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

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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. This method avoids the multiple-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 mg. We illustrate the method by applying it to the analysis of lipids A of three species of *Citrobacter* that 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.—Tirsoaga, A., A. El Hamidi, M. B. Perry, M. Caroff, and A. Novikov. **A rapid, small-scale procedure for the structural characterization of lipid A applied to *Citrobacter* and *Bordetella* strains: discovery of a new structural element. *J. Lipid Res.* 2007.** 48: **2419–2427.**

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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, 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 four to seven) 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 that includes the following main stages: LPS extraction from the bacteria, LPS purification, LPS acid hydrolysis to split the molecule into its hydrophobic and hydrophilic moieties, and lipid A extraction. This is followed by its characterization by different methods: MS, TLC, and the identification and localization of fatty acids, phosphate groups, and other substituents, 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 effi-

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Abbreviations: C_{16}, hexadecanoic acid; C_{12}, dodecanoic acid; C_{14}, tetradecanoic acid; C_{14}-OH, hydroxytetradecanoic acid; GlcN, glucosamine; HF, hydrofluoric acid; LPS, lipopolysaccharide.

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cient for lipid A liberation (8) and were usually improved by the addition of 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 developed recently (11).

We demonstrated previously that, because of steric hindrance, fatty acid ester linkages could be differentially hydrolyzed by alkaline treatment. Sequential alkaline deesterification 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 GC-MS. These conditions have been used to determine the structures of many lipid A preparations, such as those of *Bordetella* (12–15), *Helicobacter* (16), and *Yersinia* (17). We also demonstrated the importance of such a sequential release in the case of *Yersinia* lipid A structure previously erroneously described as being identical to that of *Escherichia coli*. In this special case, two pairs of fatty acids substituted different glycose positions (C-2′ and C-3′), leading to the same total molecular weight but introducing different structures. Therefore, it is 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 a 1 day step-wise use of alkali reagents (17): 10–15 min in 0.2 M NaOH for primary esters, and 1 h in hydrazine at 37°C for the secondary ester linkages. The two-step 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 of 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, our unpublished results). These structures were notable for their peculiarly high variability among species and even strains. In *B. bronchiseptica*, lipid A structural variability has been attributed to relaxed enzyme specificity (6, 13, 15). The lipids A of 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. This 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 biosynthetic originality and perhaps giving the bacteria a camouflage strategy to escape a host defense system.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains used (all from the National research council of Canada, Ottawa, Canada) were *C. sedlakii*, *C. freundii* (ATCC 51541), *C. rodentium* (19–22), *E. coli* (strain 0119), and *B. 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 liter fermenter.

B. bronchiseptica cells (two batches) were grown in 70 liter fermenters using a 3.7% brain-heart infusion containing 5% horse serum at 37°C and 200 rpm 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 at 65°C and collected by centrifugation for 15 min (25). The cooled extract was diluted with water (2 volumes), 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 0.02 M sodium acetate (pH 7), sequentially treated with RNase, DNase, and proteinase K, and cleared by centrifugation (105,000 *g*, 12 h, 4°C), and the precipitated LPS gel was dissolved in water and lyophilized (22).

Lipid A isolation from whole cells was described in detail previously (11). Briefly, lyophilized bacterial cells (10 mg) were suspended in 400 μl of isobutyric acid-1 M ammonium hydroxide mixture (5:3, v/v) and were kept for 2 h at 100°C in a screw-cap test tube under magnetic stirring. The suspension was cooled in ice water and centrifuged (2,000 *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 (2,000 *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 was used at each step.

Sequential liberation of ester-linked fatty acids by mild alkali treatment

This 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, 50, and 60°C) were also tested and followed by kinetics, and the products were 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 (1 mg/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 (50 μg) 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, and 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 at 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 deesterification. Fifty to 100 μl aliquots of the chloroform-methanol-water extracts were transferred to Eppendorf® tubes and dried with a stream of nitrogen before treatment. To define volumes of the solutions necessary for the treatment, the total mass of the isolated lipid A was estimated to be ~1% of the initial mass of the bacterial sample.

Hydrolysis procedures used to liberate lipid A

Acetic acid hydrolysis. LPS was suspended in 2% acetic acid (5 mg/ml) and kept for 2 h at 100°C under stirring. Acid was removed under vacuum, and the residue, suspended in water (5 mg/ml), was ultracentrifuged (45 min, 300,000 *g*, 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. LPS 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 1 h. 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 was suspended in 0.1 M HCl at a concentration of 5 mg/ml and kept for 15 mn at 100°C under stirring (7). The acid was neutralized 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 was suspended at 5 mg/ml in hydrofluoric acid (HF) and kept at 4°C under stirring for 48 h. After solvent removal under a stream of nitrogen under a hood, the residue was taken up in water and lyophilized before extraction.

MALDI-MS

Analyses were performed on a PerSeptive Voyager-DE STR time-of-flight mass spectrometer (Applied Biosystems) at the Institut de biochimie et biophysique moléculaire et cellulaire, Université de Paris Sud. The analysis of the small lipid A samples used was 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 (Sigma Chemical Co., St. Louis, MO) was used as a matrix. A few microliters of lipid A solution (1 μg/μl) in the extraction mixture was 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 aluminum-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). The 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 microhydrolysis of bacteria, could thus be further characterized in 1 day experiments, as shown schematically in **Fig. 1**.

Full-scale analysis of *Citrobacter* lipids A

Comparison of direct microhydrolysis of bacteria with conventional hydrolytic methods. We compared the lipid A preparations obtained by direct microhydrolysis of whole *Citrobacter* bacterial cells with those obtained by conventional hydrolytic methods applied to phenol-extracted endotoxins of *Citrobacter*. **Figure 2** shows negative-ion MALDI mass spectra of *C. sedlakii* lipid A obtained by direct microhydrolysis of bacteria (Fig. 2A), mild SDS-promoted pH 4.5 hydrolysis of the LPS, 1 h at 100°C (Fig. 2B), 2% acetic acid hydrolysis of the LPS, 2 h at 100°C (Fig. 2C), 0.1 M HCl hydrolysis of the LPS, 10 min at 100°C (Fig. 2D), and HF treatment (48 h) of lipid A isolated by direct microhydrolysis of the bacteria (Fig. 2E).

Interestingly, the direct microhydrolysis performed on bacteria (Fig. 2A) shows peaks for the four main molecular species having four to seven fatty acid 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 glucosamine

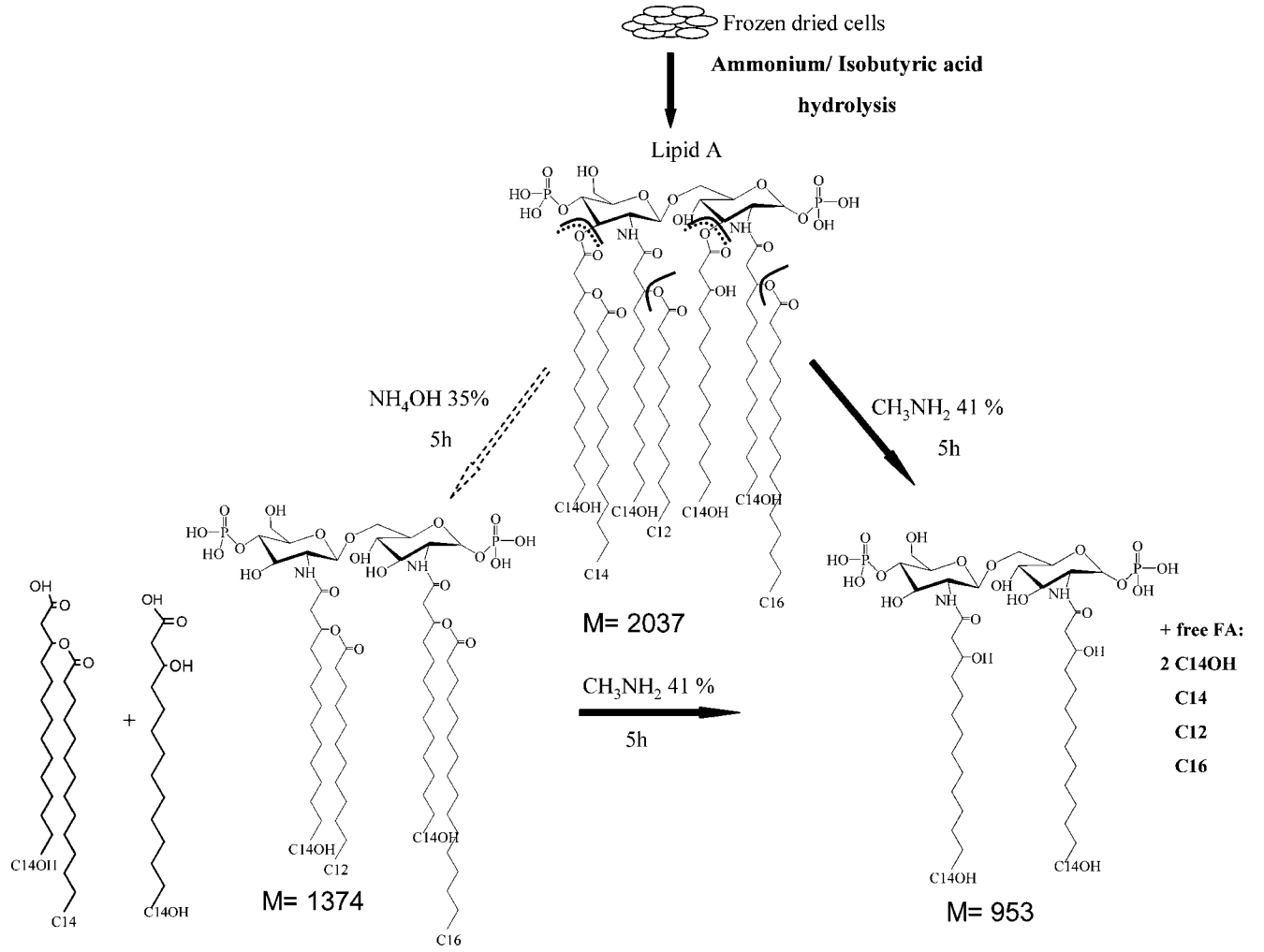

Fig. 1. Schematic representation of the analytical steps used for the analysis of lipid A isolated after hydrolysis of freeze-dried bacteria. The latter were hydrolyzed in a mixture of isobutyric acid and 1 M ammonium hydroxide (5:3) for 2 h at 100°C (11). The dotted arrow indicates alkaline treatment for 5 h at 50°C with NH_4OH, leading to partial de-*O*-acylation, and the black arrows indicate complete de-*O*-acylation with methylamine for 5 h at 37°C. Liberation of acyloxyacyl fatty acids and free fatty acids is indicated.

(GlcN) I. Although dephosphorylation has to be avoided for biological activity determination of the native lipid A molecules, complete or incomplete dephosphorylation can be useful 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 nontoxic and nonpyrogenic lipid A molecular species (27).

It was shown previously that α-glycosidic phosphate groups substituting *N*-acyl glucosamine could be distinguished from their β-anomer by their stability at pH 4.5 (9). As shown by comparing synthetic α- and β-derivatives, even the mildest conditions at pH 4.5 would liberate 100% of a β-derivative (9). The resistance of the glycosidic phosphate as observed in these conditions (Fig. 2B) allows us to conclude that an α-glycosidic phosphate group is present in *Citrobacter* lipids A, as in most lipid A structures. This α-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 dephosphorylation has taken place during hydrolysis, the mass spectrum shows satellite peaks 80 Da lower than those of nonmodified lipid A species. Partial monodephosphorylation was observed after 2% acetic acid hydrolysis for 2 h at 100°C (Fig. 2C). The penta-acylated molecular species appears as a "doublet" because of hydroxylation of some of the tetradecanoic acid (C_{14}). The monodephosphorylation is almost complete after 10 min at 100°C with 0.1 M HCl. (Fig. 2D). HF treatment leads to monodephosphorylation or bisdephosphorylation, 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 monodephosphorylated molecular peaks remaining even after 48 h of HF treatment. The bisdephosphorylation 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

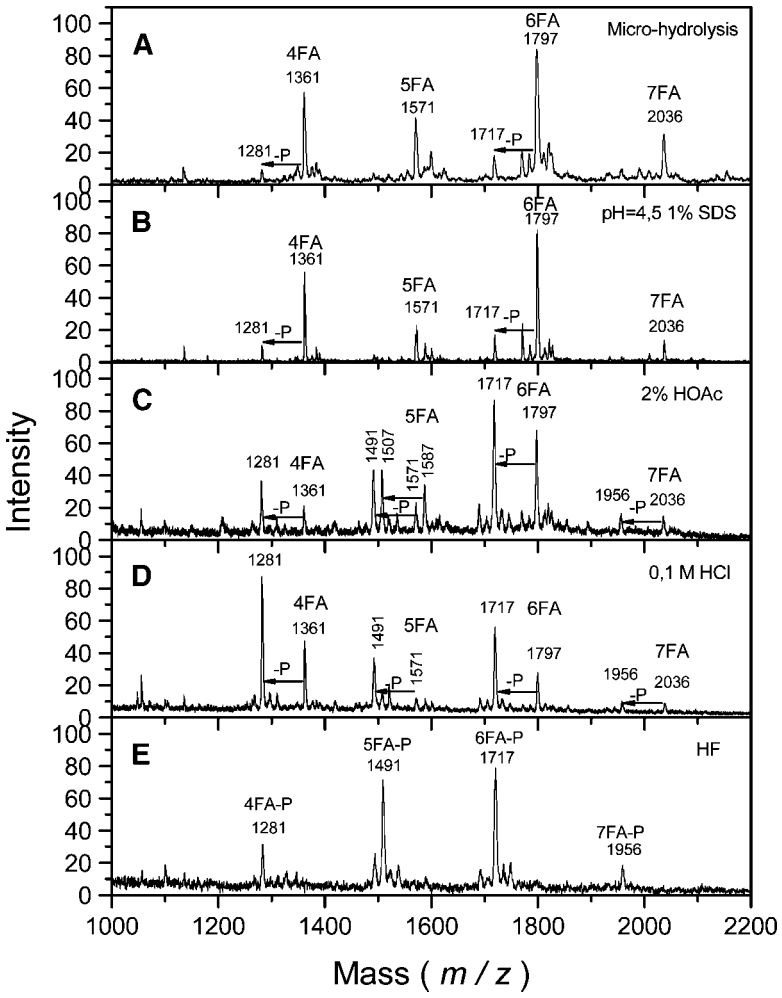

Fig. 2. Negative-ion MALDI mass spectra of *C. sedlakii* lipid A obtained by various methods. A: Hydrolysis of bacterial cells in a mixture of isobutyric acid and 1 M ammonium hydroxide (5:3, v/v) for 2 h at 100°C. B: SDS-promoted hydrolysis at pH 4.5 for 1 h at 100°C. C: Hydrolysis of lipopolysaccharide (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: A 48% hydrofluoric acid (HF) lipid A hydrolysis at 4°C for 48 h. −P, minus phosphate.

on the hydrolytic conditions used but also on the degree of substitution of the diglucosamine backbone. This allowed us to conclude that position C-3′ 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 *Salmonella* 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 previously for *Yersinia* lipid A (17). The analyses 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. **Figure 3** presents negative-ion MALDI mass spectra of the *C. freundii* nonmodified lipid A (Fig. 3A, A′) as well as the same lipid A after ammonium hydroxide (Fig. 3B, B′) and methylamine (Fig. 3C, C′) treatments. The spectra of the lipid A preparations obtained both by direct microhydrolysis of bacteria (Fig. 3A, B, C) and by SDS acetate pH 4.5 hydrolysis of phenol-water-extracted LPS (Fig. 3A′, B′, C′) were compared. Again, it can be appreciated that the qualities 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 nonmodified *C. freundii* lipid A samples gave a rough idea of the degree of molecular heterogeneity in the preparations (Fig. 3A, A′). Signals corresponding to lipid A $[M\text{-}H]^-$ ions of hepta-acyl (*m/z* 2,036), hexa-acyl (*m/z* 1,797), penta-acyl (*m/z* 1,587 and 1,571), and tetra-acyl (*m/z* 1,361) 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., hexadecanoic acid (C_{16}) (238 units), hydroxytetradecanoic acid (C_{14}-OH; 226 units), and C_{14} (210 units)]. 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 1,588 units. Small peaks of monodephosphorylated molecular species produced during hydrolysis were also observed at 80 units below those of the nonmodified molecular ions.

Negative-ion mass spectra of the de-*O*-acylated lipid A samples (Fig. 3B, B′, C, C′) gave additional information

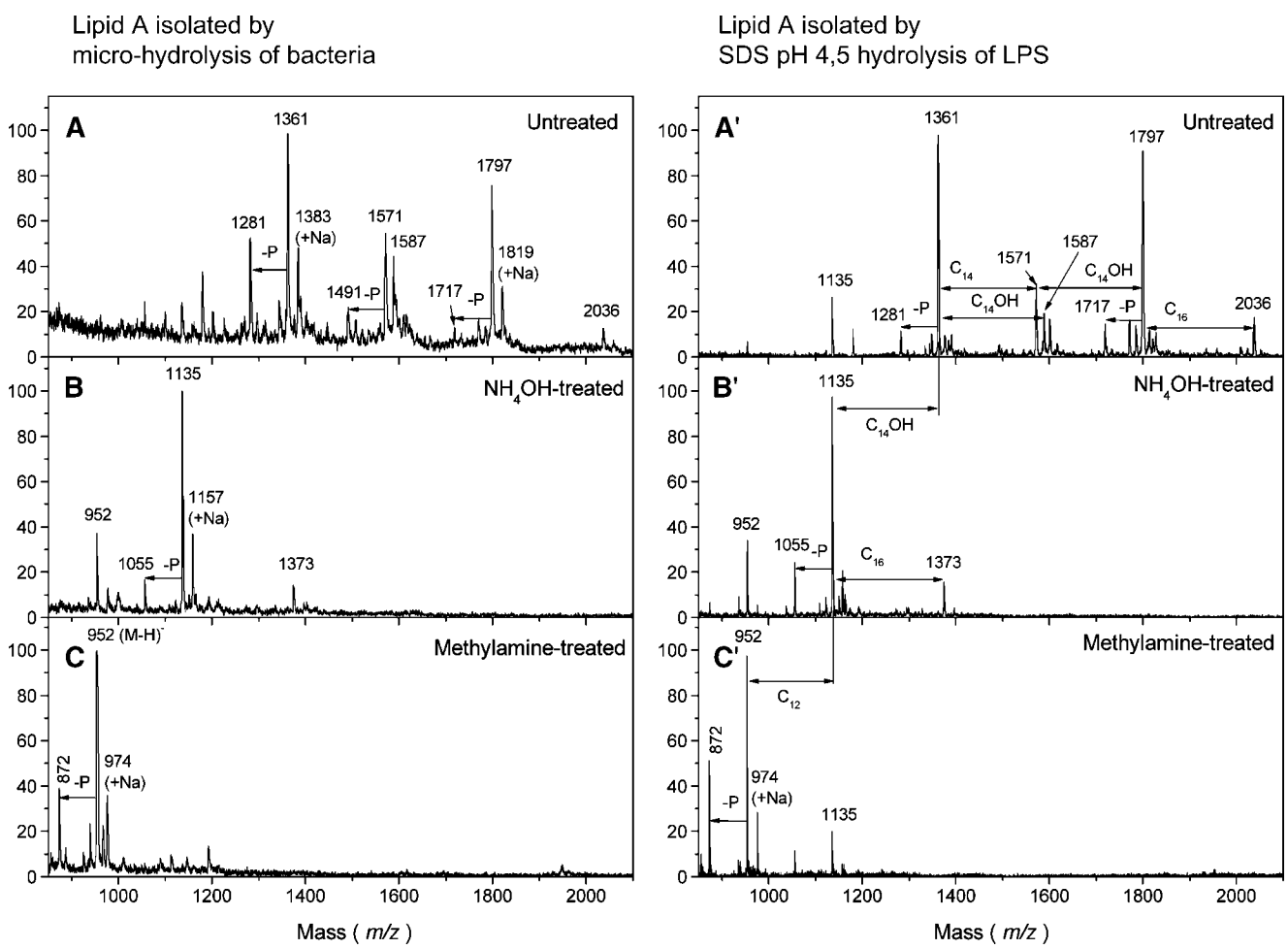

Fig. 3. Negative-ion MALDI mass spectra of untreated and de-*O*-acylated *C. freundii* lipid A isolated by direct bacterial cell microhydrolysis (A, B, C) or by the SDS-promoted hydrolysis of LPS (A′, B′, C′). The spectra in A and A′ were obtained from untreated lipid A samples; the spectra in B and B′ were from lipid A samples treated with ammonium hydroxide at 50°C for 5 h; the spectra in C and C′ were from lipid A samples treated with methylamine at 37°C for 5 h. +Na, sodiated peaks.

about the fatty acid content in the lipid A molecules. After primary ester-linked fatty acid liberation by NH_4OH de-*O*-acylation treatment, two main molecular ion peaks were observed in the negative-ion MALDI mass spectra at *m/z* 1,135 and 1,373. They correspond to triacylated and tetra-acylated molecules, respectively. The difference of 238 units between these peaks corresponds to the mass of a C_{16} fatty acid. Comparison of the de-*O*-acylated mass spectra with those of the nonmodified lipid A (Fig. 3A, A′) allowed us to conclude that NH_4OH treatment liberated one (C_{14}-OH) from the tetra-acyl, two (C_{14}-OH plus C_{14} or two C_{14}-OH) from the penta-acyl, and three (one C_{14} and two C_{14}-OH) ester-linked fatty acids from the hexa-acyl molecular species. All of these molecular ion species translated to a main basic molecular ion species appearing at *m/z* 1,135 and probably corresponding to the lipid A backbone carrying a dodecanoic acid (C_{12}) fatty acid, which had to be in secondary linkage, and two amide-linked C_{14}-OH. The hepta-acyl molecular ion species liberated three ester-linked fatty acids (one C_{14} and two C_{14}-OH) translating to the molecular species appearing at *m/z* 1,373, corresponding to the substitution of the second amide-linked C_{14}-OH by a C_{16} fatty acid.

As one can see, the acyloxyacyl ester linkage was not cleaved or was incompletely cleaved during the NH_4OH treatment. The acyloxyacyl cleavage requires stronger or extended hydrolytic conditions, such as lipid A treatment for 5 h at 37°C with methylamine (Fig. 3C, 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. 3C, C′). Minor peaks were observed at *m/z* 872 corresponding to a monodephosphorylated molecular ion and at *m/z* 974 corresponding to sodiated molecular ions. 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, analysis of the lipid A fragmentation patterns in the positive-ion mode gives the necessary information.

Positive-ion mode MALDI-MS analysis of nonmodified lipid A samples. Positive-ion MALDI mass spectra of the *C. freundii* nonmodified lipid A isolated by direct microhydrolysis of bacteria and by SDS acetate pH 4.5 hydrolysis of phenol-water-extracted LPS were compared (data not shown). Apart from signals corresponding to multisodiated quasi-molecular ions $[M+Na_n\text{-}H_{n-1}]^+$ (*m/z* 1,821, 1,843, and 1,865), a series of fragment ions was observed. A prominent signal was detected at *m/z* 1,086 (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 two C_{14}-OH, one C_{14}, and one C_{12}. The latter two had to be carried by the hydroxylated fatty acids at C-2′ and C-3′.

Citrobacter lipid A molecular structures. The totality of the data obtained allows us to propose the following structures for the lipid A molecules synthesized by the *Citrobacter* bacteria (**Fig. 4**). For tetra-acyl lipid A (1361.7 units), the bisphosphorylated diglucosamine backbone is substituted with C_{14}OH fatty acids at the amide position of GlcN I and at the amide and C-3′ 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 C-3′ position is justified by the observed resistance to the HF treatment, caused by steric hindrance, of the phosphate group at the C-4′ position. Penta-acyl lipid A (1572.1 and 1588.1 units) has two different structures both derived from the tetra-acyl lipid A structure by the addition of a C_{14} in acyloxyacyl linkage at C-3′ or by the addition of a C_{14}OH at C-3. Both of these substitutions are present in the hexa-acyl lipid A structure (1798.5 units). Hepta-acyl lipid A (2036.9 units) carries a C_{16} fatty acid esterifying the C_{14}OH at the GlcN I amide position.

Negative-ion MALDI-MS analysis of native and de-O-acylated lipids A of B. 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-MS.

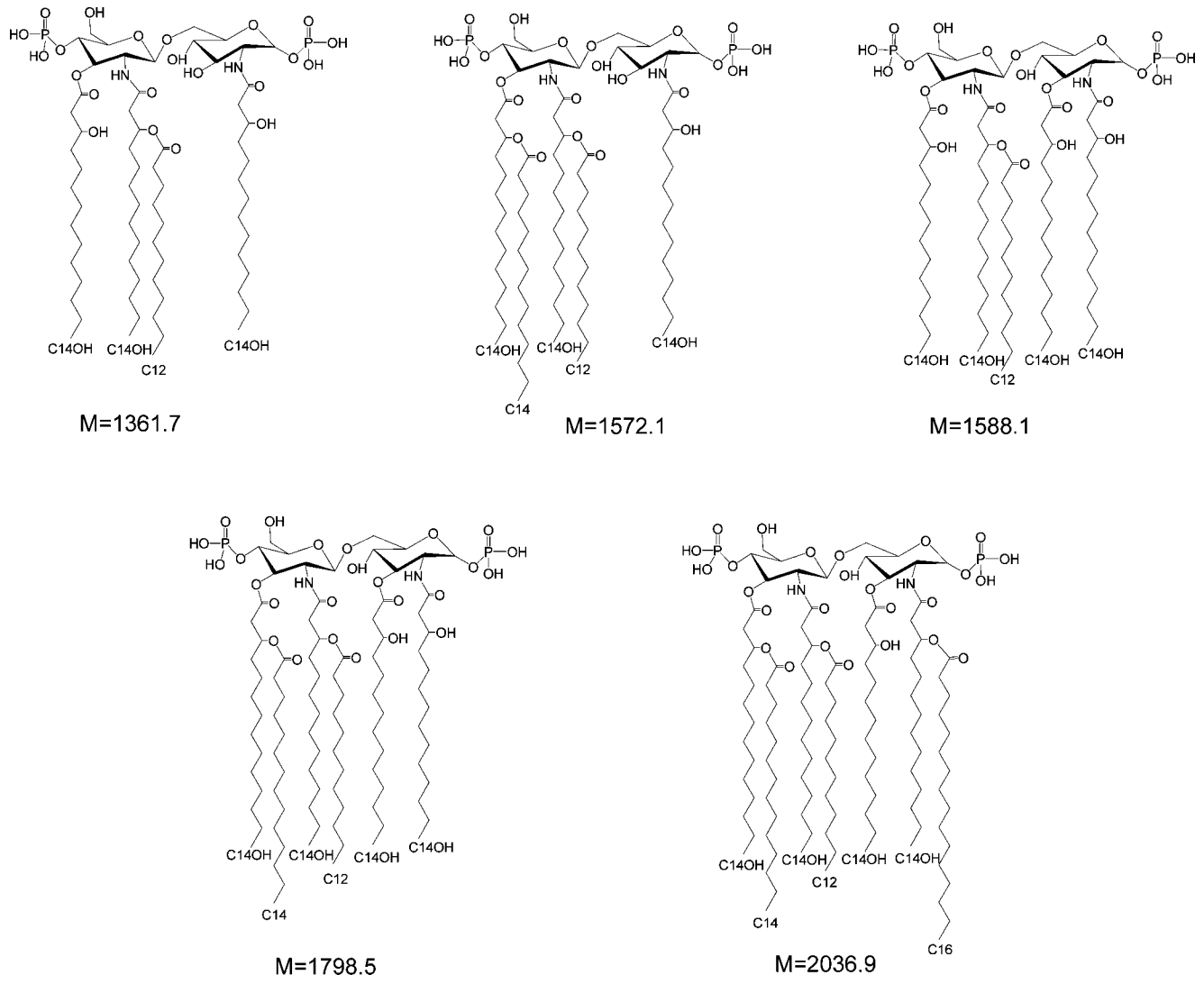

Fig. 4. *Citrobacter* lipid A molecular structures.

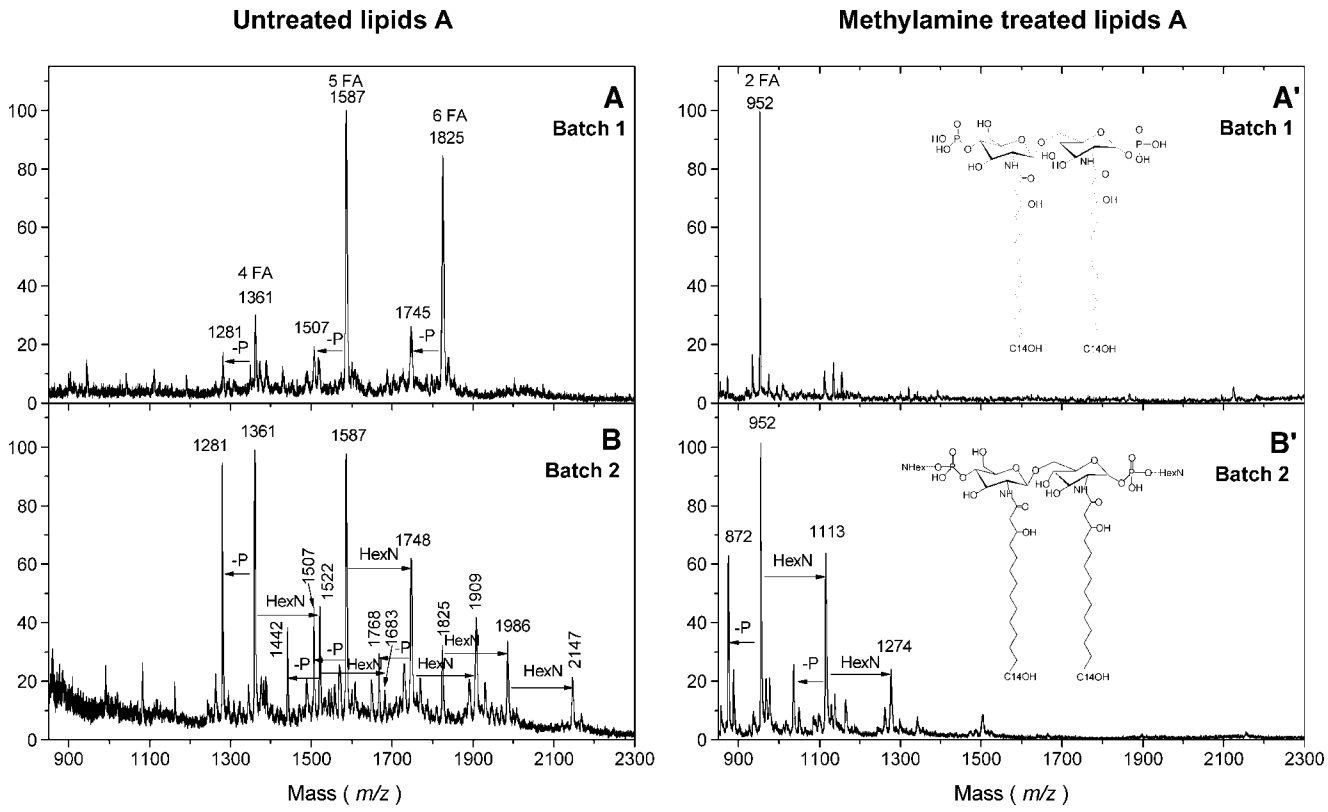

Fig. 5. Comparison by negative-ion MALDI-MS of two batches of *B. bronchiseptica* strain 4650 lipids A. A: Batch 1, untreated. A′: Batch 1, de-*O*-acylated. B: Batch 2, untreated. B′: Batch 2, de-*O*-acylated.

The spectrum in **Fig. 5A** was heterogeneous and included tetra- to hexa-acylated molecular species (at *m/z* 1,361, 1,587, and 1,825), as reported previously (13). Some mono-dephosphorylated (-P) molecular species were observed. In Fig. 5B, corresponding to batch 2 lipid A, extra peaks appeared at the same values plus 161 units (*m/z* 1,522, 1,748, and 1,986). Other peaks have two times 161 units; they appeared at *m/z* 1,909 and 2,147. After methylamine treatment of the lipids A, the spectrum corresponding to batch 1 showed 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* 1,114 and 1,275, in addition to the peak at *m/z* 952, and corresponding respectively to the addition of one and two hexosamine units esterifying the phosphate groups. As shown in Fig. 5B, 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 (1–3). 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 the 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 1,588 units, carrying four C_{14}-OH and one C_{12}, less commonly observed than the molecular species of mass 1572 units, carrying three C_{14}-OH, one C_{14}, and one C_{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 amounts could affect biological activities.

The procedure described here 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 for the phosphate groups in one batch of *B. bronchiseptica* NRCC 4650 lipid A 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 this hexosamine (13). As the same source strain 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 substituents were found at positions C-1 and C-4′ in a *Bordetella* lipid A. Other *Bordetella* species and strains will be examined by the procedure described above that allows rapid screening of lipids A.

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