A rapid, small-scale procedure for the structural characterization of lipid A applied to *Citrobacter* and *Bordetella* strains: discovery of a new structural element

Alina Tirsoaga¹,², Asmaa El hamidi¹, Malcolm B. Perry³, Martine Caroff¹ and Alexey Novikov¹*

¹ Equipe “Endotoxines”, UMR 8619 du CNRS, IBBMC, Université de Paris-Sud, 91405 Orsay, France. ² Permanent address: Department of Physical Chemistry, University of Bucharest, Romania
³ Institute for Biological Sciences, National Research Council of Canada, Ottawa, Canada

* Corresponding author
Tel. 33 1 69 15 48 29
Fax 33 1 69 85 37 15
E-mail: Alexey.Novikov@u-psud.fr

Abbreviations used: DHB, dihydroxybenzoic acid; C₁₂ dodecanoic acid; C₁₄ tetradecanoyl acid, C₁₄-OH, hydroxytetradecanoic acid; HF, hydrofluoric acid; MALDI-MS, matrix-assisted laser-desorption/ionisation mass spectrometry; LPS, lipopolysaccharide; PS, polysaccharide; TLC, thin-layer chromatography; TNF, tumor necrosis factor.
ABSTRACT

Endotoxins (lipopolysaccharides, LPSs) are part of the outer cell membrane of Gram-negative bacteria; their biological activities are associated mainly with the lipid component (lipid A) and even more specifically with discrete aspects of their fine structure. The need for a rapid and small-scale analysis of lipid A motivated us to develop a procedure that combines direct isolation of lipids A from bacterial cells with sequential release of their ester-linked fatty acids by a mild alkali treatment followed by MALDI-MS analysis. The method avoids the multi-step LPS extraction procedure and lipid A isolation. The whole process can be performed in a working day and applied to lyophilized bacterial samples as small as 1 milligram. We illustrate the method by applying it to the analysis of lipids A of three species of Citrobacter which were found to be identical. On the other hand, when applied to two batches of Bordetella bronchiseptica strain 4650, it highlighted the presence in one of them, of hitherto unreported hexosamine residues substituting the lipid A phosphate groups, possibly a new camouflage opportunity to escape a host defense system.

Key words: Bordetella, Citrobacter, de-O-acylation, Gram-negative bacteria, hexosamine, endotoxin, lipid A, lipopolysaccharide, mass spectrometry.
INTRODUCTION

Endotoxins are lipopolysaccharides (LPSs), major components of the external membrane of Gram-negative bacteria. They may cause several pathophysiological symptoms such as fever, septic shock and death but they are also able to elicit beneficial activities such as the production of Tumor Necrosis Factor (TNF), adjuvant, and radioprotection effects (1, 2).

LPS molecular architecture has three regions: a hydrophobic moiety, called lipid A, a core oligosaccharide and a serospecific O-polysaccharide composed of repeating oligosaccharide units. Lipid A is embedded in the external bacterial membrane together with phospholipids and proteins. It is responsible for the major toxic and beneficial properties characteristic of bacterial endotoxins (2, 3).

Lipid A structure generally consists of a diglucosamine backbone substituted with varying numbers (usually 4 to 7) of ester- or amide- linked fatty acids. In most cases, phosphates, with and without other substituents, are linked to carbons at the C-1 and C-4’ positions of the lipid A disaccharide unit (2-4). These and the number and chain lengths of fatty acids are highly important for the toxic effects of lipids A (5). The addition of a single fatty acid can be responsible for an increase or decrease in bacterial virulence (6).

Lipid A classical structural analysis is a rather long and complicated process including the following main stages: LPS extraction from the bacteria, LPS purification, LPS acid hydrolysis to split the molecule into its hydrophobic and hydrophilic moieties, lipid A extraction followed by its characterization by different methods: mass spectrometry (MS), thin-layer chromatography (TLC), identification and localization of fatty acids, phosphate groups and other substituants, if any, on the glucosamine backbone.

Endotoxins can be isolated from Gram-negative bacteria by a variety of different methods (2). Long and strong hydrolytic conditions, which are occasionally required to cleave the lipid A-polysaccharide bond, result in partial dephosphorylation and O-deacylation of lipid A (7). Such modifications strongly diminish the biological activities of the molecule. Milder hydrolysis conditions such as pH 4.4-4.5 in sodium acetate buffer were shown to be efficient for lipid A liberation (8) and were usually improved by the addition of sodium dodecylsulfate (SDS) when the hydrolysis kinetics were too slow or ineffective (9). These hydrolytic processes are the conventional first steps for lipid A analysis after LPS isolation from the bacteria. An interesting approach using SDS-promoted hydrolysis of intact bacteria
has been reported (10). Other new conditions for a quick extraction of lipid A directly from bacterial cells, were recently developed (11).

We demonstrated earlier that, because of steric hindrance, fatty-acid ester linkages could be differentially hydrolyzed by alkaline treatment. Sequential alkaline de-esterification conditions in combination with mass spectrometry can reveal the substitution positions of primary ester-linked fatty acids on the glycose residues as well as secondary ester-linked positions on the hydroxyl groups of other fatty acids, called acyloxyacyl acids. In mild conditions, the acyloxyacyl ester could be released without splitting the secondary ester linkage between the two fatty acids and characterized by gas chromatography/MS (GC/MS). These conditions have been used to determine the structures of many lipid A preparations such as those of Bordetella (12, 13, 14, 15), Helicobacter (16), and Yersinia (17). We also demonstrated the importance of such sequential release in the case of Yersinia lipid A structure previously erroneously described as being identical to that of E. coli. In this special case, two pairs of fatty acids substituted different glycose positions (C2’and C3’) leading to the same total molecular weight but introducing different structures. It is therefore recommended to consider this point with any lipid A having a molecular-mass similar to that of any other well-known structure. Our first demonstration of selective conditions for the release of ester-linked fatty acids in lipid A was done in one-day step-wise use of alkali reagents (17): 10-15 min in 0.2 M NaOH for primary esters and 1h in hydrazine at 37°C for the secondary ester-linkages. The two steps de-O-acylation strategy was also used by others (18) on lipids A isolated from LPS by SDS-promoted mild hydrolysis (9). Ammonium hydroxide is frequently used in de-O-acylation, but it requires hydrolyses too long for our purpose.

When the use of hydrazine was restricted for security reasons we established new conditions, described here, that are also better adapted to our small lipid A samples isolated directly from bacteria.

Citrobacter belongs to the Enterobacteriaceae group, and 11 species are known at this time. For the present study three species were selected. They are C. freundii and C. sedlakii, two human pathogens afflicting particularly neonates, the elderly, and immunocompromised patients, and C. rodentium, a strict pathogen for mice (19-22). Apart from the latter, all the Citrobacter species are opportunistic pathogens, especially in nosocomial infections.

The Bordetella genus contains nine species. The lipid A structures of seven of them have been described (15, unpublished results). These structures were notable for their peculiarly high variability among species and even strains. In Bordetella bronchiseptica lipid
A structural variability has been attributed to relaxed enzyme specificity (6, 13, 15). The lipids A of the two human pathogens, *B. pertussis* and *B. parapertussis* have been associated with their hypoacylation and short-chain fatty acids causing reduced endotoxicity (23, 24). Because of this high structural variability the Bordetella strains presented a very suitable model for testing the new method.

Here we characterize three Citrobacter and two Bordetella lipids A by a new procedure involving direct extraction from cells. The method is especially convenient when only small amounts of bacteria, LPS or lipid A are available. When applied to Bordetella strains as a routine test, the method led us to discover a new original lipid A structural element increasing the LPS structural and biosynthetical originality and perhaps giving the bacteria a camouflage strategy to escape a host defence system.

**MATERIALS AND METHODS**

**Bacterial strains**: Bacterial strains used (all from NRC, Ottawa, Canada) were *Citrobacter sedlakii*, *C. freundii* (ATCC 51541), *C. rodentium* (19-22) *Escherichia coli* (strain 0119), and *Bordetella bronchiseptica* NRCC 4650.

**Bacterial growth conditions** Cells were grown as described (13, 19-22). Briefly, Citrobacter cells were grown to late exponential phase in a brain-heart infusion (Difco) at 37°C under constant aeration, in a New-Brunswick 25-l fermenter. *B. bronchiseptica* cells (2 batches) were grown in 70-l fermenters using 3.7% brain-heart infusion containing 5% horse serum, at 37°C, 200 r.p.m. with aeration for 18 h (13). All cells were killed with phenol (1% final concentration) before harvesting.

**LPS extraction conditions** The wet bacteria were washed with 1% saline, and were extracted by stirring with 50% aqueous phenol and collected by centrifugation at 65°C for 15 min (25). The cooled extract was diluted with water (2 vol.), insoluble material was removed by centrifugation and the cleared extract was dialyzed under tap water until free from phenol. The lyophilized retentate was dissolved in 0.02M sodium acetate (pH 7) and was sequentially treated with RNase, DNase and proteinase K, cleared by low speed centrifugation (105000 g, 12h, 4°C), and the precipitated LPS gel was dissolved in water and lyophilized (22).

**Lipid A isolation from whole cells** was described in detail in (11). Briefly, lyophilized bacterial cells (10 mg) were suspended in 400 µl of isobutyric acid-1M ammonium hydroxide mixture (5:3, v:v), and were kept for 2 hours at 100°C in a screw-cap test tube under magnetic stirring. The suspension was cooled in ice water and centrifuged
(2000 g for 15 min). The supernatant was diluted with the same volume of water and lyophilized. The lyophilized sample was then twice washed with 400 µl of methanol and centrifuged (2000 g for 15 min). Finally the lipid A was extracted from the pellet in 100 to 200 µl of a mixture of chloroform, methanol and water (3:1.5:0.25, v:v:v). For 1 mg samples, 100 µl of the solvent mixtures were used at each step.

**Sequential liberation of ester-linked fatty acids by mild alkali treatment:** The method was first developed with a relatively homogeneous *E. coli* lipid A. The following reagents were tested to define convenient two-step liberation of ester-linked fatty acids: methylamine, dimethylamine, ethylamine, diethylamine, triethylamine, and ammonium hydroxide at various concentrations. Different temperatures (37°C, 50°C and 60°C) were also tested and followed by kinetics, and the products monitored by TLC and MALDI-MS.

The following conditions were then selected for the first-step liberation of primary ester-linked fatty acids: lipid A (50 µg) was suspended (1mg/ml) in 35% ammonium hydroxide and stirred for 5 h at 50°C. To liberate the secondary ester-linked fatty acids, the resulting lipid A (50µg) was suspended in 50 µl of 41% methylamine and stirred for 5 h at 37°C. The resulting samples were dried under a stream of nitrogen, the residues were taken up in a mixture of chloroform, methanol and water (3:1.5:0.25 v:v:v) and followed by TLC and MALDI-MS analyses.

Methylamine and ethylamine were similarly efficient in liberating ester-linked fatty acids. We chose to use the amine with the smallest alkyl moiety. In addition, diethylamine produced some degradation products. This was not surprising because secondary amines are known to be more strongly basic.

Micro quantities of lipid A isolated directly from the bacterial cells were used for de-esterification. Fifty to 100 µl aliquots of the chloroform-methanol-water extracts were transferred to Eppendorf® tubes and dried with a stream of nitrogen before the treatment. To define volumes of the solutions necessary for the treatment, the total mass of the isolated lipid A was estimated to be about 1 % of the initial mass of the bacterial sample.

**Hydrolysis procedures used for liberating lipid A:**

**Acetic acid hydrolysis:** LPS was suspended in 2% acetic acid (5 mg/ml) and kept two hours at 100°C under stirring. Acid was removed under vacuum and the residue, suspended in water (5 mg/ml), was ultracentrifuged (45 min, 300.000g, 4°C). The pellet containing lipid A
was lyophilized and lipid A was extracted with chloroform-methanol-water extraction mixture (3:1.5:0.25 v:v:v).

**SDS-promoted hydrolysis:** LPSs was dispersed at a concentration of 5 mg/ml in 20 mM sodium acetate - acetic acid buffer (pH 4.5) containing 1% SDS and hydrolyzed at 100°C for 1h. After removal of SDS with acidified ethanol, lipid A was isolated as described previously (9).

**Hydrolysis procedures used for liberation of the lipid A glycosidic phosphate:**

**Hydrochloric acid hydrolysis:** LPS or lipid A were suspended in 0.1M HCl at a concentration of 5 mg/ml and kept for 15 mn at 100°C under stirring (7). The acid was neutralised with a 0.1 M NaOH solution and ultracentrifuged. The pellet containing the dephosphorylated lipid A was lyophilized, and the lipid A was extracted as above.

**Hydrofluoric acid treatment:** LPS or lipid A were suspended at 5 mg/ml in hydrofluoric acid and kept at 4°C under stirring for 48h. After solvent removal under a stream of nitrogen under a hood the residue was taken up in water and lyophilized before extraction.

**MALDI mass spectrometry:** Analyses were performed on a PerSeptive Voyager-DE STR time-of-flight mass spectrometer (Applied Biosystem) at the IBBMC, Université de Paris Sud. The analysis of the small lipid A samples used were done in linear mode with delayed extraction. Both negative- and positive-ion spectra were recorded. The ion-accelerating voltage was set at 20 kV. Dihydroxybenzoic acid (DHB) (Sigma chemical Co., St Louis) was used as a matrix. A few microliters of lipid A solution (1µg/µl) in the extraction mixture were desalted with a few grains of ion-exchange resin Dowex 50W-X8 (H+), either in an Eppendorf® tube or for small samples in a single surface droplet on Parafilm®. A 1 µl aliquot of the solution was deposited on the target and covered with the same volume of the matrix dissolved at 10 mg/ml in the same solvent. Different analyte/matrix ratios were tested when necessary. *B. pertussis* or *E. coli* highly purified lipids A were used as external standards for mass calibration.

**Thin-layer chromatography:** Chromatography was performed on aluminium-backed silica TLC plates (Merck) and compounds were visualized by charring at 145°C after spraying with 10% sulfuric acid in ethanol. Mixtures of isobutyric acid and 1 M ammonium hydroxide were used for migration of oligosaccharides (3:5, v:v) and LPSs (5:3, v:v) (26).
Solvent used for lipid A migration, was a mixture of chloroform, methanol, water and triethylamine (3:1.5:0.25: 0.1, v:v:v:v) (9).

RESULTS AND DISCUSSION

The need for a rapid method for analyzing lipid A structures on small bacterial samples initiated our search for new lipid A isolation methods (11). In the present work, we applied selective mild-alkaline treatments sequentially liberating fatty acids, as well as acid dephosphorylating treatments to micro quantities of Citrobacter and Bordetella lipids A. The latter, which were isolated by micro-hydrolysis of bacteria, could thus be further characterized in one-day experiments as schematically described in Fig. 1.

Full scale analysis of Citrobacter lipids A

Comparison of direct micro-hydrolysis of bacteria with conventional hydrolytic methods: We compared the lipid A preparations obtained by direct micro-hydrolysis of the whole Citrobacter bacterial cells with those obtained by conventional hydrolytic methods applied to phenol-extracted endotoxins of Citrobacter. Fig. 2 shows negative-ion MALDI mass spectra of C. sedlakii lipid A obtained by (a) direct micro-hydrolysis of bacteria, (b) mild SDS-promoted pH 4.5 hydrolysis of the LPS, one hour at 100°C, (c) 2% acetic acid hydrolysis of the LPS, two hours at 100°C, (d) 0.1 M HCl hydrolysis of the LPS, 10 min at 100°C and (e) HF treatment (48 hours) of lipid A isolated by the direct micro-hydrolysis of the bacteria.

Interestingly, the direct micro-hydrolysis performed on bacteria (Fig. 2a) shows peaks for the four main molecular species having four to seven fatty acids (FA) structures as well preserved as, if not better than, those obtained by the SDS-promoted mild hydrolysis (Fig. 2b). The quality of the spectrum is comparable with a good signal to noise ratio for molecular-ion peaks, and a negligible level of dephosphorylation. The latter point is a great advantage for good structural and biological practice. Few lipid A preparations can be obtained without dephosphorylation especially of the phosphate in glycosidic linkage on GlcN I. While dephosphorylation has to be avoided for biological activity determination of the native lipid A molecules, complete or incomplete dephosphorylation can be useful, on the other hand, in other biological experiments or for structural modifications (2). For example, it was early
shown with *B. pertussis* lipid A, that cleavage of the glycosidic phosphate leads to a non-toxic and non-pyrogenic lipid A molecular species (27).

It was previously shown that \( \alpha \)-glycosidic phosphate groups substituting N-Acyl glucosamine could be distinguished from their \( \beta \)-anomer by their stability at pH 4.5 (9). As shown by comparing synthetic \( \alpha \)- and \( \beta \)-derivatives, even the mildest conditions at pH 4.5 would liberate 100% of a \( \beta \)-derivative (9). The resistance of the glycosidic phosphate as observed in these conditions (Fig. 2b) allows us to conclude that an \( \alpha \)-glycosidic phosphate group is present in Citrobacter lipids A as in most lipid A structures. This \( \alpha \)-glycosidic phosphate anomery was first demonstrated in *B. pertussis* (9) and the procedure is useful for samples that are too small for NMR analysis.

If some de-phosphorylation (-P) has taken place during hydrolysis, the mass spectrum shows satellite peaks 80 Da lower than those of non-modified lipid A species. Partial mono de-phosphorylation was observed after 2 % acetic acid hydrolysis for 2 hours at 100°C (Fig.2c). The pentaacylated molecular specie appears as a “doublet” because of hydroxylation of some of the C14. The mono dephosphorylation is almost complete after 10 min at 100°C with 0.1 M HCl. (Fig.2d). HF treatment leads to mono- or bis- dephosphorylation depending on steric hindrance (7). When position C-3’ is substituted with fatty acid(s), the HF treatment does not release the phosphate group at C-4’ but only that at C-1. This is illustrated in the spectrum shown in Fig. 2e by the presence of the mono de-phosphorylated molecular peaks remaining even after 48 hours of HF treatment. The bis- dephosphorylation would have drastically changed the ionization of the molecules leading to the complete disappearance of the molecular-ion peaks in the negative-ion mode. Therefore the degree of dephosphorylation depends not only on the hydrolytic conditions used but also on the degree of substitution of the diglucosamine backbone. This allowed us to conclude that position C3’ of GlcN II was substituted in the molecular species present in Citrobacter lipid A.

The spectra obtained for the other two Citrobacter lipid A isolates were similar (data not shown) and at first glance were comparable to those of *E. coli* or Salmonellae lipid A spectra with the presence of molecular species having four (4FA) to seven (7FA) fatty acids. However, this comparison was not sufficient to describe their structures accurately, as we demonstrated earlier for *Yersinia* lipid A. (17). The analysis of de-O-acylated lipids A as well as of lipid A fragmentation patterns in the positive-ion mode were required for this purpose.

**Negative-ion MALDI MS analysis of untreated and de-O-acylated lipid A samples:** Fig. 3 presents negative-ion MALDI mass spectra of the *C. freundii* non-modified
lipid A (Fig. 3 a, a’) as well as of the same lipid A after ammonium hydroxide (Fig. 3 b, b’) and methylamine (Fig. 3 c, c’) treatments. The spectra of the lipid A preparations obtained both by direct microhydrolysis of bacteria (Fig. 3 a, b, c) and by SDS-acetate pH 4.5 hydrolysis of phenol-water extracted LPS were compared (Fig. 3 a’, b’, c’). Again it can be appreciated that the quality of the spectra obtained for the two preparations are comparable and the same peaks were observed in both cases.

**Negative-ion mass spectra of the non-modified *C. freundii* lipid A samples** gave a rough idea of the degree of molecular heterogeneity in the preparations (Fig. 3 a, a’). Signals corresponding to lipid A [M-H] ions of hepta-acyl (m/z 2036), hexa-acyl (m/z 1797), penta-acyl (m/z 1587 and m/z 1571) and tetra-acyl (m/z 1361) molecular species were observed. It is well-known that these peaks correspond to molecular species naturally present in lipid A samples and not to MS fragmentation in the negative-ion mode. They can result from different levels of biosynthetic steps and from late enzymatic modification of the structure (1). Mass differences between the adjacent molecular-ion peaks gave a first idea of the fatty acid components i.e. C\textsubscript{16} (238 u), C\textsubscript{14}-OH (226 u), C\textsubscript{14} (210 u). The only difference observed relative to the *E. coli* lipid A spectrum was the presence of the second penta-acyl molecular species with the mass of 1588 u. Small peaks of monodephosphorylated molecular species produced during hydrolysis were also observed at 80 u below those of the non-modified molecular ions.

**Negative-ion mass spectra of the de-O-acylated lipid A samples** (Fig 3 b, b’, 3 c, c’) gave additional information about the fatty-acid content in the lipid A molecules. After primary ester-linked fatty acid liberation by NH\textsubscript{4}OH de-O-acylation treatment, two main molecular-ion peaks were observed in the negative-ion MALDI mass spectra at m/z 1135 and 1373. They correspond respectively to tri- and tetra-acylated molecules. The difference of 238 u between these peaks corresponds to the mass of a C\textsubscript{16} fatty acid. Comparison of the de-O-acylated mass-spectra with those of the non-modified lipid A (Fig. 4a, a’) allowed us to conclude that NH\textsubscript{4}OH treatment liberated, one (C\textsubscript{14}-OH) from the tetra-acyl, two (C\textsubscript{14}-OH plus C\textsubscript{14} or two C\textsubscript{14}-OH) from the penta-acyl, and three (one C\textsubscript{14} and two C\textsubscript{14}-OH) ester-linked fatty acids from the hexa-acyl molecular species. All these molecular-ion species translated to a main basic molecular-ion species appearing at m/z 1135 and most probably corresponding to the lipid A backbone carrying a C\textsubscript{12} fatty acid which had to be in secondary linkage and two amide-linked C\textsubscript{14}-OH. The hepta-acyl molecular-ion species liberated three ester-linked fatty acids (one C\textsubscript{14} and two C\textsubscript{14}-OH) translating to the molecular species appearing at m/z 1373 corresponding to the substitution of the second amide-linked C\textsubscript{14}-OH by a C\textsubscript{16} fatty acid.
As one can see, the acyloxyacyl ester linkage was not or incompletely cleaved during the \( \text{NH}_4\text{OH} \) treatment. The acyloxyacyl cleavage requires stronger or extended hydrolytic conditions such as the lipid A treatment for 5 hours at 37°C with methylamine (Fig 3 c, c’). In these conditions, secondary ester-linked fatty acids were also liberated and the major molecular-ion species, after liberation of C\(_{12}\) and C\(_{16}\), appeared at \( m/z \) 952 (Fig. 4 c and 4 c’). Minor peaks were observed at \( m/z \) 872 corresponding to a mono-dephosphorylated molecular ion and at \( m/z \) 974 corresponding to sodiated ones. The major peak observed at \( m/z \) 952 could be attributed to a molecular species corresponding to the bisphosphoryl GlcN disaccharide substituted by two C\(_{14}\)-OH. Their stability in the given conditions allows us to deduce their location on the two amide groups. To establish the distribution of the ester-linked fatty acids on the glucosamine backbone, the analysis of the lipid A fragmentation patterns in the positive-ion mode gives the necessary information.

**Positive-ion mode MALDI-MS analysis of non-modified lipid A samples:**
Positive-ion MALDI mass spectra of the *C. freundii* non-modified lipid A isolated respectively, by direct microhydrolysis of bacteria, and by SDS acetate pH 4.5 hydrolysis of phenol-water extracted LPS were compared (not shown). Apart from signals corresponding to multi-sodiated quasi-molecular ions \([\text{M}+\text{Na}^n-\text{H}^n]^+\) (\( m/z \) 1821, 1843, and 1865), a series of fragment ions was observed. A prominent signal at \( m/z \) 1086 (28) obtained only in the positive-ion mode and derived from the intact protonated GlcN II part of the lipid A molecule with its phosphate ester and four fatty ester substituents but not the oxygen atom linkage to GlcN I. The \( m/z \) 886 signal indicated the loss of the C\(_{12}\) by this fragment-ion in the form of neutral acid (1086-200). It could be concluded that GlcN-II bears 2 C\(_{14}\)-OH, 1 C\(_{14}\) and 1 C\(_{12}\). The two latter had to be carried by the hydroxylated fatty acids at C-2’ and C-3’.

**Citrobacter lipid A molecular structures:** The ensemble of the data obtained allows us to propose the following structures for the lipid A molecules synthesized by the Citrobacter bacteria (Fig. 4):

Tetra-acyl lipid A (Fig. 4 a): The bis-phosphorylated di-glucosamine backbone is substituted with C\(_{14}\)OH fatty acids at the amide position of GlcN I and at the amide and C3’ ester positions of GlcN II. The GlcN II amide-linked C\(_{14}\)OH is substituted with a C\(_{12}\) fatty acid. Localization of one of the C\(_{14}\)OH fatty acids at the C3’position is justified by the observed resistance to the HF treatment, due to steric hindrance, of the phosphate group at the C4’ position. Penta-acyl lipid A (Fig. 4 b, c) has two different structures both deriving from
the tetra-acyl lipid A structure by addition of a C$_{14}$ in acyloxyacyl linkage at C3’ or by addition of a C$_{14}$OH at C3. Both of these substitutions are present in the hexa-acyl lipid A structure (Fig. 4 c). Hepta-acyl lipid A (Fig. 4 d) carries a C$_{16}$ fatty acid substituting the C$_{14}$OH at the GlcN I amide position.

**Negative-ion MALDI MS analysis of natives and de-O-acylated lipids A of**

*Bordetella bronchiseptica* strain 4650 batches 1 and 2

Lipids A of two different batches of strain *B. bronchiseptica* 4650, grown in similar conditions, were compared by MALDI Mass spectrometry. Spectrum 5 a was heterogeneous and included tetra- to hexa-acylated molecular species (respectively at $m/z$ 1361, 1587, and 1825) as previously reported (13). Some mono-dephosphorylated (-P) molecular species were observed. In Fig 5 b, corresponding to batch 2 lipid A, extra peaks appeared at the same values plus 161u ($m/z$ 1522, 1748, and 1986). Other peaks have two times 161u, they appeared at $m/z$ 1909 and 2147. After methylamine treatment of the lipids A, the spectrum corresponding to batch 1 shows only one peak at $m/z$ 952 attributed to a molecular-ion species corresponding to the bisphosphoryl GlcN disaccharide substituted by two C$_{14}$-OH units. The corresponding batch 2 spectrum presented two other major peaks at $m/z$ 1114 and 1275 in addition to the peak at $m/z$ 952 and corresponding respectively to the addition of one and two hexosamines units substituting the phosphate groups. As shown in Fig. 5 b, peaks at (-P) carry only one hexosamine, confirming their position if necessary.

**CONCLUSION**

Lipid A structural elucidation is usually done by following a complex series of chemical and physical experiments described by different authors (1,2,3). The procedure usually starts after LPS extraction and acidic hydrolysis. The method presented here allows a rapid and effective determination of the major characteristics of lipid A structure. It is thus convenient for following the reproducibility of bacterial LPS extractions and for identifying possible late enzymatic structural modifications causing variability with important effects on biological activities. PagP palmitoylation of lipid A (6,29,30) is one such modification that leads to decreased signaling through TLR-4 (31) and increased bacterial resistance to cationic peptides. PagL enzyme was described as a deacylase acting at the C-3 position (6). The best known example of phosphate group substitution was observed in *Salmonella minnesota* Re
595 strain with phosphorylethanolamine attached to the glycosidic phosphate and 4-amino-4-deoxy-L-arabinose on the phosphate at C-4’ of GlcN II. The presence of these positively-charged substituents is known to increase resistance of the bacteria to cationic antibiotics (32,33). The presence of a galactosamine at position C-1 in *Francisella tularensis* lipid A (34) is another example of phosphate substitution.

Because of antigenic similarities, it is often difficult to differentiate the Citrobacter from Salmonella or Escherichia species. However, they can be differentiated by their growth characteristics. The lipid A structures of three different Citrobacter species, described here were identical and very similar to the well-known *E. coli* lipid A structure. The only difference from the *E. coli* structure was observed at the level of the penta-acyl molecular species with molecular mass 1588 u carrying four C\textsubscript{14}-OH and one C\textsubscript{12} less commonly observed than the molecular species of mass 1572 u carrying three C\textsubscript{14}-OH, one C\textsubscript{14} and one C\textsubscript{12}, (Fig.4). It is known that the presence of different molecular species in a lipid A preparation leads to quantitative differences in biological activities and some molecular species have been shown to act as antagonists (5,31) for the other structures in the preparation (4). The presence of this molecular species in higher amount could affect biological activities.

The described procedure was found to be particularly useful for comparing *B. bronchiseptica* lipids A isolated from various strains and batches available in this laboratory. The discovery of two hexosamine residues substituting the phosphate groups in one batch of *B. bronchiseptica* lipid A NRCC 4650 could represent a possibility for the bacteria to escape the host defense system by neutralizing the negative charges of the phosphate group(s). Other batches tested previously did not have the hexosamine (13). As the same source strain was employed and identical growth conditions were used, we consider that the process is regulated in a late biosynthetic step in only one of the tested preparations. Further experimental evidence and genetic data will be required to explain this structural feature.

This is the first time that hexosamine substituants were found at positions C-1 and C-4’ in a Bordetella lipid A. Other Bordetella species and strains will be examined by the above procedure allowing rapid screening of lipids A.
References


Figure legends

**Figure 1**: Schematic representation of the analytical steps used for analysis of lipid A isolated after hydrolysis of freeze-dried bacteria. The latter were hydrolyzed in a mixture of isobutyric acid and 1 M ammonium hydroxide (5/3) for two hours at 100°C (11). The dotted arrow indicates alkaline treatment for 5h at 50°C with NH₄OH leading to partial de-O-acylation and the black arrow, to complete de-O-acylation with methylamine for 5h at 37°C. Liberation of acyloxyacyl fatty acids and free fatty acids is indicated.

**Figure 2**: Negative-ion MALDI mass spectra of *C. sedlakii* lipid A obtained by various methods: (a) hydrolysis of bacterial cells in a mixture of isobutyric acid and 1 M ammonium hydroxide (5:3, v:v) for 2h at 100°C; (b) SDS-promoted hydrolysis at pH 4.5 for 1h at 100°C; (c) hydrolysis of LPS in 2% acetic acid for 2 h at 100°C; (d) hydrolysis of LPS in 0.1 M HCl for 10 min at 100°C, (e) 48% HF lipid A hydrolysis at 4°C for 48h. (-P) means minus phosphate and (FA) fatty acid.

**Figure 3**: Negative-ion MALDI mass spectra of untreated and de-O-acylated *C. freundii* lipid A isolated by direct bacterial cell micro-hydrolysis (a, b, c) or by the SDS-promoted hydrolysis of LPS (a’, b’, c’). Spectra (a) and (a’) were obtained from untreated lipid A samples; spectra (b) and (b’) – from lipid A samples treated with ammonium hydroxide at 50°C for 5h, spectra (c) and (c’) – from lipid A samples treated with methylamine at 37°C for 5h. (+Na) indicates sodiated peaks.

**Figure 4**: Citrobacter lipid A molecular structures.

**Figure 5**: Comparison by negative-ion MALDI MS of 2 batches of *B. bronchiseptica* strain 4650 lipids A.
Batch 1: a- untreated; a’-O-deacylated
Batch 2: b- untreated; b’-O-deacylated
Figure 1

Frozen dried cells

Ammonium/Isobutyric acid

hydrolysis

Lipide A

NH\textsubscript{2}OH 35%

5h

CH\textsubscript{3}NH\textsubscript{2} 41 %

5h

[M]= 2037

CH\textsubscript{3}NH\textsubscript{2} 41 %

5h

[M]= 1374

[M]= 953

+ free FA:

2 C14OH
C14
C12
C16

[M]= 2037

[M]= 953

Figure 1
Figure 2
Figure 3
Figure 4
Figure 5