methods

Microextraction of bacterial lipid A: easy and rapid method for mass spectrometric characterization

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Abstract Endotoxins (lipopolysaccharides) are the main components of Gram-negative bacterial outer membranes. A quick and simple way to isolate their lipid region (lipid A) directly from whole bacterial cells was devised. This method using hot ammonium-isobutyrate solvent was applied to small quantities of cells and proved to be indispensable when a rapid characterization of lipid A structure by mass spectrometry was required. Biological activities of endotoxins are directly related to the lipid A structures, which vary greatly with cell growth conditions. This method is suitable for rough- and smooth-type bacteria and very efficient for screening variations in lipid A structures. Data are acquired in a few hours and avoid the use of phenol in extraction.—El Hamidi, A., A. Tirsoaga, A. Novikov, A. Hussein, and M. Caroff. **Microextraction of bacterial lipid A: easy and rapid method for mass spectrometric characterization.** ***J. Lipid Res.*** **2005.** 46: **1773–1778.**

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Lipopolysaccharides (LPSs) are the major components of the external membrane of almost all Gram-negative bacteria (1, 2); they are known as endotoxins and may cause several pathophysiological symptoms, such as fever, diarrhea, blood pressure decrease, septic shock, and death (3).

The LPS molecular architecture consists of three different regions. The innermost, hydrophobic region, lipid A, is responsible for the major toxic and beneficial properties of bacterial endotoxins (4). Lipid A is the least variable part of the molecule among the different species of a genus, and its structure generally consists of a diglucosamine backbone substituted with varying numbers (usually four to seven) of ester- or amide-linked fatty acids. Phosphate and/or other substituents are linked to carbons at the C-1 and C-4′ positions of the glucosamine disaccharide (5). A 2-keto-3-deoxyoctonate (Kdo) unit links the lipid A to a core oligosaccharide (OS) composed of ~10 sugar residues divided into two regions: a best conserved inner core part and a distal outer core. The core is linked to a third outermost region of a highly immunogenic and variable O-chain polysaccharide (PS) or O-antigen made up of repeating OS units. The latter region of the LPS molecule is responsible for bacterial serological strain specificity (6) and is present only in smooth-type bacteria. The so-called rough-type bacteria produce LPSs lacking O-antigens.

Endotoxin can be isolated from Gram-negative bacteria by different methods, the most efficient and commonly used one being the hot phenol-water extraction procedure introduced by Westphal and Lüderitz (7). It was later modified by different authors (8, 9), and specific methods were developed for rough-type endotoxin extractions (10, 11). However, each method requires several days for the extraction and purification of endotoxins and further steps to isolate the lipid A moiety. New methods have been described to extract LPS from small quantities of cells, such as by mini phenol extraction (12) or using an RNA-isolating reagent, but these methods still require 2 or 3 days and the use of phenol (13).

In early experiments, mineral acid hydrolysis was used to liberate lipid A from endotoxins, splitting the acido-labile ketosidic bond of Kdo. It was followed in 1963 by a milder hydrolysis treatment using acetic acid (14). Excessively long and strong hydrolytic conditions, which are sometimes necessary to release the lipid A-PS, results in some dephosphorylation and O-deacylation of lipid A (15). Such modifications strongly diminish the biological activities of the molecule. Milder hydrolysis conditions, such as pH 4.5 sodium acetate buffer, were often shown to be efficient for lipid A liberation (16) and were usually improved by adding SDS (17) when the hydrolysis kinetics were too slow or totally ineffective.

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Abbreviations: Kdo, 2-keto-3-deoxyoctonate; MALDI, matrix-assisted laser desorption/ionization; LPS, lipopolysaccharide; OS, oligosaccharide; PS, polysaccharide.

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It is often necessary to check the reproducibility of bacterial LPS extractions and to characterize structural modifications having potential effects on biological activities. Such characterization may also be necessary to determine the consequences of variability in cell growth conditions (temperature, salt concentration, etc.).

We recently developed new conditions for extracting rough-type endotoxins with ammonium isobutyrate in a quick and simple way (Caroff, M. patent 2004/062690 A1). The method used the solubility capacities of a solvent originally used for paper chromatography of peptidoglycan fragments or ^{14}C-labeled LPSs (18). Here, we present a new lipid A microisolation procedure performed directly on bacterial cells using this solvent. The method is broadly applicable to different Gram-negative bacteria and is appropriate for the analysis of small amounts of cells.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains used were *Haemophilus influenzae* (strain b Eagan) and the smooth-type bacteria *Bordetella holmesii* (ATCC 51541), both from National Research Council (Ottawa, Canada), *Escherichia coli* (strain C60) from Insitut de Biochimie, Biophysique Moléculaire et Cellulaire (Orsay, France), and *Bordetella pertussis* (strain Bp1414) from the Institut Mérieux (Lyon, France).

Thin-layer chromatography

Chromatography was performed on aluminum-backed silica TLC plates (Merck), and compounds were visualized by charring (145°C after spraying with 10% sulfuric acid in ethanol). Solvent 1 was a mixture of isobutyric acid-molar ammonium hydroxide [3:5 (v/v) for OSs and 5:3 (v/v) for LPS] (19). The latter was also used for kinetics when lipid A, LPS, and PSs had to be seen on the same plate. Solvent 2 used for lipid A sample migration was a mixture of chloroform-methanol-water-triethylamine (3:1.5:0.25:0.1, v/v), as described previously (17).

LPS hydrolyses

Acetic acid hydrolysis. The LPSs were cleaved by hydrolysis in 1% acetic acid at 100°C for 1.5 h at a concentration of 5 mg/ml. Lipid A was recovered from the lyophilized residue by two extractions with a volume of 0.5 ml of a chloroform-methanol-water (3:2:0.25, v/v) mixture.

Detergent-promoted hydrolysis. The LPSs were cleaved by hydrolysis in 20 mM Na acetic acid-sodium acetate buffer, pH 4.5, and 1% Na dodecyl sulfate at 100°C for 1 h at a concentration of 5 mg/ml, and lipid A was isolated as described previously (17).

Matrix-assisted laser desorption/ionization negative ion mass spectrometry

Analyses were performed on a PerSeptive Voyager-DE STR model time-of-flight mass spectrometer (Applied Biosystems; Institut de Biochimie, Biophysique Moléculaire et Cellulaire, IFR46, Université de Paris XI) in linear mode with delayed extraction. 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 suspension (1 mg/ml) was desalted with a few grains of ion-exchange resin (Dowex 50W-X8; H^+) either in an Eppendorf tube or as a single droplet onto a piece of Parafilm® for small samples. A 1 μl aliquot of the suspension (50–100 μl) was deposited on the target and covered with the same amount of the matrix suspended at 10 mg/ml in a 0.1 M solution of citric acid (20). Different ratios between the samples and dihydroxybenzoic acid were tested when necessary. *B. pertussis* or *E. coli* lipids A were used as external standards.

Cell wash

This procedure was advisable with *E. coli* but not *B. pertussis* lipid A, so it should be used only when a high content of phospholipid contaminants appears in the matrix-assisted laser desorption/ionization (MALDI) spectra. To remove most of the membrane phospholipids, lyophilized bacterial cells (10 mg) were washed twice with 400 μl of a fresh, single-phase mixture of chloroform-methanol (1:2, v/v) and once with 400 μl of chloroform-methanol-water (3:2:0.25, v/v). The insoluble material, corresponding to washed cells, was recovered by centrifugation in the pellet, and the supernatants were discarded.

Lipid A isolation from whole cells

Lyophilized crude or freshly washed cells (10 mg) were suspended in 400 μl of isobutyric acid-ammonium hydroxide 1M (5:3, v/v) and were kept for 2 h at 100°C in a screw-cap test tube under magnetic stirring. The mixture was cooled in ice water and centrifuged (2,000 *g* for 15 min). The supernatant was diluted with water (1:1, v/v) and lyophilized. The sample was then washed twice with 400 μl of methanol and centrifuged (2,000 *g* for 15 min). Finally, the insoluble lipid A was solubilized and extracted once in 100–200 μl of a mixture of chloroform-methanol-water (3:1.5:0.25, v/v). For 1 mg samples, 100 μl of the different solvent mixtures were used at each step.

Monitoring the kinetics of lipid A release by TLC

After repeating the above procedure up to the methanol wash and centrifugation, the residues were suspended in water or small volumes of isobutyric acid-ammonium hydroxide (1M) (5:3, v/v), deposited on the plate, chromatographed in the same mixture of solvents, dried, and charred (19).

RESULTS AND DISCUSSION

The need for a rapid method to check the variability of lipid A structures with growth conditions of bacteria initiated our search for new isolation methods. The mild SDS promoted hydrolysis at pH 4.5 (17) and had been applied directly on bacterial cells with success (21). However, this method was too time-consuming for our purpose.

The extraction of LPS with methanol as described in the literature was limited to deep-rough LPSs (11). The tri-reagent extraction method (13) used for all types of LPS was also time-consuming and did not avoid the use of phenol. In this work, we examined the capacity of ammonium-isobutyrate to simultaneously extract and hydrolyze the endotoxin directly from bacterial cells.

Different conditions of concentrations, temperatures (40, 60, 80, and 100°C), time (30 min, 1 h, 1.5 h, 2 h, 3 h, and 4 h), and mixtures of solvents [isobutyric acid-water (1:1), isobutyric acid: (1M and 2 M)-ammonium hydroxide (5:3), and pure isobutyric acid] were tested. Splitting kinetics were visualized by thin-layer chromatography (19), followed by more accurate analysis by MALDI mass spectrometry (20).

The best results were obtained with the 5:3 (M) mixture for the endotoxins tested. Good splitting results were ob-

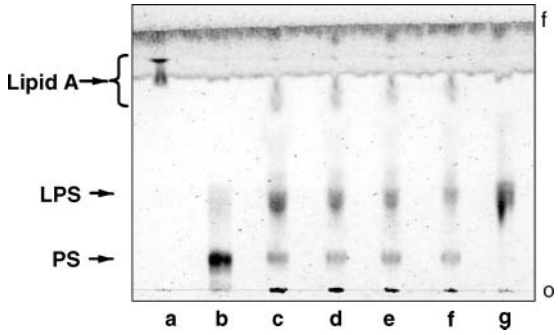

Fig. 1. Monitoring, by TLC, of the kinetics of *B. pertussis* cell hydrolysis at 100°C in a mixture of isobutyric acid-molar ammonium hydroxide (5:3). The TLC was run in the same solvent (19). *B. pertussis* lipid A (lane a), polysaccharide (PS; lane b), and lipopolysaccharide (LPS; lane g) were used as standards. LPS and its fragments (lipid A and LPS) released upon hydrolysis at 1 h (lane c), 1.5 h (lane d), 2 h (lane e), and 3 h (lane f) were compared. f, front; o, origin.

tained at 100°C in a few hours without degradation of LPS components. If any dephosphorylation occurred during the testing of the conditions, it was indicated in the spectra by the appearance of peaks at m/z −80 Da, and the duration of hydrolysis was reduced. At lower temperatures, no or incomplete splitting was observed. It is highly recommended that the proper mixture of solvents and hydrolysis conditions be determined for each bacterium.

The isobutyrate TLC solvent system 1 (19) allowed quick visualization of the uncleaved native *B. pertussis* endotoxin and of the released OS and lipid A moieties as a function of time. As expected and shown in **Fig. 1**, the LPS spots decreased progressively as those of OS and lipid A increased, giving a first indication of the splitting rate. This easy test allowed a preselection of the hydrolytic conditions. Lipid A is rarely composed of a single molecular species: in the case of the *B. pertussis* cells used in this laboratory, it consists of two to three molecular species, as seen in the spectra (22). Therefore, on thin-layer chromatography, the lipid A preparation gives two or three spots, more or less discrete. There is also a slight difference between the migration of pure isolated lipid A and that of lipid A liberated in the solvent system, because of the presence of salts in the latter. The charring method is also less sensitive for lipid A than for LPS or PS because it has fewer hydroxyl groups.

This experiment was followed by extraction of the lipid A with a mixture of solvents (17) and TLC of the concentrated extracts in solvent system 2, as well as MS analyses. These data confirmed the choice of 2 h of hydrolysis at 100°C in the solvent mixture isobutyric acid-ammonium hydroxide (1M) (5:3).

Figure 2 shows a comparison by TLC of *B. pertussis* lipid A thus obtained (Fig. 2A), with lipids A obtained by hydrolysis of the *B. pertussis* endotoxin by the SDS-pH 4.5 method (Fig. 2B) and by the 1% acetic acid method for 1.5 h at 100°C (Fig. 2C). Less degradation was observed with the methods shown in Fig. 2A, B, as shown by the absence of fast-migrating products corresponding to dephosphorylated lipid A molecular species. These results

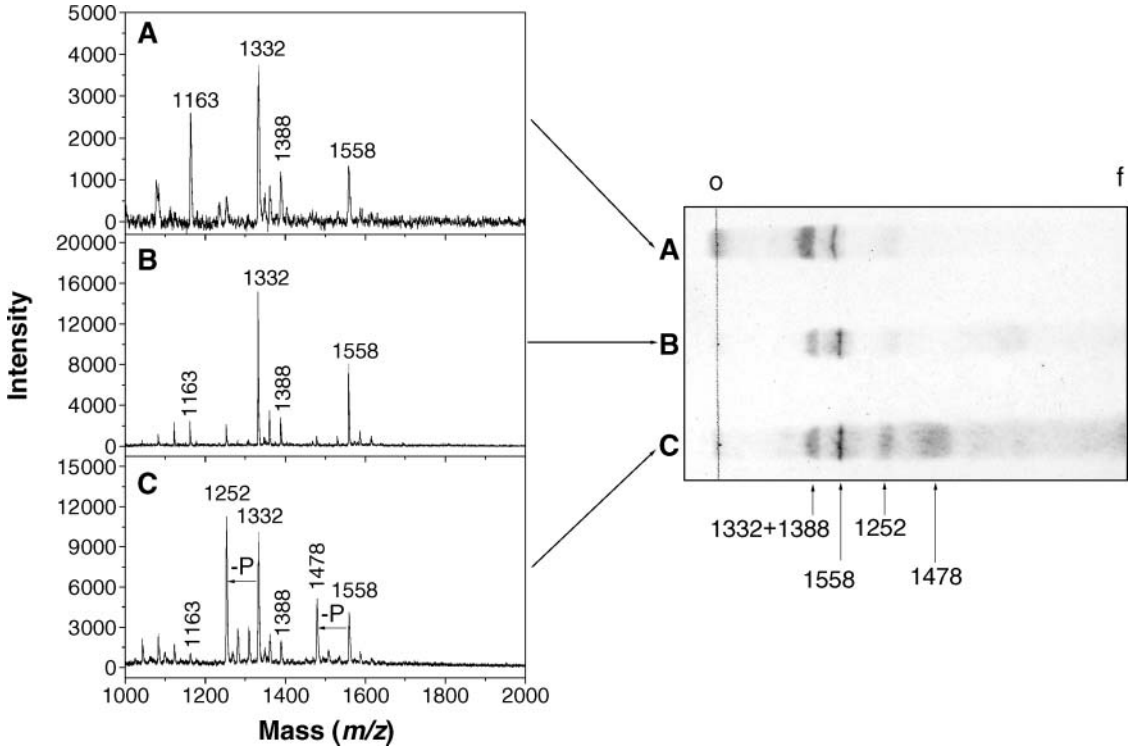

Fig. 2. Negative-ion matrix-assisted laser desorption/ionization (MALDI) mass spectrometry and TLC comparison of *B. pertussis* lipid A obtained by different methods. A: Hydrolysis of *B. pertussis* cells by isobutyric acid-molar ammonium hydroxide (5:3, v/v) for 2 h at 100°C. B: Hydrolysis of *B. pertussis* LPS by SDS-pH 4.5 for 1 h at 100°C. C: Hydrolysis of *B. pertussis* LPS by 1% acetic acid for 1.5 h at 100°C. Molecular species visualized by TLC were labeled with the corresponding masses obtained in MALDI.

were confirmed by MALDI analyses of the lipid A samples. The spectrum shown in Fig. 2A was obtained by the present method and shows two peaks corresponding to the migrating molecular species of the penta-acyl and tetra-acyl *B. pertussis* lipid A (1,558Da and 1,332Da, respectively). The presence of these two molecular species in lipid A obtained by mild hydrolysis has already been described for this hypoacylated lipid A (22). It is very similar to the spectrum shown in Fig. 2B obtained for lipid A after the mild SDS-pH 4.5 hydrolysis (17). The lipid obtained by hydrolysis in acetic acid showed some degradation (Fig. 2C), especially by the loss of phosphate groups from the two main molecular species with peaks at *m/z* 1,252 and 1,478. The presence of the glycosidic phosphate in MALDI spectra was a good indicator of structure integrity, the latter being the more labile component of such molecules (17).

Different rough-type bacteria of known lipid A structure were tested: *B. pertussis* (22), *H. influenzae* (23), and *E. coli* (24). MALDI spectra in the negative-ion mode demonstrated that no structural elements were lost upon hydrolysis. Each spectrum obtained with the new method was compared with the corresponding spectrum obtained after SDS-promoted hydrolysis of the phenol-extracted endotoxins. They were found to give similar results in terms of lipid A structural integrity.

Dephosphorylation and O-deacylation of lipid A (25) are modifications that strongly diminish the biological activities of lipid A, as shown, for example, by the loss of toxicity and pyrogenicity of the *B. pertussis* lipid A after release of the glycosidic phosphate (26). The use of SDS to disrupt micellar LPS suspensions during hydrolysis (17) proved to be effective in favoring hydrolysis of the ketosidic bond of Kdo at pH 4.5. In the present experiments, the solvent used for extraction of endotoxins combines the effect of solubilization of the endotoxin and gives a final pH of 4.2–4.3, somewhat lower than the conventional pH of acetic acid-sodium acetate buffer, but a weaker acid that is ideal for preserving linkages involving more labile structural elements. It is recommended that the conditions to be used on the same or similar types of cells be tested before their use in a routine procedure. In some cases, partial dephosphorylation can help with the structural determination of unknown lipids A, a more common difference of 80Da suggesting the loss of a glycosidic phosphate. Differences corresponding with the molecular weight of other compounds such as sugars can be expected with other structures (4). Thus, as already mentioned, precise experimental conditions have to be defined for each particular purpose and bacterium.

Spectra obtained for the three tested bacteria under the new conditions of hydrolysis are presented in **Fig. 3**. Figure 3A shows the spectrum obtained for *E. coli* lipid A (24) under the new conditions with the classical hexa-acylated molecular species at *m/z* 1,797 followed by penta- and tetra-acylated molecular species at *m/z* 1,570 and 1,361, respectively. For *B. pertussis* lipid A (Fig. 3B), two main peaks are present corresponding to the tetra- and penta-acylated molecular species at *m/z* 1,332 and 1,558, respectively (22,

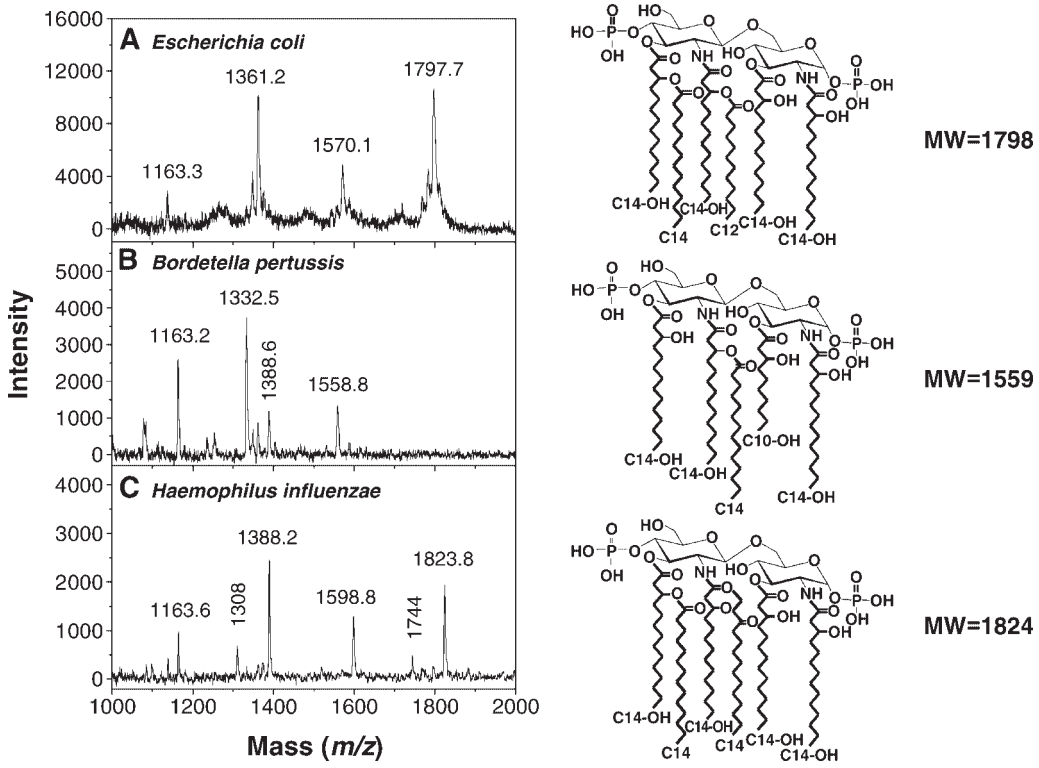

Fig. 3. Negative-ion MALDI mass spectra and structures of the main molecular species present in *E. coli* (A), *B. pertussis* (B), and *H. influenzae* (C) lipids A obtained after hydrolysis of the cells by isobutyric acid-molar ammonium hydroxide (5:3, v/v) for 2 h at 100°C. MW, molecular weight.

27). Some minor peaks correspond to the presence of fatty acids with two additional carbons. *H. influenzae* lipid A (23) extracted under the new conditions gave the spectrum shown in Fig. 3C. Three main peaks are observed at *m/z* 1,824, 1,599, and 1,388, corresponding, respectively, to hexa-, penta-, and tetra-acylated molecular species. Small peaks at *m/z* 1,308 and 1,744 are attributable to a slight dephosphorylation of the main molecular species. The peak at *m/z* 1,163 present in the three spectra corresponds to a triacylated molecular species.

The sensitivity of the method was tested on *E. coli* cells and found to be reproducible and to give good quality spectra on samples of lyophilized cells as small as 250 μg. In this case, the concentrations were decreased to facilitate handling of the samples (see Materials and Methods). No variations in the results were observed with the new concentrations. The limit of detection was found to be 50–100 μg depending on the cells tested.

The experimental conditions used with the above bacteria having known structures were then tested on cells of a recently isolated smooth-type *Bordetella* species: *B. holmesii* (28). Most of the *Bordetella* species examined to date have displayed different lipid A structures. The *B. holmesii* lipid A spectrum isolated by the new conditions was compared with that of *B. pertussis*, the "prototype" of the genus that is responsible for whooping cough, and to those of other *Bordetella* species. The spectrum revealed structural differences from that of the *B. pertussis* lipid A. The heterogeneous spectrum, however, did give mass peaks corresponding to molecular species found in other *Bordetella* species, such as *B. bronchiseptica* (29) and *B. hinzii* (30). The detailed structure will be described in a future publication.

Comparisons of lipid A structures are essential when they are associated with and related to different biological activities or bacterial resistance. Good examples are the presence or absence of a single fatty acid (31–33) or of substituents on the C-1 and/or C-4′ phosphates (34). Such structural differences could be easily demonstrated by the present method on small cell samples.

In conclusion, this method proved to be rapid and efficient in providing information on lipid A structures. It can be used to check the stability of bacterial extracts, to determine structural variations resulting from varied culture conditions, and to characterize structures obtained in low yield.

The gifts of *H. influenzae* cells from James C. Richards and Adèle Martin, of *E. coli* C60 cells from Dominique Mengin-Lecreulx, and of *B. holmesii* cells from Malcolm B. Perry are greatly appreciated. Dr. D. Karibian is acknowledged for constructive discussions, and Malcolm B. Perry is thanked for help with the manuscript. This work was part of the Action Intégrée Franco-Marocaine MA 02/52 and was supported by the Agence Universitaire de la Francophonie.

REFERENCES

1. Raetz, C. R. 1990. Biochemistry of endotoxins. *Annu. Rev. Biochem.* **59:** 129–170.
2. Rietschel, E. T, O. Holst, A. J. Ulmer, H. Brade, and H. Flad. 1996. Biochemistry and cell biology of bacterial endotoxin. *FEMS Immunol. Med. Microbiol.* **16:** 83–104.
3. Morrison, D. C., and J. L. Ryan. 1987. Endotoxins and disease mechanisms. *Annu. Rev. Med.* **38:** 417–432.
4. Caroff, M., and D. Karibian. 2003. Structure of bacterial lipopolysaccharides. *Carbohydr. Res.* **338:** 2431–2447.
5. Takayama, K., and N. Qureshi. 1992. Chemical structure of lipid A. *In* Bacterial Endotoxic Lipopolysaccharides. Molecular Biochemistry and Cellular Biology. D. C. Morrison and J. L. Ryan, editors. CRC Press, Boca Raton, FL. 43–65.
6. Weynants, V., D. Gilson, A. Cloeckaert, A. Tibor, P. A. Denoel, F. Godfroid, J. N. Limet, and J. J. Letesson. 1997. Characterization of smooth lipopolysaccharides and O polysaccharides of *Brucella* species by competition binding assays with monoclonal antibodies. *Infect. Immun.* **65:** 1939–1943.
7. Westphal, O., and O. Lüderitz. 1954. Chemische Erforschung von Lipopolysacchariden Gram-negativer Bakterien. *Angew. Chem.* **66:** 407–417.
8. Westphal, O., and K. Jann. 1965. Bacterial lipopolysaccharide. Extraction with phenol-water and further application of the procedure. *Methods Carbohydr. Chem.* **5:** 83–91.
9. Johnson, K. G., and M. B. Perry. 1997. Improved techniques for the preparation of bacterial lipopolysaccharides. *Can. J. Microbiol.* **1:** 29–34.
10. Galanos, C., O. Luderlitz, and O. Westphal. 1968. A new method for the extraction of R lipopolysaccharides. *Eur. J. Biochem.* **9:** 245–249.
11. Nurminen, M., and M. Vaara. 1996. Methanol extracts LPS from deep rough bacteria. *Biochem. Biophys. Res. Commun.* **219:** 441–444.
12. Li, J., P. Thibault, A. Martin, J. C. Richards, W. W. Wakarchuk, and W. Van der Wilp. 1998. Development of an on-line preconcentration method for the analysis of pathogenic lipopolysaccharides using capillary electrophoresis-electrospray mass spectrometry. Application to small colony isolates. *J. Chromatogr. A.* **817:** 325–336.
13. Yi, C. E., and M. Hackett. 2000. Rapid isolation method for lipopolysaccharide and lipid A from Gram-negative bacteria. *Analyst.* **125:** 651–656.
14. Osborn, M. J. 1963. Studies on the Gram-negative cell wall. I. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. USA.* **50:** 499–506.
15. Karibian, D., C. Deprun, and M. Caroff. 1995. Use of plasma desorption mass spectrometry in structural analysis of endotoxins: effect on lipid A of different acid treatments. *In* Bacterial Endotoxins: Lipopolysaccharides for Genes to Therapy. J. Levin, C. R. Alving, R. S. Munford, and H. Redl, editors. Wiley-Liss, New York. 103–111.
16. Rosner, M. R., J. Tang, I. Barzilay, and H. G. Khorana. 1979. Structure of the lipopolysaccharide from an *Escherichia coli* heptose-less mutant. I. Chemical degradations and identification of products. *J. Biol. Chem.* **254:** 5906–5917.
17. Caroff, M., A. Tacken, and L. Szabo. 1988. Detergent-accelerated hydrolysis of bacterial endotoxins and determination of the anomeric configuration of the glycosyl phosphate present in the "isolated lipid A" fragment of the *Bordetella pertussis* endotoxin. *Carbohydr. Res.* **175:** 273–282.
18. Boman, H. G., and D. A. Monner. 1975. Characterization of lipopolysaccharides from *Escherichia coli* K-12 mutants. *J. Bacteriol.* **121:** 455–464.
19. Caroff, M., and D. Karibian. 1990. Several uses for isobutyric acid-ammonium hydroxide solvent in endotoxin analysis. *Appl. Environ. Microbiol.* **56:** 1957–1959.
20. Thérisod, H., V. Labas, and M. Caroff. 2001. Direct microextraction and analysis of rough-type lipopolysaccharides by combined thin-layer chromatography and MALDI mass spectrometry. *Anal. Chem.* **73:** 3804–3807.
21. Que, N. L., S. Lin, R. J. Cotter, and C. R. Raetz. 2000. Purification and mass spectrometry of six lipid A species from the bacterial endosymbiont *Rhizobium etli*. Demonstration of a conserved distal unit and a variable proximal portion. *J. Biol. Chem.* **275:** 28006–28016.
22. Caroff, M., C. Deprun, J. C. Richards, and D. Karibian. 1994. Structural characterization of the lipid A of *Bordetella pertussis* 1414 endotoxin. *J. Bacteriol.* **176:** 5156–5159.
23. Helander, I. M., B. Lindner, H. Brade, K. Altmann, A. A. Lindberg, E. T. Rietschel, and U. Zähringer. 1988. Chemical structure of the

lipopolysaccharide of *Haemophilus influenzae* strain I-69 Rd−/b+. Description of a novel deep-rough chemotype. *Eur. J. Biochem.* **177:** 483–492.
24. Karibian, D., C. Deprun, and M. Caroff. 1993. Comparison of lipids A of several *Salmonella* and *Escherichia* strains by ^{252}Cf plasma desorption mass spectrometry. *J. Bacteriol.* **175:** 2988–2993.
25. Caroff, M., D. Karibian, N. Haeffner-Cavaillon, and J-M. Cavaillon. 2002. Structural and functional analyses of bacterial lipopolysaccharides. *Microbes Infect.* **4:** 915–926.
26. Caroff, M., J-M. Cavaillon, C. Fitting, and N. Haeffner-Cavaillon. 1986. Inability of pyrogenic, purified *Bordetella pertussis* lipid A to induce interleukin-l release by human monocytes. *Infect. Immun.* **54:** 465–471.
27. Caroff, M., J-R. Brisson, A. Martin, and D. Karibian. 2000. Structure of the *Bordetella pertussis* 1414 endotoxin. *FEBS Lett.* **477:** 8–14.
28. Weyant, R. S., D. G. Hollis, R. E. Weaver, M. F. Amin, A. G. Steigerwalt, S. P. O'Connor, A. M. Whitney, M. I. Daneshvar, C. W. Moss, and D. J. Brenner. 1995. *Bordetella holmesii* sp. nov., a new gram-negative species associated with septicemia. *J. Clin. Microbiol.* **33:** 1–7.
29. Zarrouk, H., D. Karibian, S. Bodié, M. B. Perry, J. C. Richards, and M. Caroff. 1997. Structural characterization of the lipids A of three *Bordetella bronchiseptica* strains: variability of fatty acid substitution. *J. Bacteriol.* **179:** 3756–3760.
30. Aussel, L., J. R. Brisson, M. B. Perry, and M. Caroff. 2000. Structure of the lipid A of *Bordetella hinzii* ATCC 51730. *Rapid Commun. Mass Spectrom.* **14:** 595–599.
31. Tanamoto, K., and S. Azumi. 2000. Salmonella-type heptaacylated lipid A is inactive and acts as an antagonist of lipopolysaccharide action on human line cells. *J. Immunol.* **164:** 3149–3156.
32. Raetz, C. R. H. 2001. Regulated covalent modifications of lipid A. *J. Endotoxin Res.* **7:** 73–78.
33. Preston, A., E. Maxim, E. Toland, E. J. Pishko, M. Caroff, and D. J. Maskell. 2003. *Bordetella bronchiseptica* PagP is a Bvg-regulated lipid A palmitoyl transferase that is required for persistent colonization of the mouse respiratory tract. *Mol. Microbiol.* **48:** 725–736.
34. Gunn, J. S. 2001. Bacterial modification of LPS and resistance to antimicrobial peptides. *J. Endotoxin Res.* **7:** 57–62.