Helicobacter mustelae lipid A structure differs from that of *Helicobacter pylori*

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Abstract The lipid A structure of the Gram-negative bacterium *Helicobacter mustelae*, a ferret gastric pathogen responsible for the onset of gastric diseases in its host, was investigated. Two variant lipid A structures were found in the same strain. One structure contained a bisphosphorylated β-(1→6)-linked D-glucosamine backbone disaccharide with hydroxytetradecanoic acid in amide linkages. Unlike the structure described for the lipid A of the related human *Helicobacter pylori* gastric pathogen, which contains a C1 phosphate moiety, this lipid A presented phosphate groups at both the C1 and C4′ positions, and contained no octadecanoyl fatty acid, which is present in *H. pylori*. The second lipid A structure had a different fatty acid composition in that 3-OH C_{16} replaced most of the amide-linked 3-OH C_{14}.

Key words: Endotoxin; Lipid A; Structure; *Helicobacter mustelae*

1. Introduction

Endotoxins are powerful immunomodulators and the major components of the outer membrane of Gram-negative bacteria. These molecules are heterogeneous mixtures of lipopolysaccharides (LPS) (O-chain→core→lipid A) from which both the lipid and saccharide domains can be heterogeneous. Some endotoxins are not toxic, but among those that are, the lipid A region has been recognized to be the principal toxic component. Lipid A structures from the ubiquitous human gastric pathogen, *Helicobacter pylori* (Fig. 1a), have been described as having some unusual features, such as hypoacylation with relatively long chain fatty acids, and absence of the ester-linked phosphate group at C4′ [1,2].

Helicobacter mustelae colonizes the gastric mucosa of ferrets and, like *H. pylori* in humans, induces gastritis-like symptoms in its host. *H. mustelae* has become an important research pathogen and is often used as a model of *Helicobacter* infections [3]. The outer core region of the LPS isolated from this bacterium was shown to express the monofucosyl A type 1 histo-blood group epitope, αD-Gal*p*NAc-(1→3)-[αL-Fuc*p*-(1→2)]-βD-Gal*p*-(1→3)-βD-Glc*p*NAc [4], in mimicry of ferret gastric cell surface glycoforms [5].

Structural analysis of the lipid A moiety of *H. mustelae* represents an application of the plasma desorption mass spectrometry (PDMS) method described in a recent article [6] completed by data from nuclear magnetic resonance (NMR), gas chromatography (GC)/mass spectrometry (MS), and MS by matrix-assisted laser desorption ionization (MALDI), to the hitherto undescribed lipid A of the LPSs of *H. mustelae* type strain ATCC 43772. Two different lipid A structures were produced from cells obtained in culture preparations that were ostensibly identical.

2. Materials and methods

2.1. MS

PDM spectra were obtained using a Depil time of flight (TOF) 21 mass spectrometer (IPN, Orsay, France) [7]. Flight distance was 90 cm, acceleration voltage was + or −15 kV, and time bins, 1 or 2 ns/channel with a multistop time digital converter. Counting time varied between 15 min and 4 h. Lipid A samples (40 μg) were dissolved in 20 μl of chloroform:methanol:water (16:8:1 by volume), desalted with a few grains of Dowex 50 $(Me_2NH)^+$, and 5 μl were deposited on an aluminized mylar disk previously electrosprayed with nitrocellulose [8]. *m/z* values were determined by centroid analysis.

MALDI spectra were acquired in the negative mode using a TOF Voyager STR (Perseptive, Applied-Biosysteme, France) equipped with a 337-nm nitrogen laser and set at 20 kV extraction voltage. Spectra were obtained with an average of 64 shots. 2,5-Dihydroxybenzoic acid was used as a matrix in water (10 mg/ml). Lipid As were dissolved in a mixture of chloroform/methanol (2:1, v/v) and deposited on the target followed by the same volume of the matrix, and the mixtures were allowed to dry at room temperature. The masses were average masses.

2.2. Bacterial strain and growth conditions

Four batch growths of *H. mustelae* ATCC 43772 cells were analyzed. Batches one and two were grown in solid medium [4], and batches three and four in liquid medium. Broth-grown cells were inoculated from plates of brain heart infusion (BHI) agar (1.2% w/v) supplemented with 0.5% (w/v) yeast extract and 0.5% (v/v) fetal bovine serum. These *H. mustelae* strains were allowed to grow for 3 days under micro-aerobic conditions at 37°C and subcultured into BHI broth. Cells were isolated by centrifugation. For plate-grown cells, bacteria were grown for 48 h at 37°C in antibiotic supplemented trypticase soy agar containing 5% horse blood in a micro-aerophilic environment. Cells were scraped off plates and washed with phosphate buffered saline.

2.3. LPS

The cells were killed in 2% phenol before harvesting. The LPS were extracted by the modified phenol–water method [9], and were obtained as precipitated gels by ultracentrifugation (105 000×*g*, 4°C,

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Abbreviations: BHI, brain heart infusion; GC, gas chromatography; GlcN, glucosamine; HMQC, heteronuclear multiple quantum correlation; LPS, lipopolysaccharide; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; NMR, nuclear magnetic resonance; PDMS, plasma desorption mass spectrometry; TLC, thin layer chromatography; TOF, time of flight

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12 h). The preparations were further purified by extraction of phospholipids, repeated treatment with proteases and nucleases, and centrifugation, until thin layer chromatography (TLC) and UV spectra showed no detectable contaminants [10]. Lipid A was prepared by hydrolyzing LPS in 1% acetic acid at 100°C for 1 h, centrifugation, and extraction of the pellet with chloroform:methanol:water 12:6:1 [11]. Alternatively, lipid A was prepared by hydrolyzing LPS in 20 mM Na acetate–acetic acid pH 4.5–1% Na dodecyl sulfate at 100°C for 1 h, lyophilization, removal of the detergent by extraction with acidified ethanol, centrifugation, and extraction of the pellet with chloroform:methanol:water 12:6:1 [11].

2.4. TLC

TLC was done on aluminum-backed silica gel plates (Merck) in the solvent mixture chloroform:methanol:water:triethylamine (12:6:1:0.04) [11]. Spots were visualized by charring after spraying with 5% sulfuric acid in ethanol.

2.5. Identification of glycose absolute configurations

Lipid A (1 mg) was hydrolyzed with 1 ml of 4 M HCl at 100°C for 2 h. After cooling and extraction of fatty acids with chloroform, the mixture was concentrated to dryness under reduced pressure, water was added and repeatedly evaporated from the residue until it became neutral. After *N*-acetylation the residue was treated with trifluoroacetic acid–*R*-(−)-2-butanol [12], peracetylated, and analyzed by GC with an HP-5 (Hewlett Packard, 30 m×0.32 mm) GC column at 200°C and 0.6 kPa.

2.6. Chemical analyses

Hexosamines were assayed as described by Rondle et al. [13] and phosphate as described by Chen et al. [14]. Fatty acids of the LPS or lipid As were analyzed after hydrolysis with 4 M HCl for 2 h at 100°C, neutralization followed by treatment with 0.5 M NaOH for 1 h at 100° [15], extraction with ethyl acetate, methylation of the extract by diazomethane, and identification by GC retention time on an HP-5 column using a temperature gradient of 3°C per min from 130°C to 160°C, then 4°C per min to 240°C. GC/MS analyses were performed as previously described [16] using an internal standard of eicosanoic acid for quantitation. Synthetic acyloxyacyl fatty acids provided by D. Charon (CNRS, Châtenay, France) were used as reference.

2.7. NMR analysis of intact lipid A

The lipid A samples were dissolved in a mixture of deuterated chloroform and methanol (2:1). ^{1}H NMR experiments were performed on a Varian 500 spectrometer at 300 K employing standard Bruker software. Acetone was used as the internal reference at δ_H 2.225 and chemical shifts for ^{31}P NMR were measured with reference to external orthophosphoric acid (δ_P 0.0). The mixing times used in the ^{1}H–^{31}P heteronuclear multiple quantum correlation (HMQC) experiments were optimized for the anomeric (60 ms) and aglyconic (40 ms) phosphate.

2.8. Preparation of O-deacylated lipid A

Mild alkali treatment of lipid As (0.25 M NaOH at 37°C for 15 min) was used as previously described [17] to remove selectively the ester-linked fatty acids at the C3 and C3′ positions. Hydrazine treatment was employed to liberate all the ester-linked fatty acids. 2.7 mg of lipid A was treated, as previously described [18], with 0.5 ml of anhydrous hydrazine at 37°C for 1 h. The reaction mixture was cooled to 0°C, and the hydrazine destroyed by addition of 1.5 ml of cold acetone (−20°C). The *O*-deacylated product (1 mg) was recovered by centrifugation and washed with 1 ml of cold acetone. The product was monitored by TLC and PDMS.

2.9. Substitution groups on lipid A

PDM spectra in the negative ion mode were examined for evidence of groups other than glucosamine (GlcN), phosphate, and fatty acids. Ninhydrin on TLC plates was used for the detection of free amino groups.

3. Results and discussion

Two different lipid A structures were obtained from four different batch growths of *H. mustelae* (ATCC 43772) cells. They were designated lipid A I and lipid A II. The first and fourth batches in order of analysis, obtained from solid and liquid growth, furnished the same structure (lipid A I, Fig. 1b). The second and third batches, also produced under the same growth conditions, gave the other structure (lipid A II, Fig. 1c). Temperature growth conditions, which are known to affect fatty acid composition [19], were, in our work, conscientiously maintained.

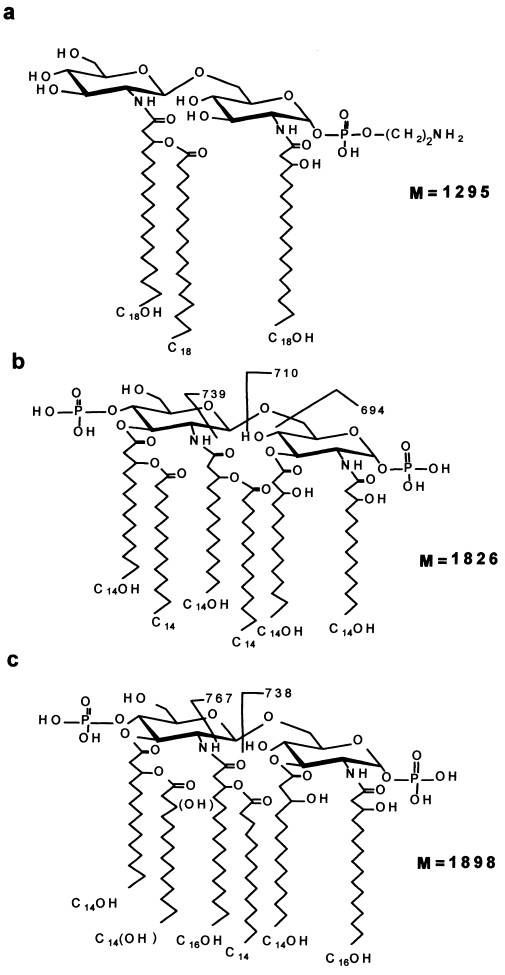

Fig. 1. Major molecular structures of: (a) *H. pylori* lipid A [1,2]; (b) *H. mustelae* lipid A I; (c) *H. mustelae* lipid A II.

3.1. Chemical composition of lipid As

The lipid A was shown to contain GlcN and phosphorus in a 1.17:1 ratio. The GlcN units were found to have the D configuration and no pyrophosphate or additional sugars were detected. PDM spectra contained no signals indicating the presence of phosphorylethanolamine, nor was there any evidence for other substituents, except for fatty acids.

3.2. Determination of the total fatty acid composition of lipid A I

GC/MS analyses revealed that tetradecanoic acid (C_{14}) and 3-hydroxytetradecanoic acid (3-OH C_{14}) occurred in the proportions 1:1.7 corresponding to two units of C_{14} and four units of 3-OH C_{14}, for a major molecular species (see below).

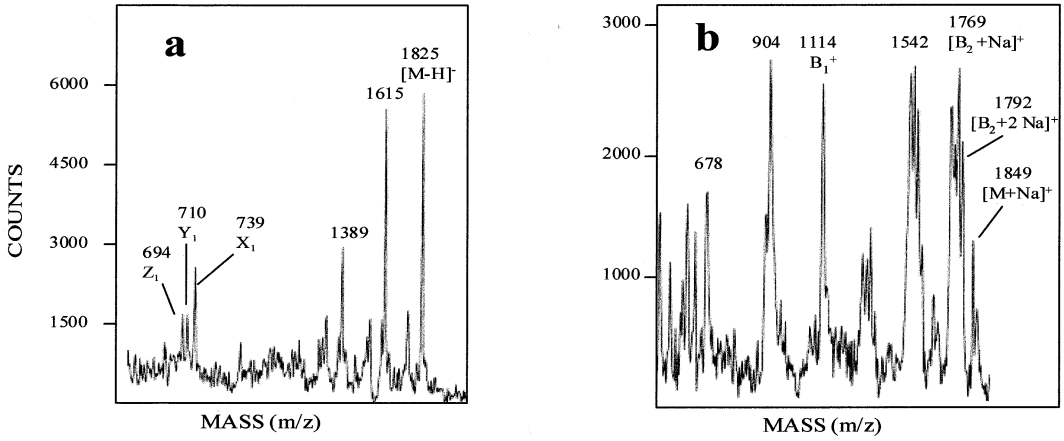

Fig. 2. *H. mustelae* lipid A I. a: Negative ion PDM spectrum. b: Positive ion PDM spectrum.

3.3. Distribution of the fatty acids on the D-GlcN residues in lipid A I

3.3.1. Negative ion mode PDM spectrum of lipid A I. The spectrum displayed in Fig. 2a had two main molecular ion signals at *m/z* 1615 and *m/z* 1825. The composition of the corresponding molecular species was attributed on the basis of the determined overall chemical composition. The molecular ion signal at *m/z* 1825 corresponded to a bisphosphorylated di-GlcN substituted with four 3-OH C_{14} and two C_{14}. The signal at *m/z* 1615 differed by the mass of a C_{14} (210 U) and is due to natural heterogeneity since no such fragmentation was observed in the PDMS process [6].

Less intense signals at *m/z* 739 (X_1), *m/z* 710 (Y_1) and *m/z* 694 (Z_1) were observed and attributed to the GlcN I moiety [6]. They are the peaks similar to those observed in the spectrum of *Escherichia coli* lipid A [6], suggesting the identity of that part of the molecule: one GlcN, one phosphate, and two 3-OH C_{14}, thus pointing to the linkage of one phosphate, two 3-OH C_{14} and two C_{14} to GlcN II. *m/z* 710 and *m/z* 694 corresponded to GlcN I with and without the aglycone oxygen atom that joins it to GlcN II. The peak at *m/z* 739 was generated by the double cleavage of C5′–O and C1′/C2′ (GlcN I and OHCO: 694+45 U).

3.3.2. Positive ion mode PDM spectrum of lipid A I (Fig. 2b). Following the fragmentation pattern previously described in [6], B_1 corresponded to the GlcN II (non-reducing) end of the lipid A molecules that arises by glycosidic cleavage and B_2 to the disaccharide fragment without the glycosidic phosphate. The spectrum shown in Fig. 2b yielded a signal at *m/z* 1849 corresponding to the intact lipid A quasimolecular ion as a Na^+ adduct (1826+23 U). The large signal at *m/z* 1769 $(B_2+Na)^+$ showed that the glycosidic phosphate on GlcN I was lost. *m/z* 1792 corresponded to $(B_2+2Na)^+$. The signal at *m/z* 1542 differed from *m/z* 1769 by the mass of a hydroxytetradecanoic acid (226.36 U). Three peaks were observed in the lower mass range at *m/z* 678, *m/z* 904, and *m/z* 1114 (B_1). The peak at *m/z* 1114 was interpreted to correspond to an ion containing one GlcN, one phosphate, two hydroxytetradecanoic acids, and two tetradecanoic acids (anhydro masses = 161, 80, 226.36, and 210.36, respectively). The peak at *m/z* 904 would correspond to *m/z* 1114 minus one tetradecanoic acid unit and the ion at *m/z* 678 to *m/z* 1114 minus one unit of each fatty acid. With position C6′ being unsubstituted, as shown by NMR, and position C4′ being substituted by a phosphate group, both C2′ and C3′ would carry a $C_{14}OC_{14}$.

3.3.3. Linkage of fatty acids in H. mustelae lipid A I. Mild alkali treatment of the lipid As, which removes esterified substituents at the C3 and C3′ positions, released one equivalent of tetradecanoic acid and two equivalents of hydroxytetradecanoic acid as measured by GC analysis, consistent with the MS data. Esterified $C_{14}OC_{14}$ was isolated under mild conditions and compared to a synthetic sample by GC/MS. Since it was shown that GlcN I has no C_{14}, the $C_{14}OC_{14}$ must esterify the C3′ position. Hydrazinolysis of this material gave a negative ion signal at *m/z* 952 (data not shown) corresponding to two GlcN, two phosphates, and two hydroxytetradecanoic acids. Thus the latter fatty acids must substitute the amino groups of the disaccharide backbone since they can be liberated and characterized only after strong acid treatment.

3.3.4. Determination of the total fatty acid composition of lipid A II. GC/MS analyses revealed that tetradecanoic acid (C_{14}), 3-hydroxytetradecanoic acid (3-OH C_{14}) and 3-hydroxyhexadecanoic acid (3-OH C_{16}) occurred in roughly the proportions 1:2.5:2 corresponding to one unit of C_{14}, three units of 3-OH C_{14}, and two units of 3-OH C_{16}, for a major molecular species (see below).

3.4. Distribution of the fatty acids on the D-GlcN residues in lipid A II

The negative ion PDM spectrum of the lipid A II (Fig. 3a) had five main molecular ion signals at *m/z* 1445, *m/z* 1655, *m/z* 1825, *m/z* 1881, and *m/z* 1897. The composition of the corresponding molecular species was attributed on the basis of the determined overall chemical composition. The signal corresponding to the hexaacylated molecular ion signal at *m/z* 1825 was also found in lipid A I and corresponded to four 3-OH C_{14} and two C_{14} linked to the bisphosphorylated disaccharide. The signal at *m/z* 1881 corresponded to a similar molecular ion species with two 3-OH C_{16} in amide linkage instead of the two 3-OH C_{14}. The signal at *m/z* 1897 would correspond to a molecular species with a 3-OH C_{14} replacing a C_{14}. The fatty acids in the amide linkage was inferred after liberation of all ester-linked fatty acids by hydrazine treatment and PDMS analysis of the residual material.

The signals at *m/z* 1671 and *m/z* 1655 differed from *m/z* 1897 and *m/z* 1881 by the mass of 3-OH C_{14} (226.36 U).

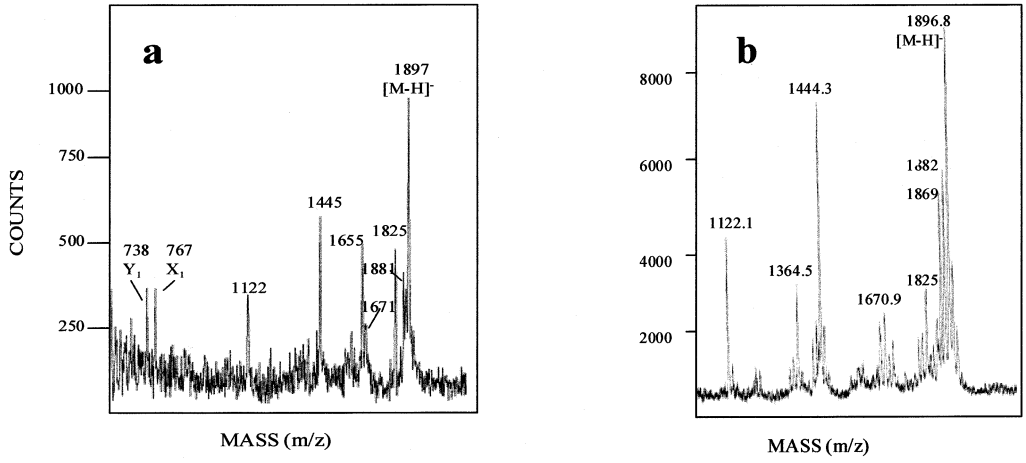

Fig. 3. *H. mustelae* lipid A II. a: Negative ion PDM spectrum. b: Negative ion MALDI-TOF spectrum.

The molecular ion species at *m/z* 1445 differed by the mass of a C_{14} unit (210 U) from that at *m/z* 1655. These ions are all due to natural heterogeneity as such fragmentations were shown not to occur in the PDMS process [6].

Smaller ions appearing 28 U higher than in lipid A I, at *m/z* 767 (X_1) and *m/z* 738 (Y_1), arising from fragmentation, were attributed to the GlcN I moiety, and were consistent with the presence of a 3-OH C_{16} replacing 3-OH C_{14} at C2 in lipid A I. They corresponded to one GlcN, one phosphate, one 3-OH C_{16} and one 3-OH C_{14}.

3.5. Linkage of fatty acids in H. mustelae lipid A II

Mild alkali treatment of lipid A II, releasing ester-linked fatty acids at C3 and C3′, liberated 2.5 equivalents of hydroxytetradecanoic acid and 0.5 equivalent of tetradecanoic acid as measured by GC analysis and confirmed by the mass spectral data. The partially *O*-deacylated lipid (data not shown) furnished a signal at *m/z* 1218 showing that the GlcN II moiety carried the mass of $C_{16}OC_{14}$ in amide linkage. From these observations we also concluded that the heterogeneity observed in the native lipid and lost during alkaline treatment involved variability in secondary acylation at the C3′ position. Hydrazinolysis of this material gave a negative ion signal at *m/z* 1008 corresponding to two GlcN, two phosphates, and two hydroxyhexadecanoic acids (data not shown).

3.6. MALDI-TOF analysis

All samples were tested with both PDMS and MALDI-TOF methods. The MALDI gave better resolution and, as expected, was more sensitive. Only 0.5 μg of lipid A was used in each spectrum compared to 10 μg with PDMS. However, only the PDMS method gave in one spectrum direct structural information on each of the GlcN moieties as well as proportionality between peak intensities and the relative abundances of the corresponding molecular species [17]. The MALDI spectrum presented in Fig. 3b corresponded to the lipid A isolated from bacteria grown in liquid culture. It was found to be similar for molecular ion species to the PDM spectrum obtained for lipid A II and prepared on solid medium as shown in Fig. 3a.

3.7. NMR analysis

The ^1H NMR spectra of the lipid A molecules examined showed one β anomeric resonance at δ 4.51 ($J_{1,2}$, 7.1 Hz) and another resonance in the typical α anomeric chemical shift region, as a quartet, at δ 5.26. The ^{31}P NMR spectrum afforded one broad resonance at δ 1.357, characteristic of monoester phosphates (at pD = 7, pD = 5). No signals characteristic of diester phosphate substituents were observed. Two-dimensional ^1H–^{31}P HMQC experiments showed two resonances at δ_H 5.26/δ_P 1.35 and at δ_H 3.85/δ_P 1.35, and these two signals represented, respectively, the location of a monoester phosphate at the α anomeric position of the reducing GlcN I unit and at O-4 of the distal GlcN II residue [18].

The ensemble of the presented results generated the structures shown in Fig. 1b,c for the two main molecular species of lipid A I and lipid A II respectively. Lipid A I's structure is similar to that of *Haemophilus influenzae* and, with the exception of the aminoarabinose decoration, to that of *Proteus mirabilis* [20,21]. However, these structures differed from those described for the *H. pylori* lipid A [1,2] which lacked the esterified phosphate, was less acylated, but has longer chain fatty acids. The unusual 3-OH C_{18} fatty acid found in ester and amide linkages in *H. pylori*, as well as the ester-linked C_{18}, were not detected in *H. mustelae* lipid A where the amide-linked fatty acids were found to be 3-OH C_{14} and 3-OH C_{16} in lipid A I and lipid A II respectively. In the lipid A II spectrum (Fig. 3a), a minor molecular ion species (*m/z* 1825) corresponding to lipid A I was found showing heterogeneity at the amide-linked positions. This type of heterogeneity corresponds to the first described example of non-enzyme selectivity at this position probably due to the activity of a bifunctional enzyme. Three members of the *Bordetella* genus showed unusual qualitative variability of the ester-linked fatty acid at positions C3 and C3′ (*B. pertussis, B. parapertussis* and *B. bronchiseptica*) [22]. In *Bordetella hinzii* the variability was extended to a new ester-linked position (C2′) [17], showing a certain non-conservatism during the biosynthesis of the lipid moiety in this genus. However, this variability was found to be characteristic of each species and reproducible. In the case of *H. mustelae*, the variability was observed with different batches of the same strain with no critical change in culture condition. Structural variability has already been observed in studies on total fatty acid content in *H. mustelae* cells from different laboratories [23,24]. The variability observed in the *H. mustelae* lipid A structure was in good agreement with the results showing an extraordinary degree of variation in total fatty acid composition. This

kind of variability was not however observed with *H. pylori* [23,25]. To our knowledge this is the first reported variation involving the amide-linked fatty acids. However, the polysaccharide parts of the LPSs corresponding to lipid A I and lipid A II both expressed the same structure as shown by MS (data not shown).

In 1989, *Campylobacter pylori* and *Campylobacter mustelae* were transferred to the new genus *Helicobacter* on the basis of five major groups of taxonomic features including: ultrastructure and morphology, cellular fatty acid composition, menaquinone content, growth characteristics, and enzyme activities [23]. The results presented here revealed great differences between their lipid A structures. Lipid A molecules are often highly conserved within a given genus, and it has been suggested that their fatty acid patterns are constant enough to serve as a taxonomic tool [26]. Recent examples of non-conservatism in *Bordetella* [22] as well as in *Yersinia* [16] run counter to this idea. The *Helicobacter* genus represents yet another example of the non-conservatism character of lipid A moieties.

However, the differences in lipid A structures were not the only differences observed between the two species. There are also differences in phospholipid composition [24]. In some regards, *H. mustelae* stands apart from other *Helicobacter* species. The pathogenically important ureases of *H. pylori*, *Helicobacter felis* and *Helicobacter heilmannii* have greater similarity to each other than that of *H. mustelae* [27], and this is also true for phylogenetic studies based on 16S rRNA sequences [23].

H. pylori LPSs induce a weak immunological response which could be related to their monophosphorylation and hypoacylation, and long length of the fatty acid chains of their lipid As [28]. Preliminary comparative biological tests performed on *H. mustelae* LPSs, i.e. interleukin-1 induction and tumor necrosis factor production on human monocytes, support these data. The LPS of *H. mustelae* follows the paradigm of *H. pylori* LPSs in producing glycan antigens with structural homology to histo-blood group antigens in mimicry of host gastric molecules [5], however, marked differences in lipid A structures observed here between *H. mustelae* and *H. pylori* may directly affect the pathogenic behavior of these two related species in their environments.

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