

Detailed characterization of the lipid A fraction from the nonpathogen *Acinetobacter radioresistens* strain S13

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Abstract The genus *Acinetobacter* is composed of ubiquitous, generally nonpathogen environmental bacteria. Interest concerning these microorganisms has increased during the last 30 years, because some strains, belonging to the so-called *A. baumannii*-*A. calcoaceticus* complex, have been implicated in some severe pathological states in debilitated and hospitalized patients. The involvement of lipopolysaccharides (LPSs) as virulence factors in infections by *Acinetobacter* has been proven, and ongoing studies are aimed toward the complete serological characterization of the O-polysaccharides from LPSs isolated in clinical samples. Conversely, no characterization of the lipid A fraction from *Acinetobacter* strains has been performed. Here, the detailed structure of the lipid A fraction from *A. radioresistens* S13 is reported for the first time. *A. radioresistens* strains have never been isolated in cases of infectious disease. Nevertheless, it is known that the lipid A structure, with minor variations, is highly conserved across the genus; thus, structural details acquired from studies of this nonpathogen strain represent a useful basis for further studies of pathogen species.—Leone, S., L. Sturiale, E. Pessione, R. Mazzoli, C. Giunta, R. Lanzetta, D. Garozzo, A. Molinaro, and M. Parrilli. Detailed characterization of the lipid A fraction from the nonpathogen *Acinetobacter radioresistens* strain S13. *J. Lipid Res.* 2007. 48: 1045–1051.

Supplementary key words lipopolysaccharide • matrix-assisted laser-desorption ionization mass spectrometry • fatty acid

Bacteria of the genus *Acinetobacter* are ubiquitous, generally nonpathogen microorganisms belonging to the family Moraxellaceae (1). These bacteria have gained interest, in the last 30 years, because of the identification of some *Acinetobacter* species causing severe nosocomial infections, particularly in compromised patients. Diseases commonly associated with *Acinetobacter* infections include nosocomial pneumonia, urinary tract infections, meningitis, and in

some cases sepsis (2), depending on the site of infection and on the patient's susceptibility. Occasionally, these infections have a lethal course, as a result of the frequently encountered pan-resistance to antibiotic therapies exhibited by *Acinetobacter* virulent strains. Among the 19 genomic species identified within the genus to date, those detected in human clinical isolates usually belong to the so-called *A. calcoaceticus*-*A. baumannii* complex (3, 4), a set of four distinct genomic species of ascertained virulence. Other *Acinetobacter* genomovars are only occasionally associated with human infections (i.e., *Acinetobacter lwoffii*, a common colonizer of food, which is able to trigger gastritis in debilitated patients with no history of *Helicobacter pylori* colonization) (5).

The mechanisms by which *Acinetobacter* expresses its pathogenicity are not yet completely elucidated. Nevertheless, it is known that a crucial role is played by capsular polysaccharides and lipopolysaccharides (LPSs). These two moieties act in synergy by blocking the access of the human complement to the bacterial cell wall, thus preventing its lytic activity on bacterial membranes (6). Furthermore, the hydrophilicity conferred by the presence of capsular polysaccharides, together with other nonspecific adherence factors (i.e., fimbriae), prompts the colonization of human epithelial cells by pathogen strains (3, 4). Enzymes able to damage tissue lipids are also produced during infectious stages of the colonization (7). Interestingly, the action of LPSs in the pathogenicity of *Acinetobacter* is not limited to the protection of bacterial cells from host defenses; these molecules are in fact provided with a powerful endotoxic activity, and their production *in vivo* is thought to be related to *Acinetobacter* septicemia symptoms, given the ability of *Acinetobacter* LPSs to induce lethal toxicity in mice and pyrogenicity in rabbits. It is well known that a close correlation exists between LPS structure and pathogenicity, so it seems clear

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that a deeper knowledge of the structural details of these molecules constitutes an essential basis to understand the mechanisms that lead to the infectious course.

LPSs are characteristic and vital molecules constituting the outer leaflet of the Gram-negative bacteria outer membrane (8). Their chemical nature affects the physical properties of the cell envelope, thus determining the interactions with the surrounding environment as well as the host-pathogen recognition mechanism. From a structural point of view, these amphiphilic molecules can be divided in two categories: smooth-type LPSs, composed of the glycolipid portion of lipid A, the core oligosaccharide, and the *O*-polysaccharide or *O*-specific chain; and rough-type LPSs (R-LPSs), in which the *O*-polysaccharide is missing (9, 10). The *O*-polysaccharide represents the most variable portion of the molecule, possessing intraspecies variability, and constitutes the antigenic determinant of LPSs, being recognized by specific antibodies of the host acquired immune system. The structural variability that characterizes the polysaccharide moiety decreases in the core oligosaccharide and primarily in lipid A, whose architecture is usually conserved within the genus. All virulent strains of *Acinetobacter* have been found to possess smooth-type LPSs, and ongoing studies are attempting to realize a complete *O*-serotyping of clinical *Acinetobacter* isolates (11–15). Nevertheless, it is known that the most powerful immunostimulator center of the LPS is the lipid A glycolipid moiety, which is able to activate the innate immune system (16–18), triggering cytokine production that, when uncontrolled, can lead to septic shock. To date, only partial data have been available on *Acinetobacter* lipid A structure (19), and in old studies the occurrence and nature of amide-linked (*R*)-3-acyloxyacyl residues was defined. Nevertheless, no recent investigation has been performed, using state-of-the-art analytical techniques, aimed at the complete characterization of this moiety.

In a previous report, we described the structure of the core-lipid A saccharide backbone from *A. radioresistens* S13 (20), a nonpathogen strain isolated from the soil surrounding an activated sludge plant in Torino (Italy) and selected for its ability to efficiently metabolize phenol and benzoate (21). It is also able to produce a powerful extracellular emulsifier, alasan, strictly involved in the solubilization of hydrophobic molecules and structurally related to the outer membrane protein OmpA (22). Structural investigation of the core-lipid A region from the R-LPS of *A. radioresistens* showed close similarity, especially in the inner core-lipid A portion, with oligosaccharides from other *Acinetobacter* strains, and particularly with the core region of the virulent *A. baumannii* strain ATCC 19606 (23). Remarkable features of the isolated oligosaccharide are the absence of heptoses and the occurrence of phosphorylation only at the lipid A glucosamine backbone. In this work, the full structure of the lipid A of *A. radioresistens* S13 is investigated and the complete acylation/phosphorylation pattern of the lipid A fraction from *A. radioresistens* S13 is reported. This represents the first detailed investigation of a lipid A fraction from an *Acinetobacter* species; it is reasonable to assume that, with very minor variations,

this structure represents a frame to which other lipid A from pathogen strains are strictly related.

EXPERIMENTAL PROCEDURES

Cell growth and LPS extraction

Cells were cultured in Sokol and Howell (24) minimal medium supplemented with phenol (400 mg/L) as the sole carbon source. Cultures were incubated in Erlenmeyer flasks at 30°C in a Gallenkamp oscillating shaker (210 rpm). Biomass was harvested by centrifugation (3,000 g, 15 min, 4°C) at the end of the exponential growth phase. Isolation of the R-LPS was performed from the dried cells (3.520 g) by aqueous 90% phenol-chloroform-petroleum ether (2:5:8, v/v/v) extraction (25). After removal of the light solvents under vacuum, R-LPS was precipitated from the phenol with water and washed with aqueous 80% phenol and then with acetone, each time centrifuged and lyophilized. The extract was then suspended in 20 ml of 4.2 mM MgCl₂ and 2 mM CaCl₂ buffer and treated with 2 mg of DNase and 2 mg of RNase (5 h, 25°C) and subsequently with proteinase K (16 h, 25°C). After dialysis and lyophilization, the R-LPS (40 mg, 1.1% of the dry mass) was analyzed by silver-stained SDS-PAGE as described (26).

Isolation of lipid A and chemical characterization

Free lipid A was obtained after treatment of the R-LPS (18 mg) with 1% acetic acid (100°C, 2 h). After centrifugation (8,000 g, 60 min, 4°C), the lipid A was collected as precipitate, washed twice with water, and lyophilized (8 mg). Monosaccharides were analyzed as acetylated *O*-methyl glycoside derivatives obtained after methanolysis (2 M HCl/methanol, 85°C, 16 h) and acetylation with acetic anhydride in pyridine (85°C, 30 min). The absolute configuration of the monosaccharides was obtained according to a published method (27).

Methylation analysis was performed using the Hakomori procedure (28) modified by Ciucanu and Kerek (29). After chloroform-water extraction, the organic phase was evaporated and hydrolyzed with 4 M trifluoroacetic acid (100°C, 3h), carbonyl reduced with NaBD₄, acetylated with 1:1 acetic anhydride and pyridine, and analyzed by GC-MS.

Total fatty acid content was determined after strong hydrolysis of lipid A, first with 4 M HCl (100°C, 4 h) and subsequently with 5 M NaOH (100°C, 30 min). Fatty acids were then extracted with chloroform, methylated with diazomethane, and analyzed by GC-MS. Ester-bound fatty acids were analyzed after selective alkaline hydrolysis with 0.5 M NaOH/methanol (1:1, v/v; 85°C, 2 h). After acidification and extraction with chloroform, fatty acids were methylated with diazomethane and analyzed by GC-MS. Fatty acids were identified by comparison of typical retention times and MS fragmentation with the standard bacterial fatty acid methyl esters (Supelco). Absolute configurations of fatty acids were determined as described (30).

Mass spectrometry

Mass spectrometry of the native, the partially *O*-deacylated and the dephosphorylated lipid A was performed on a 4800 Proteomics analyzer MALDI time-of-flight/time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA) in reflector mode, both in negative and in positive polarity. Compounds were dissolved in CHCl₃/CH₃OH (50:50, v/v) at a concentration of 1 μg/μl. Matrix solution was prepared by dissolving trihydroxyacetophenone (THAP) in CH₃OH/0.1% trifluoroacetic acid/CH₃CN (7:2:1, by volume) at a concentration of 75 mg/ml⁻¹. One microliter of

the sample/matrix solution (1:1, v/v) was deposited onto a Opti-TOF™ 384 well plate and allowed to dry at room temperature. Mass spectra, resulting from the sum of 1250 laser shots, were obtained with a resolution higher than 10,000 (as the ratio between the mass of the peak and its full width at half maximum intensity) and with mass accuracy below 100 ppm.

RESULTS AND DISCUSSION

The R-LPS from *A. radioresistens* S13 was extracted from a bacterial culture, supplied with phenol as the sole carbon and energy source, by the described phenol-chloroform-petroleum ether procedure (25) and purified from nucleic acids and contaminant proteins by means of enzymatic digestion with DNase, RNase, and proteinase K. Silver-stained SDS-PAGE of the extracts revealed the typical low-migrating bands observed for R-LPS. Lipid A was obtained after mild acid hydrolysis with acetic acid to selectively cleave the linkage between the core oligosaccharide and the lipid A glucosamine backbone and underwent chemical analyses for sugar and fatty acid determination. Monosaccharide and methylation analyses showed the occurrence of 6-substituted-glucosamine and terminal glucosamine, both possessing the D configuration, in agreement with what was observed previously in the core-lipid A structure (20), from which we know that the disaccharide backbone is composed of two 2-amino-2-deoxy-glucose residues (GlcN I and GlcN II) linked by the typical β -(1-6) glycosidic linkage and phosphorylated at positions

1 and 4', as unambiguously confirmed by either NMR and MALDI MS analyses performed on the partially degraded as well as the intact R-LPS from *A. radioresistens* S13.

Total and O-linked fatty acids were analyzed by GC-MS of their O-methyl ester derivatives and revealed the presence of amide-linked (*R*)-3-hydroxy-tetradecanoic acid [14:0(3-OH)], (*R*)-3-hydroxy-dodecanoic acid [12:0(3-OH)], both in ester and amide linkage, secondary dodecanoic acid (12:0), and, in a minor percentage, (*S*)-2-hydroxy-dodecanoic acid [12:0(2-OH), ~5% over 12:0(3-OH)].

The lipid A fraction was then analyzed by MALDI MS. The negative ion mass spectrum recorded on the intact lipid A fraction showed a peculiar appearance, attributable to the presence of distinct groups of ions representative of the microheterogeneity of the sample. Three main series of signals (A–C) were detectable, indicating molecular species ranging from penta- to hepta-acyl substitution (Fig. 1). Acyl substitution for the main peak of species A, in accordance with the ion peak at m/z 1,838.18, was realized by four 12:0(2-OH) and three 12:0 residues. A less intense peak at m/z 1,866.23 ($\Delta m/z = 28$) was originated by the molecular species possessing three 12:0(3-OH), one 14:0(3-OH), and three 12:0 residues. Both peaks showed minor related signals with $\Delta m/z = 16$, indicating non-stoichiometric substitution of one 12:0 residue with one 12:0(3-OH) residue. The same diversity in fatty acid substitution was observable for molecular species B (hexa-acyl), at m/z 1,656.05 and 1,684.07, differing from the main two species A for a missing secondary 12:0 residue. Another peak at m/z 1,640.04, corresponding to a hexa-acyl substi-

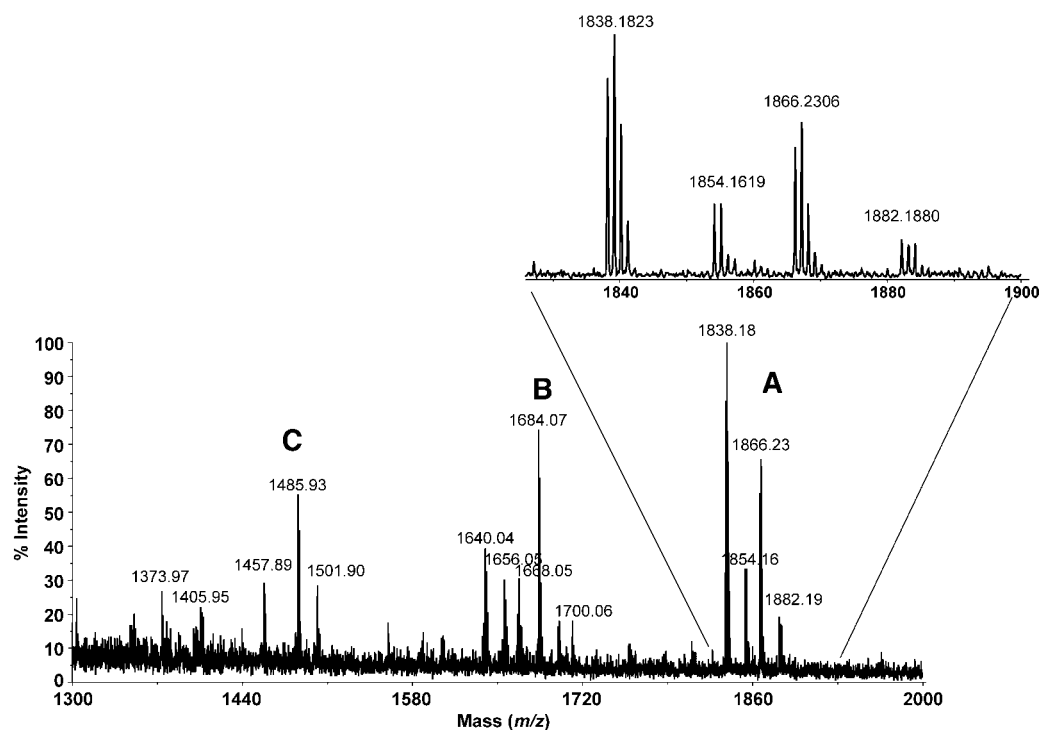


Fig. 1. Negative ion matrix-assisted laser-desorption ionization (MALDI) mass spectrum of the intact lipid A fraction from *A. radioresistens* S13. Uppercase letters refer to the relevant ion peak clusters described in Table 1 and in the text.

tution with three 12:0(3-OH) and three 12:0 fatty acids, was also present. Notably, the latter hexa-acylated species must have three primary and three secondary fatty acids. The ions belonging to series **C** were generated by penta-acyl species, the components of which were a peak at m/z 1,485.93 having two 12:0(3-OH), one 14:0(3-OH), and two 12:0 fatty acids, the related peak at $\Delta m/z = 16$ (m/z 1,501.90) with a 12:0 unit substituted from a 12:0(2-OH) residue, and the species at m/z 1,457.89 with three 12:0(3-OH) and two 12:0 fatty acids. The molecular masses for all of the assigned species are listed in **Table 1**.

To locate the fatty acid residues, lipid A underwent two parallel and complementary treatments: mild hydrolysis with ammonium hydroxide and total dephosphorylation with 48% hydrofluoric acid. The first procedure leaves unaffected the amide-linked acyloxyacyl groups and selectively removes ester-linked acyloxyacyl and acyl moieties (31). The negative ion MALDI mass spectrum (**Fig. 2**) of the obtained product showed three main ion groups (**D–F**). The most abundant species (**E**), at m/z 1,105.60, was generated by a tri-acyl species carrying one 14:0(3-OH), one 12:0(3-OH), and one 12:0 residue. Also in this case, $\Delta m/z = 28$ was observable with the peak at m/z 1,077.56, indicating the nonstoichiometric replacement of the 14:0(3-OH) unit present as one of the two amide-linked primary fatty acids by a 12:0(3-OH). The peak at m/z 1,121.59 ($\Delta m/z = 16$), again, showed the occurrence of a nonstoichiometric substitution of the secondary 12:0 with one 12:0(2-OH) residue. A less intense series of peaks (**D**), at m/z 1,259.73 and 1,287.76, was generated by the species possessing one additional secondary 12:0 residue, whereas the peaks at m/z 923.42 and 895.37 (**F**) were generated by the GlcN disaccharide carrying one 12:0(3-OH) and one 14:0(3-OH) residue or two 12:0(3-OH) residues, respectively. In correspondence with the tetra-acyl species (**D**), minor peaks were observable at m/z 1,275.73 and 1,303.76, representative of nonstoichiometric secondary substitution by one or two 12:0(2-OH) secondary fatty acids replacing the 12:0 residues. Obviously, this also means that 14:0(3-OH) is present only as an amide substituent.

Interestingly, the relative intensity ratio between the peaks differing by 28 m/z in correspondence with species **E**

(tri-acyl) and **D** (tetra-acyl) resembles the intensity ratio of the corresponding peaks for species **B** (hexa-acyl) and **A** (hepta-acyl) in the intact lipid A mass spectrum, proving that in the fully acylated species substitution by two 12:0(3-OH) residues on the amide position is a more frequent event than the corresponding substitution by one 12:0(3-OH) and one 14:0(3-OH) residue, as observed in the other molecular species within the natural blend. The positive ion MALDI mass spectrum of the same ammonium-treated sample (data not shown) enabled the detection, besides the molecular peaks of the already observed species, of the oxonium ion, generated by the in-source cleavage of the glycosidic linkage of the nonreducing GlcN residue (GlcN II) at m/z 622.1. This value was in accord with the protonated molecular ion of a species composed of one GlcN residue carrying one phosphate group, one 12:0(3-OH), and one 12:0 residue. The absence of signals for the species with one 14:0(3-OH) residue suggested that the heterogeneity in amide substitution was localized on the reducing residue of GlcN (GlcN I).

A second aliquot of lipid A was analyzed after dephosphorylation treatment with 48% HF by MALDI MS. In fact, the data still missing, concerning primary and secondary *O*-acylation, could only be deduced from the oxonium ion peak for GlcN II in the positive MALDI mass spectrum on the fully acylated species, which could be observed only after removal of the negative charges from the molecule. The positive ion spectrum (**Fig. 3**) showed at higher mass range the sodiated molecular ion series corresponding to the intact lipid A mixture at different degrees of acylation, as already described, whereas the spectrum at lower mass range revealed the presence of a peak at m/z 740.49, assignable to the oxonium ions GlcN II tri-acyl substituted by two 12:0(3-OH) and one 12:0 residue as well as the occurrence of two tetra-acyl oxonium ions at m/z 922.65 and 938.65 with one additional 12:0 or one 12:0(2-OH) residue, likely linked as acyloxyacyl to the ester-linked 12:0(3-OH) residue. Also in this case, there was no evidence for substitution by 14:0(3-OH) on GlcN II, indicating that this fatty acid must selectively link GlcN I.

The definition of the complete acylation pattern for GlcN I was made possible by comparison of the data

TABLE 1. Proposed acyl and phosphate contents of the molecular species composing the intact lipid A fraction from *A. radioresistens* S13

Theoretical Monoisotopic Mass [M-H] ⁻	Observed Monoisotopic Mass [M-H] ⁻	Experimental Error	Species	Acyl Substitution	Fatty Acids and Phosphate Substitution
		<i>ppm</i>			
1,882.2488	1,882.1880	32	A	Hepta-acyl	GlcN ₂ -P ₂ -[12:0(3-OH)] ₄ -(12:0) ₂ -14:0(3-OH)
1,866.2539	1,866.2306	12	A	Hepta-acyl	GlcN ₂ -P ₂ -[12:0(3-OH)] ₃ -(12:0) ₃ -14:0(3-OH)
1,854.2175	1,854.1619	19	A	Hepta-acyl	GlcN ₂ -P ₂ -[12:0(3-OH)] ₅ -(12:0) ₂
1,838.2225	1,838.1823	22	A	Hepta-acyl	GlcN ₂ -P ₂ -[12:0(3-OH)] ₄ -(12:0) ₃
1,700.0818	1,700.0653	10	B	Hexa-acyl	GlcN ₂ -P ₂ -[12:0(3-OH)] ₄ -12:0-14:0(3-OH)
1,684.0869	1,684.0656	13	B	Hexa-acyl	GlcN ₂ -P ₂ -[12:0(3-OH)] ₃ -(12:0) ₂ -14:0(3-OH)
1,668.0920	1,668.0513	26	B	Hexa-acyl	GlcN ₂ -P ₂ -[12:0(3-OH)] ₂ -(12:0) ₃ -14:0(3-OH)
1,656.0555	1,656.0490	4	B	Hexa-acyl	GlcN ₂ -P ₂ -[12:0(3-OH)] ₄ -(12:0) ₂
1,640.0607	1,640.0387	13	B	Hexa-acyl	GlcN ₂ -P ₂ -[12:0(3-OH)] ₃ -(12:0) ₃
1,501.9198	1,501.9022	12	C	Penta-acyl	GlcN ₂ -P ₂ -[12:0(3-OH)] ₃ -12:0-14:0(3-OH)
1,485.9249	1,485.9281	2	C	Penta-acyl	GlcN ₂ -P ₂ -[12:0(3-OH)] ₂ -(12:0) ₂ -14:0(3-OH)
1,457.8937	1,457.8921	1	C	Penta-acyl	GlcN ₂ -P ₂ -[12:0(3-OH)] ₃ -(12:0) ₂

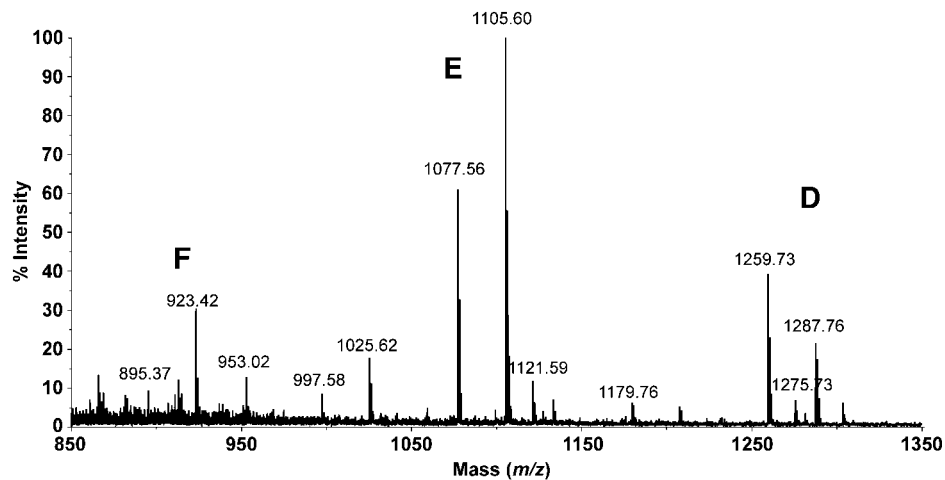


Fig. 2. Negative ion MALDI mass spectrum of the ammonium-treated lipid A fraction from *A. radioresistens* S13. Upper case letters refer to the identified molecular species discussed in the text.

concerning the partially degraded lipid A with the peaks in the intact species spectrum, finally allowing the delineation of the definitive acylation pattern of lipid A from *A. radioresistens* S13. In the highest fatty acid-containing species, namely the hepta-acyl lipid A, three residues are present on GlcN I, namely one 14:0(3-OH) or 12:0(3-OH) in amide linkage with a secondary 12:0, replaced, in a very minor amount, by a 12:0(2-OH), as observed in the ammonium-treated sample, and one 12:0(3-OH) in ester linkage, whereas GlcN II must be present in the tetra-acyl form. Hexa- and penta-acyl lipid A differ from this molecular species by one or two 12:0 residues, namely the acyloxyacyl ester on GlcN II, as proven by the ion peak in the positive spectrum of the dephosphorylated sample, and/or the acyloxyacylamide on GlcN I. Finally, the minor

tetra-acyl species differs from the penta-acyl lipid A for one 12:0(3-OH) residue, the ester at *O*-3 of GlcN I. These structures are summarized in **Fig. 4**.

Conclusion

In the last 30 years, bacteria belonging to genus *Acinetobacter* have earned importance because of the role they play in triggering severe nosocomial infections. Despite the great importance of their LPSs in the start of the pathogenic process, the studies performed to date were aimed mainly toward a full structural and serological characterization of the *O*-polysaccharide moiety, commonly associated with *Acinetobacter* virulent strains. To date, no detailed investigation of the lipid A fraction from

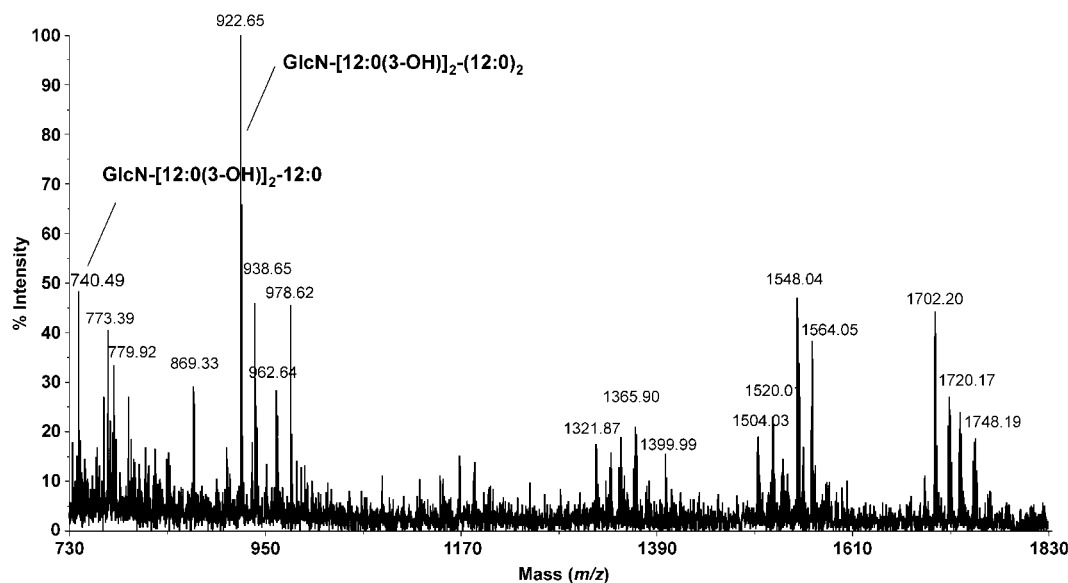


Fig. 3. Positive ion MALDI mass spectrum of the dephosphorylated lipid A fraction from *A. radioresistens* S13. The structures of the main oxonium ions are reported at low mass range.

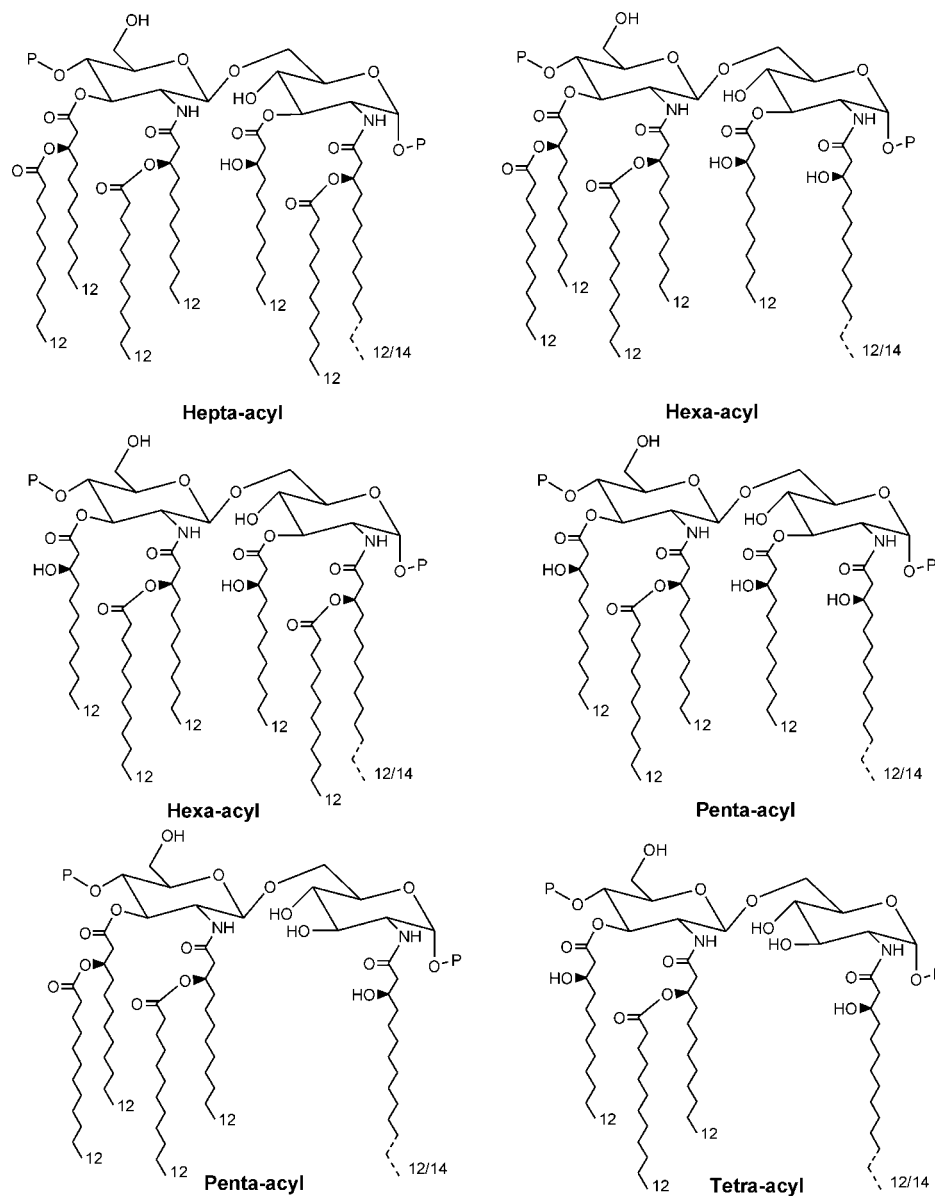



Fig. 4. Complete structures of the different lipid A species from *A. radioresistens* S13. Dotted linkages indicate nonstoichiometric fatty acid chain lengths.

Acinetobacter LPSs was performed, even though this portion of the molecule has been recognized as the most powerful activator of host immune responses during infection and colonization, representing the actual endotoxic principle within the molecule. Moreover, it is known that the strength of the observed immune response, in the case of Gram-negative bacteria infection, is closely related to three-dimensional conformation effects exerted by the lipid A moiety (32). Studies performed on *Escherichia coli* lipid A showed that the highest biological activity is associated with a high degree of acylation, with the asymmetrical distribution of the alkyl chain with respect to the two glucosamine residues, and with phosphate content. These factors lead to conformational changes in lipid A that are closely related to the ability to induce the cytokine host reaction.

This study is a first step in understanding the mechanisms of pathogenesis correlated to highly virulent strains within the *Acinetobacter* genus. In this work, we demonstrated that lipid A from *A. radioresistens* S13 is composed of a heterogeneous blend of molecules differing in degree of acylation, among which the more abundant is represented by the di-phosphoryl hepta-acyl species. This molecular species is associated with the highest level of activation of the human immune system (32). Nonetheless, the symmetrical hexa-acyl species is also present in a comparable amount and is associated with low toxicity levels. Given the intragenus regularity of lipid A, it is possible to surmise that a similar architecture, with minor structural modifications, is conserved in other *Acinetobacter* strains of recognized toxicity. The higher pathophysiological impact, associated, for example, with the *A. baumannii*-*A. calcoaceticus*

complex, could be ascribed in part to structural changes in this lipid A skeleton (e.g., a further acylation). Besides the effects of the other and most variable LPS portions in triggering the responses of the host acquired immune system, the biological activity of the different strains within the genus depends on the acylation pattern of lipid A. The activation of the innate immune system, in fact, is performed by the natural blend of variously acylated lipid A species produced by the bacterium; pathogen strains likely express higher percentages of hepta-acyl lipid A, and this may be one of the reasons for the increased response observed in the colonized organisms.

This hypothesis is in full agreement with what was recently demonstrated for *Porphyromonas gingivalis* (33). This bacterium produces a LPS with structural types of lipid A differing in acylation pattern, namely penta-acylated and tetra-acylated, that is able to induce opposite effects in the immune system. By altering the relative amount of the two lipid A structures, *P. gingivalis* is able to modulate the innate host response, and a similar mechanism could also regulate the virulence of *Acinetobacter* strains. 

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