectly, e.g., by changing conformation of the molecule or changing solubility. Since it has been suggested that just one phosphate group is present in *H. pylori* lipid A, most probably a deviating phosphorylation of the molecule also contributed to the difference between it and *C. jejuni* or *S. typhimurium* lipid A.

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