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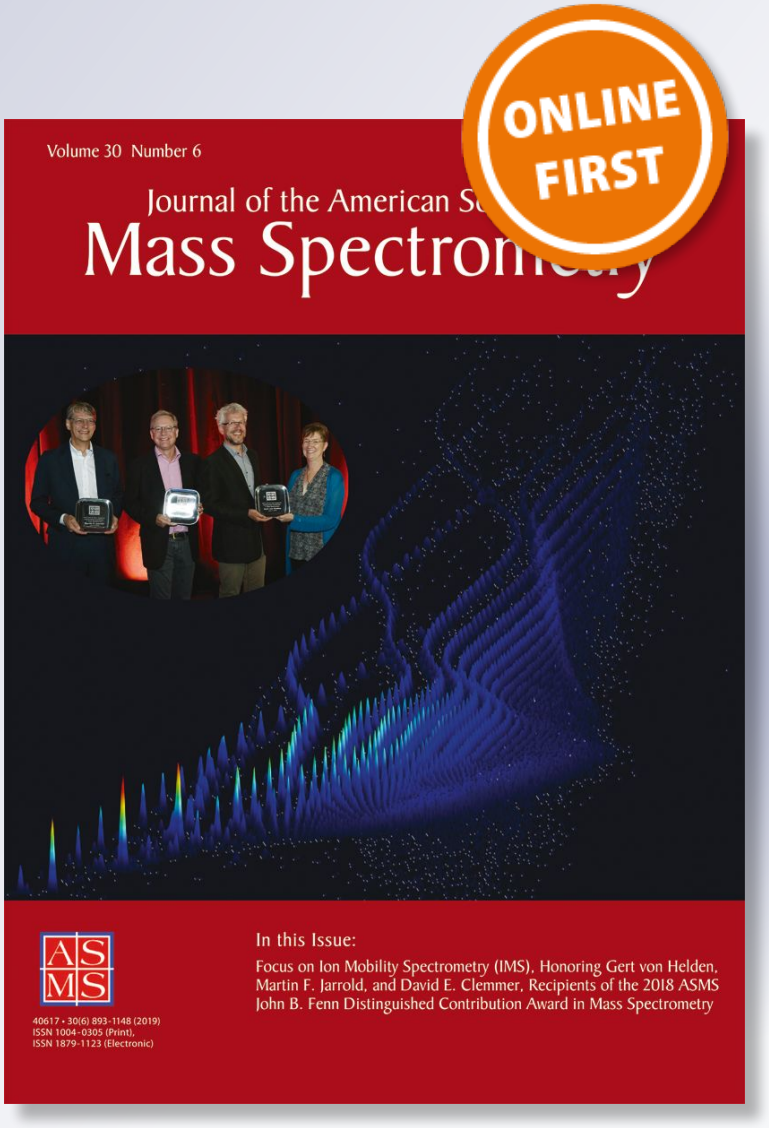

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RESEARCH ARTICLE

Bordetella bronchiseptica Glycosyltransferase Core Mutants Trigger Changes in Lipid A Structure

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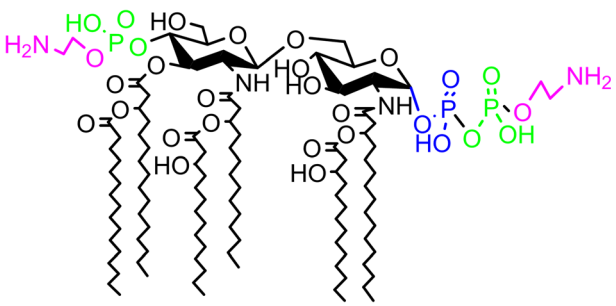

Abstract. *Bordetella bronchiseptica*, known to infect animals and rarely humans, expresses a lipopolysaccharide that plays an essential role in host interactions, being critical for early clearance of the bacteria. On a *B. bronchiseptica* 9.73 isolate, mutants defective in the expression of genes involved in the biosynthesis of the core region were previously constructed. Herein, a comparative detailed structural analysis of the expressed lipids A by MALDI-TOF mass spectrometry was performed. The *Bb*3394 LPS defective in a 2-amino-2-deoxy-D-galacturonic acid lateral residue of the core presented a penta-acylated diglucosamine backbone modified with two glucosamine phosphates, similar to the wild-type lipid A. In contrast, *Bb*LP39, resulting in the interruption of the LPS core oligosaccharide synthesis, presented lipid A species consisting in a diglucosamine backbone N-substituted with C14:0(3-O-C12:0) in C-2 and C14:0(3-O-C14:0) in C-2′, O-acylated with C14:0(3-O-C10:0(3-OH) in C-3′ and with a pyrophosphate in C-1. Regarding *Bb*3398 also presenting a rough LPS, the lipid A is formed by a hexa-acylated diglucosamine backbone carrying one pyrophosphate group in C-1 and one phosphate in C-4′, both substituted with ethanolamine groups. As far as we know, this is the first description of a phosphoethanolamine modification in *B. bronchiseptica* lipid A. Our results demonstrate that although gene deletions were not directed to the lipid A moiety, each mutant presented different modifications. MALDI-TOF mass spectrometry was an excellent tool to highlight the structural diversity of the lipid A structures biosynthesized during its transit through the periplasm to the final localization in the outer surface of the outer membrane.

Keywords: *B. bronchiseptica*, Lipopolysaccharide, UV-MALDI-TOF MS, Lipid A modifications

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Introduction

The lipopolysaccharide (LPS) is a ubiquitous component of the Gram-negative bacterial surface and is important for pathogenesis [1]. LPS is a complex polymer composed by two or three main parts: a glycolipid region called lipid A anchored in the outer membrane, an oligosaccharide (OS) region named core, and a polysaccharide region attached to the core called *O* antigen [2]. Lipid A consists of a β-D-GlcNp(1 6)-α-D-GlcNp disaccharide with 4 to 7 fatty acids and it may be also substituted with one or two negatively charged phosphates. Bacteria can modify the lipid A structure to optimize survival or enhance virulence [3]. Modifications in acyl chain composition, phosphate groups' localization, or other functional groups attached

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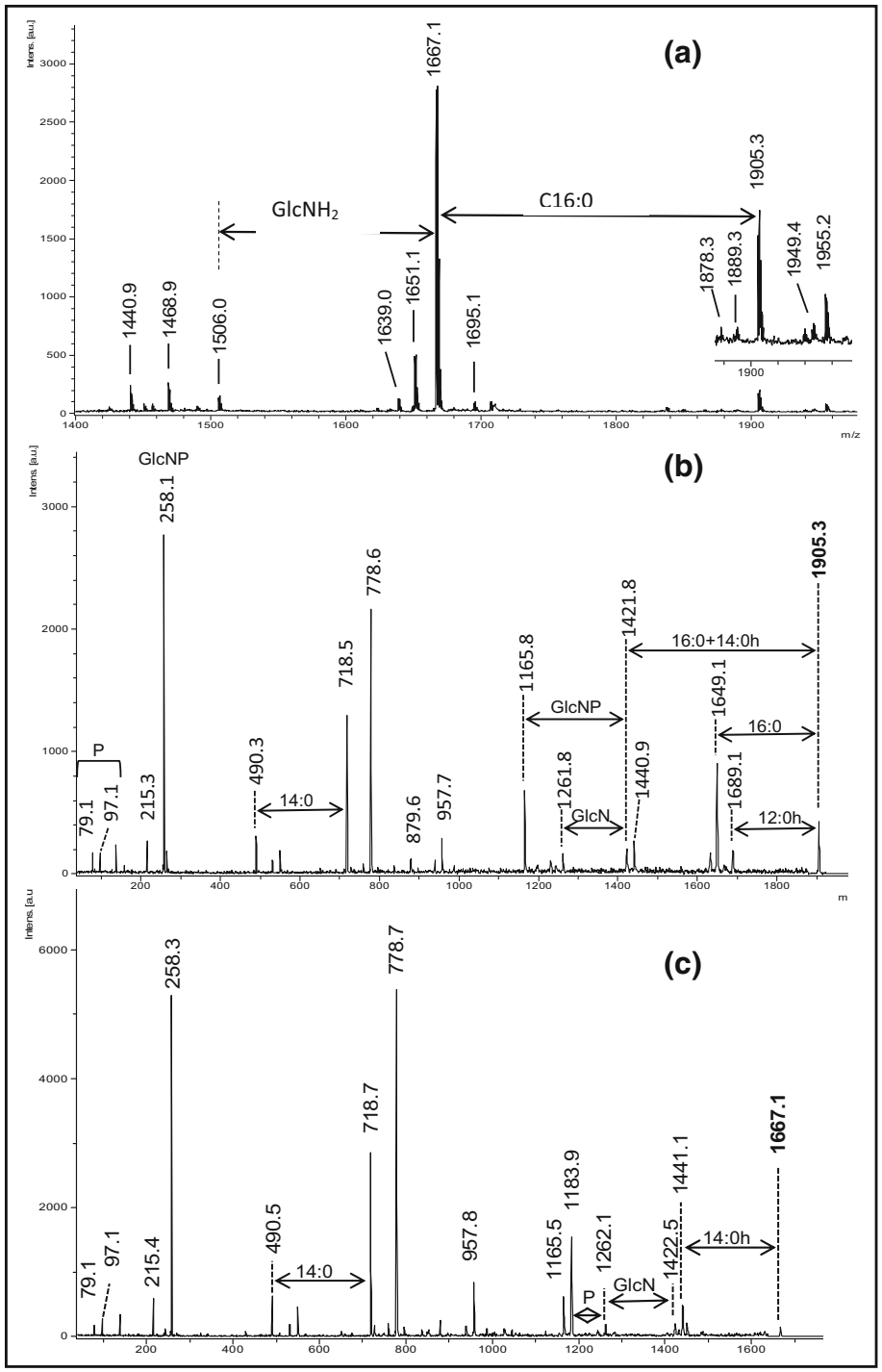


Figure 1. MALDI-TOF mass spectrum of the lipid A released from the wild-type LPS. (**a**) Spectrum performed in the negative reflectron ion mode. (**b**) LID-MS/MS analysis in the negative ion mode of precursor ion *m/z* 1905 shown in (**a**). (**c**) LID-MS/MS analysis in the negative ion mode of precursor ion *m/z* 1667shown in (**a**)

to lipid A can induce changes in biological activity. Thus, it was reported that *N. gonorrhoeae* mutant strains that carry an inactivated *msbB* gene, producing a penta-acylated lipid A instead of the hexa-acylated structure, elicit reduced inflammation and increased host survival [4].

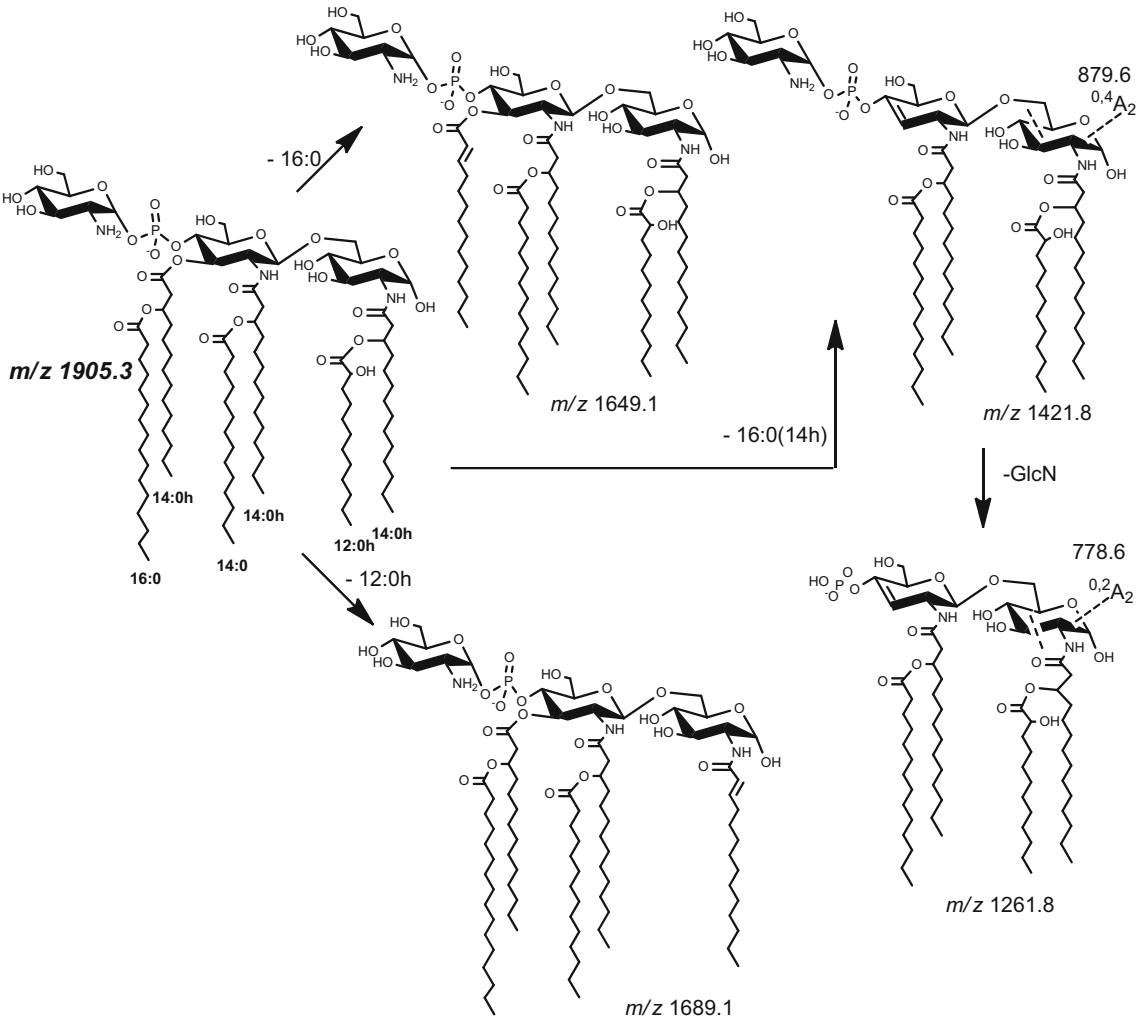
The genus *Bordetella* includes species that causes respiratory infection in different hosts. *B. pertussis* and *B. parapertussis* cause whooping cough in humans while *B. bronchiseptica* infects animals, and rarely humans [5–7]. *B. bronchiseptica* expresses a smooth LPS consisting in a lipid A domain linked to a branched-chain core nonasaccharide (Hep)GlcN-(GalNA)Glc-(GlcAGlcNHep)HepKdo where Hep accounts for L-*glycero*-D-manno-heptose. A distal trisaccharide links the O antigen composed by a homopolymer of 2,3-dideoxy-2,3-di-N-acetylgalactosaminuronic acid (GalNAc3NAcA) to the core region [8]. Oligosaccharide portion plays a fundamental role in

Table 1. Proposed Substituent Distribution of the Lipid A Species Observed by MALDI-TOF m.s and MS/MS Analysis Released from *B. bronchiseptica* Wild-Type and Mutant LPSs

	Acylation degree	Positions on the diglucosamine backbone						$[M-H]^-$ *m/z*
		C4	C3′	C2′	C3	C2	C1	
*Bb*9.73	Hexa	GlcNP	C14:0(*3-O*-C16:0)	C14:0(*3-O*-C14:0)	–	C14:0(*3-O*-C12:0)	–	1905.3
*Bb*LP39	Hexa	–	C14:0(*3-O*-C10:0h)	C14:0(*3-O*-C14:0h)	–	C14:0(*3-O*-C12:0)	PP	1756.1
*Bb*3394	Penta	GlcNP	C14:0(*3-O*-C16:0)	C14:0(3-OH)	–	C14:0(*3-O*-C16:0)	GlcNP	1976.5
*Bb*3398	Penta	PEtN	C14:0(*3-O*-14:0h)	C14:0(3-OH)	–	C14:0(*3-O*-14:0h)	PPEtN	1795.7

host interaction, presumably in the first steps of infection and in immune response regulation [9–10].

A bisphosphorylated β-D-GlcNp(1 6)-α-D-GlcNp disaccharide backbone with two N-acyl C14:0 (3-OH) substituents in C2 and C2′ and O-linked in C-3′ was found in all *B. bronchiseptica* lipid A known to the present [11–12]. The nature and distribution of ester-linked fatty acids vary among different strains [13–15]. One of the unusual features of *Bordetella* lipid A is the absence of substitution at C-3 position in the mature lipid A due to the activity of the PagL enzyme [16]. *B. bronchiseptica* lipid A species, modified with a C16:0 ester linked to the primary C14:0 (3-OH) in C-3′ position, were detected in wild-type strains [17]. It was determined that the presence of a single C16:0 is not required for initial colonization of the respiratory tract or persistence within it during the early stages of infection and this lipid A structural variability has been attributed to relaxed enzyme specificity [11, 13–17]. More recently, the modification of the lipid A backbone with an additional GlcN was related to successful transmission between mice and colonization at lower infectious doses [18–19].



Scheme 1. Proposed LID fragmentation routes for ion *m/z* 1905.3 from mass spectrum of *Bb*9.73 lipid A performed in the negative ion mode

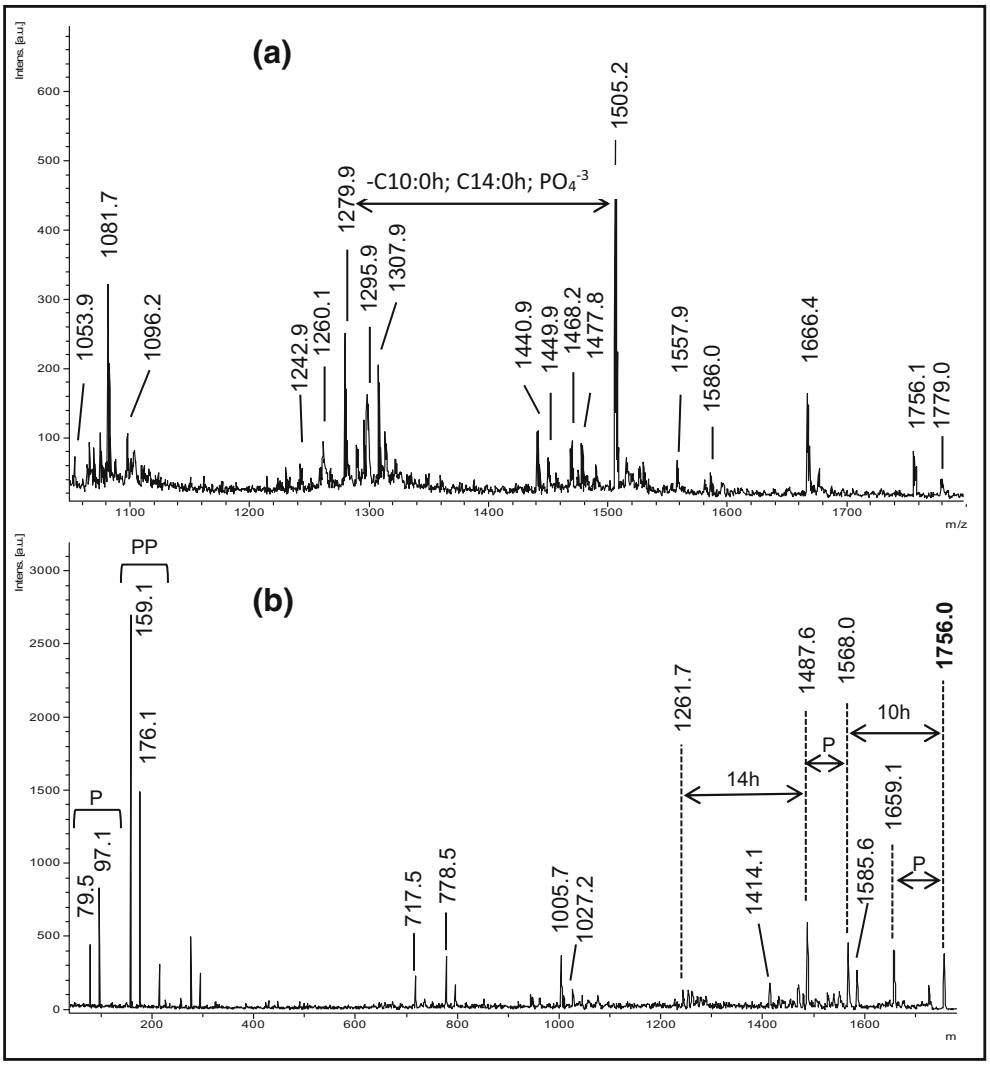


Figure 2. MALDI-TOF mass spectrum of the lipid A released from the *Bb*LP39 LPS. (**a**) Spectrum in the negative reflectron mode. (**b**) LID-MS/MS spectrum of the precursor ion *m/z* 1756.1 from (**a**)

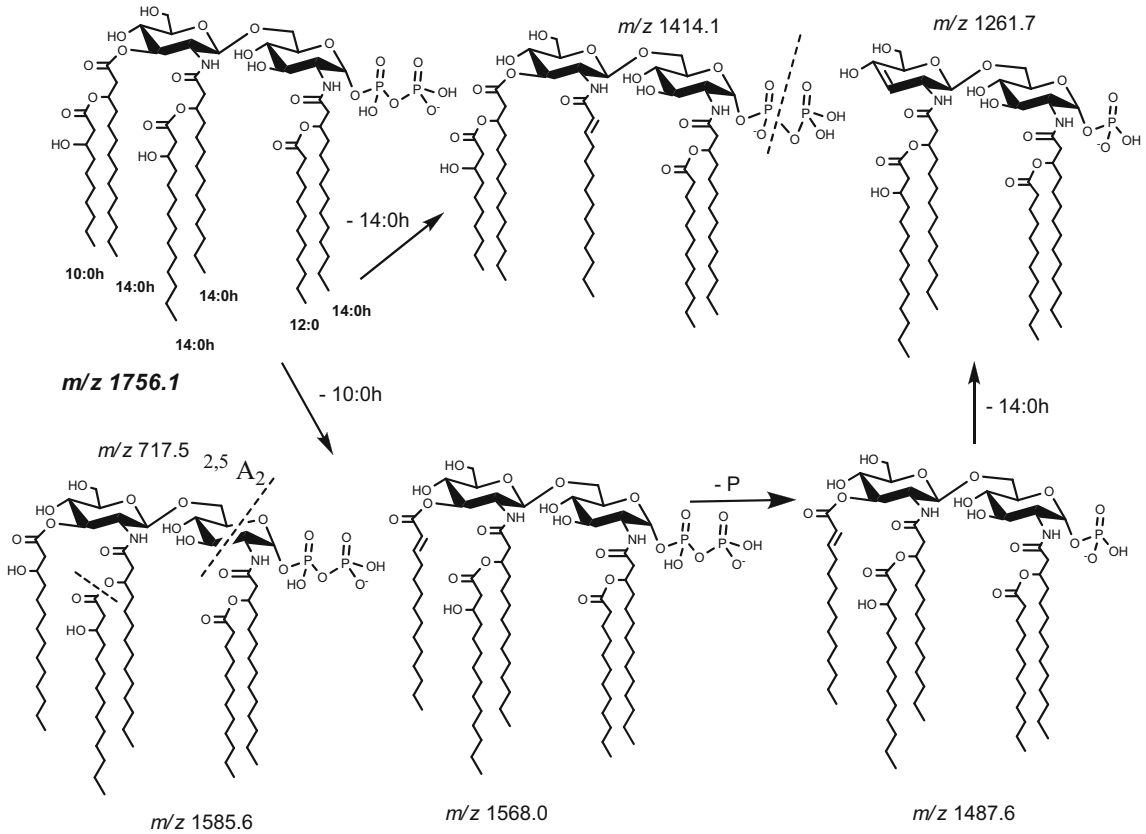
The LPS role in bacteria-host interactions has shown progress thanks to the identification of enzymes required for its synthesis. Deletion of enzyme-coding genes allowed an important advance in understanding LPS structure [9, 20]. Previously, on a non-human *B. bronchiseptica* isolate (*B. bronchiseptica* 9.73, *Bb*9.73), different mutants defective in the expression of genes involved in the biosynthesis of the LPS core region were constructed: *B. bronchiseptica* LP39 (*Bb*LP39), defective in the expression of *waaC* gene which codes for a heptosyltransferase of the core region [9]; *B. bronchiseptica* LP3394 (*Bb*3394), defective in gene *BB3394* involved in core substitution with a branching 2-amino-2-deoxy-D-galacturonic acid (GalNA); and *B. bronchiseptica* LP3398 (*Bb*3398), defective in the glycosyl transferase gene *BB3398* responsible for the linkage of the lateral L-glycero-D-manno-heptose (Hep) to the core and causing the absence of the *O* antigen [21].

A comparative analysis of the expressed lipids A was performed by MALDI-TOF MS, showing different lipid A modifications. Thus, two rough and two smooth structures bearing diversity not only in the acylation pattern but also in the substitution of the lipid A diglucosamine backbone were detected. This diversity highlights the importance of the whole LPS structure in its transit through the periplasm to the final localization in the outer surface of the outer membrane, and as it has been reported for other Gram-negative bacteria [22], these extra-cytoplasmic decorations may be suitable reporters to survey nascent LPS transit.

Experimental

Bacterial Strains and Growth Conditions

Bordetella bronchiseptica strain 9.73 (Collection de l'Institut Pasteur designation, *Bb*9.73) was grown on BGA medium (Difco, Sparks, MD, USA) at 36 °C for 48 h. *B. bronchiseptica* LP39 (*Bb*LP39), a deep rough mutant derived from *Bb*9.73 [10], was grown in BGA medium supplemented with streptomycin (200 µg/mL) and kanamycin (80 µg/mL). Both strains were replated in the same medium for 24 h. For LPS extraction, subcultures were grown in Stainer-Scholte



Scheme 2. Proposed LID fragmentation routes for ion *m/z* 1756.1 from mass spectrum of lipid A released from *Bb*LP39 LPS performed in the negative ion mode

liquid medium [21] for 20 h at 36 °C until the optical density measured at 650 nm reached 1.0.

LPS and Lipid A Isolation and Purification

B. bronchiseptica strains were grown at 36 °C in Stainer-Scholte medium for 20 h, centrifuged (10,000×*g*, 15 min, 4 °C) and washed twice in distilled water. LPSs were extracted by the hot-phenol-water method of Darveau and Hancock [23]. Isolated LPSs were sequentially treated with DNAse and proteinase K (10 µg/ml and 1 mg/ml overnight). The resultant LPS samples were dialyzed and lyophilized. The quality of each sample was checked by SDS-polyacrylamide gel electrophoresis. The isolated LPSs were stored at − 20 °C until used.

LPSs from mutants and from *Bb*9.73 were hydrolyzed with 2% acetic acid for 2 h at 100 °C. Precipitated lipids A were recovered by ultracentrifugation at 4 °C, 9.000×*g* for 60 min. The solutions containing the sugar moieties (called oligosaccharide) were separated and lyophilized. The solids were washed with water at 4 °C, ultracentrifuged, and freeze-dried. The lipids A were further purified by extraction with $CHCl_3$/MeOH/water (12:6:1) and were stored at − 20 °C.

Mass Spectrometry

MALDI-TOF MS was performed using an Ultraflex II MALDI-TOF/TOF mass spectrometer equipped with an ultraviolet high-performance solid-state laser (λ = 355 nm) and a reflector. The system was operated by Flexcontrol 3.3 software package (Bruker Daltonics GmbsH, Germany). The spectra recorded were the result of 1000–1500 laser shots. All samples were measured in the linear and the reflectron mode, and as routine in both positive and negative polarity. Laser power was typically 40–60% of its maximum intensity, the accelerating voltage 20 kV.

External calibration was carried out using commercial proteins bradykinin 1–7 (MW 757.399); angiotensin I (MW 1296.685); renin substrate (MW 1758.933); and insulin β-chain (MW 3494.6506) with CHCA as matrix and β-cyclodextrin (cycloheptaamylose, MW1135.0), and γ-cyclodextrin (cyclooctaamylose, MW 1297.1) with *nor*-harmane as matrix, in positive and negative ion mode.

For MALDI MS/MS analysis, the Ultraflex II MALDI-TOF/TOF MS spectrometer was used. For all experiments using the tandem-time-of-flight LIFT mode, the ion source voltage was set at 8.0 kV with a precursor ion mass window of 3 Da. Precursor ions generated by LID (laser-induced dissociation) were accelerated at 19.0 kV in the LIFT cell. The reflector voltage was set at 29.5 kV.

The samples were loaded onto a ground steel sample plate (MTP 384 ground steel; Bruker Daltonics GmbsH) using the sandwich method or by the classic dried drop method: a sample/matrix solution mixture 1 µl, 1:1 (*v*/*v*) was deposited

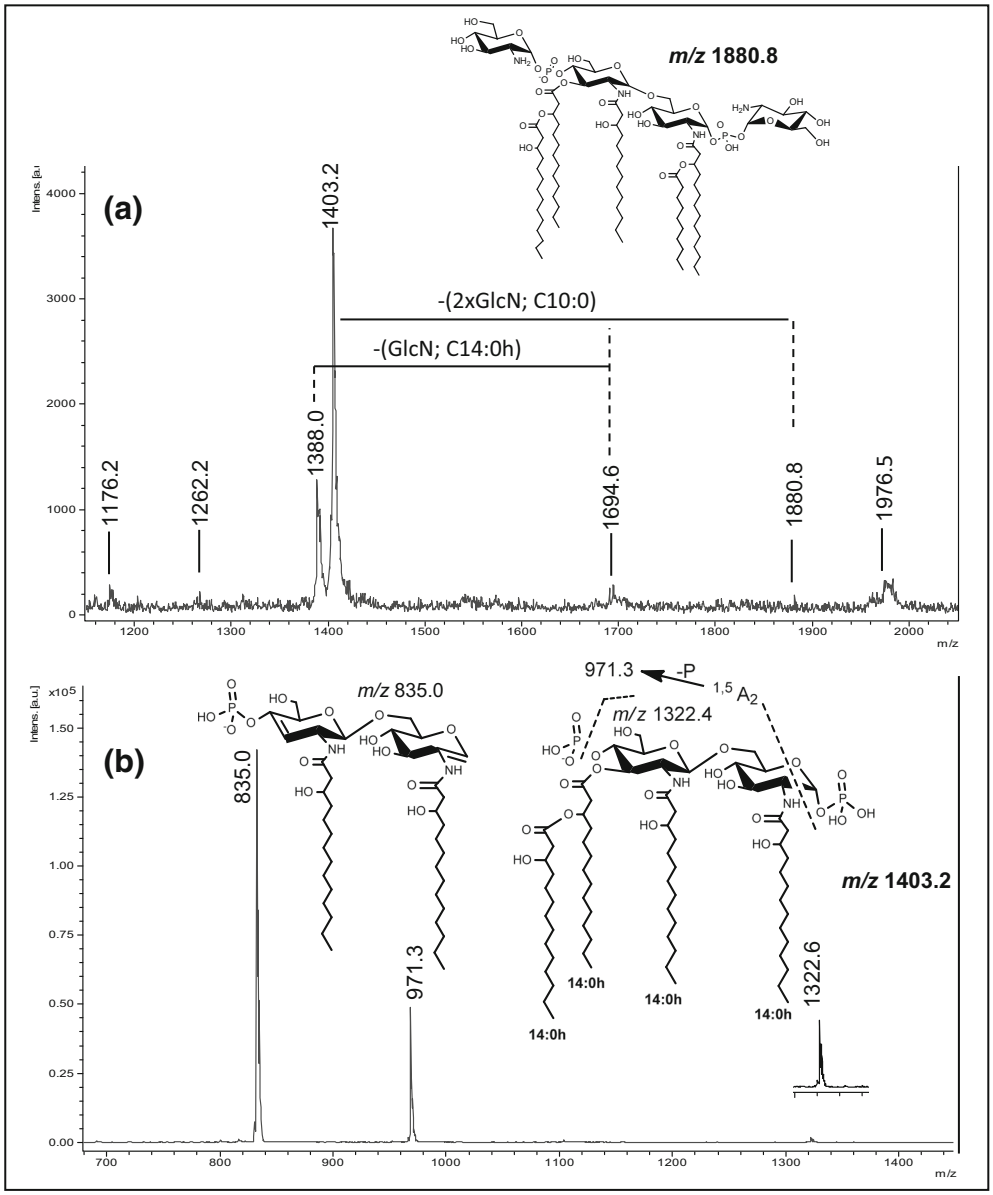


Figure 3. MALDI-TOF mass analysis of the lipid A obtained from mutant *Bb*3394 LPS. (**a**) Spectrum performed in the lineal negative ion mode. (**b**) LID-MS/MS spectrum of the selected precursor ion *m/z* 1403.2 from (**a**). Interpretation of the observed fragment ions is presented in the inset structures

on the target plate and allowed to dry under ambient conditions. Adequate spectra were obtained when *nor*-harmane (9*H*-pyrido-[3,4-*b*] indole) 2 mg/ml in acetonitrile:water (3:2) was used as matrix. The lipid A samples were dissolved in chloroform/methanol, 4:1.

Results and Discussion

Previously, on a *Bb*9.73 strain, we have constructed three mutants: *Bb*LP39, defective in the expression of *waaC* gene which codes for a heptosyltransferase of the core region [9]; *Bb*3394, defective in a gene involved in core substitution with a GalNA; and *Bb*3398, defective in the glycosyl transferase gene, responsible for the linkage of the lateral heptose to the core and causing the absence of the *O* antigen [21]. The SDS-PAGE LPS profile from *Bb*LP39 exhibited no O-antigen band and only a single LPS band [10] migrating considerably faster than that corresponding to the *Bb*9.73 wild-type strain. In addition, LPS-*Bb*3398 presented also a deep rough phenotype while LPS-*Bb*3394 was similar to parental LPS [21]. To determine the structure of the lipid A profiles, LPSs from the mutant strains were isolated, purified, and further subjected to acid hydrolysis. The released lipids A were purified and analyzed by UV-MALDI-TOF MS. The resulting spectra show signals due to the numerous related lipid A moieties biosynthesized by a single strain and also to "in source" fragmentation occurring during mass spectrometry analysis. Therefore, diagnostic

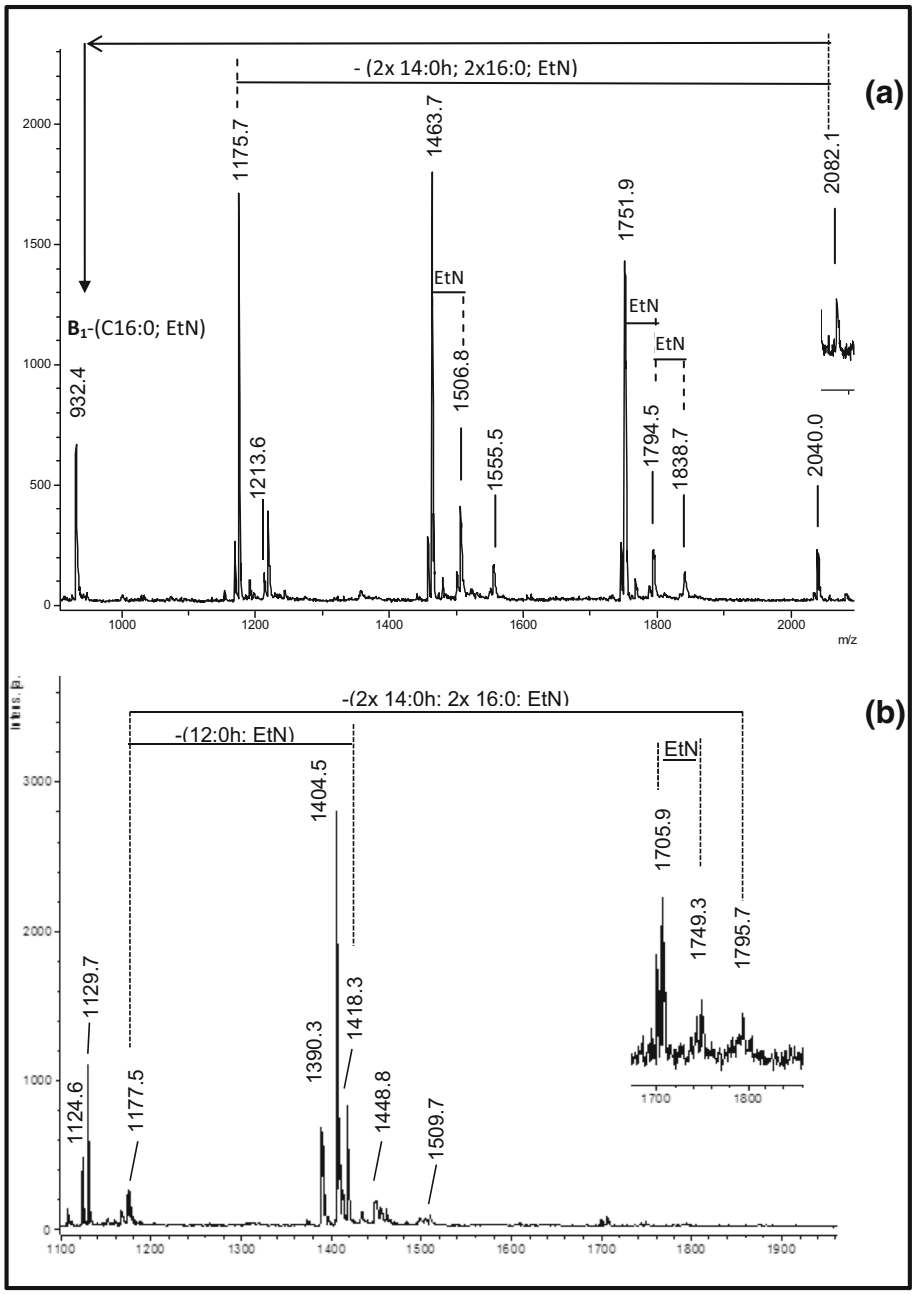


Figure 4. MALDI-TOF mass spectrum of lipid A obtained from *Bb*3398 LPS. (**a**) Spectrum performed in the positive ion mode. (**b**) Spectrum performed in the reflectron negative ion mode

signals from each spectrum were selected to highlight the structural differences among mutants. In parallel for comparison, a deep reinvestigation of the lipid A structure from the wild-type LPS obtained from strain *Bb*9.73 was performed.

MALDI-TOF MS in the negative reflectron ion mode (Fig. 1a) showed major signals at *m/z* 1667.1 (calc. *m/z* 1667.12, $C_{86}H_{161}N_3O_{25}P^-$) and 1651.1 (calc. *m/z* 1651.20, $C_{86}H_{161}N_3O_{26}P^-$) corresponding to the deprotonated penta-acylated diglucosamine backbone carrying C14:0 (3-OH) N-acyl groups at C-2, C-2′, and O-linked at C-3′, acylated with C12:0 (2-OH) or C12:0, respectively, on the fatty acid at C-2, and with C14:0 on the fatty acid at C-2′ ("secondary acylations") and a glucosamine phosphate (GlcNP) unit in the C-4′ position in agreement with previous report [19]. In accordance, *m/z* 1905.3 (calc. *m/z* 1905.35, $C_{102}H_{191}N_3O_{26}P^-$) was attributed to *m/z* 1667.1 bearing an additional palmitate as a secondary acylation at 3′ position (Table 1). The presence of a GlcNP modification was also shown in signals at *m/z* 1949.4 (calc. *m/z* 1949.37, $C_{104}H_{195}N_3O_{27}P^-$) and *m/z* 1955.2 (calc. *m/z* 1955.36, $C_{104}H_{194}N_3NaO_{26}P^-$).

The corresponding MALDI LID-MS/MS spectrum of ion *m/z* 1905.3 (Fig. 1b, Scheme 1) showed at *m/z* 1649.1 (calc. *m/z* 1649.11) the diagnostic loss of C16:0 at C-3′ indicating the presence of the labile "secondary" ester-linked palmitic acid

Table 2. Assignments of Molecular Ions Detected in the Negative and in the Positive Reflectron Ion Mode Mass Spectra of Lipid A Obtained from *Bb*3398 LPS. Sugar Backbone: [GlcN-GlcN]

[M-H] measured	[M-H] calculated	Acylation and phosphorylation patterns						[M + Na]$^+$ measured	[M + Na]$^+$ calculated
		C4'	C3'	C2'	C3	C2	C1		
		PEtN	C14:0(*3-O*-C16:0)	C14:0(*3-O*-C16:0)	–	C14:0(*3-O*-C16:0)	PPEtN	2082.1	2082.39
		P[a]	C14:0(*3-O*-C16:0)	C14:0(*3-O*-C16:0)	–	C14:0(*3-O*-C16:0)	PPEtN	2040.0	2039.35
1795.7	1796.10	PEtN	C14:0(*3-O*-C14:0h)	C14:0(*3*-OH)	–	C14:0(*3-O*-C14:0h)	PPEtN		
		PEtN	C14:0(*3-O*-C16:0)	C14:0(*3-O*-C14:0)	–	C14:0(3-OH)	PPEtN	1838.7[b]	1838.11
1749.3	1749.00	P[a]	C14:0(*3-O*-C16:0)	C14:0(*3-O*-C14:0)	–	C14:0(3-OH)	PPEtN	1794.5[b]	1795.07
1705.9	1706.05	P	C14:0(*3-O*-C16:0)	C14:0(*3-O*-C14:0)	–	C14:0(3-OH)	PP	1751.9[b]	1752.03
		P	C14:0(*3-O*-C16:0)	C14:0(3-OH)	–	C14:0(*3-O*-C14:0h)	PP	1745.9	1746.04
1509.7	1509.88	PEtN		C14:0(*3-O*-C14:0)	–	C14:0(*3-O*-C12:0)	PPEtN	1555.5[b]	1555.86
		P	C14:0(3-OH)	C14:0(3-OH)	–	C14:0(*3-O*-C12:0)	PPEtN[a]	1506.8	1506.83
		P	C14:0(3-OH)	C14:0(3-OH)	–	C14:0(*3-O*-C12:0h)	PP	1463.7	1463.78
1418.3	1418.86	PEtN[c]	C14:0(3-OH)	C14:0(3-OH)	–	C14:0(*3-O*-C12:0h)	P		
1404.5	1403.85	P	C14:0(3-OH)	C14:0(3-OH)	–	C14:0(*3-O*-C14:0h)	P[d]		
1177.5	1177.65	P	C14:0(3-OH)	C14:0(3-OH)	–	C14:0(3-OH)	P[d]		
1129.7	1129.55	PEtN			–	C14:0(*3-O*-C16:0)	PPEtN	1175.7[b]	1175.53
1124.6	1124.73	PEtN		C14:0(*3-O*-C14:0)	–	C14:0(3-OH)			

[a]Or 1 × PP and 1 × PEtN
[b]Phosphate as sodium salt
[c]Or 1 × PPEtN
[d]Or 1 × PP

[24]. The presence of the GlcNP unit accounts for ion at *m/z* 258.1 (calc. *m/z* 258.04, $C_6H_{13}NO_8P^-$) and its location at C-4′ position was shown by ions *m/z* 879.6 (calc. 879.50) corresponding to $^{0,4}A_2$ fragment from *m/z* 1421.8 according to the nomenclature of Domon and Costello [25], and *m/z* 718.5 due to the loss of a glucosamine unit from the latter.

The MALDI LID-MS/MS spectrum of ion *m/z* 1667.1 (Fig. 1c) shows diagnostic loss of C14:0 (3-OH) from position C-3′. As expected, the fragment pattern below *m/z* 1440 is coincident with the one of *m/z* 1905.3 previously described.

When the MALDI-TOF mass spectrum of the lipid A obtained from the wild-type LPS was performed in the reflectron positive ion mode (Suppl. Fig. S1), ions at *m/z* 1713.0 (calc. *m/z* 1713.1, $C_{86}H_{161}N_3Na_2O_{25}P$) and *m/z* 1951.3 (cal. *m/z* 1951.32, $C_{102}H_{191}N_3Na_2O_{26}P$) represent the disodiated adducts *m/z* 1667.1 and *m/z* 1905.3 from the negative ion mode spectrum and *m/z* 1530.0 (calc. *m/z* 1530.04, $C_{80}H_{151}N_2NaO_{21}P$) corresponds to the $[M+Na]^+$ adduct of *m/z* 1506.0. Besides, minor ion at *m/z* 1991.3 (calc. *m/z* 1991.39) corresponds to a structure similar to *m/z* 1951.3 differing in a secondary linked C16:0 at C-2 [17].

Regarding the MALDI-TOF mass spectrum, in the negative reflectron mode of the lipid A released from *Bb*LP39 LPS (Fig. 2a), in the high mass range, signals at *m/z* 1779.0 (calc. *m/z* 1778.13) and *m/z* 1756.1 (calc. *m/z* 1756.14) (ΔNa), as well as *m/z* 1666.4 (calc. *m/z* 1665.98), present a common diglucosamine backbone bearing primary N-linked C14:0 (3-OH) at C-2 and C-2′, O-linked at C-3′ position, modified with a pyrophosphoryl group at C-1 (Table 1). While *m/z* 1779.0 bears secondary linked a C12:0 at C-2 and a C10:0 at C-3′, *m/z* 1666.4 carries a secondary linked C12:0 at C-2 and a phosphate group at C-4′.

The presence and the location of the pyrophosphate group was assured by LID-MS/MS spectrum of ion *m/z* 1756.1 (Fig. 2b, Scheme 2). Main fragment ions at *m/z* 159.1 ($H_2O_6P_2$) and *m/z* 176.1 ($H_3O_7P_2$) support the presence of the pyrophosphate group [26]. Ion *m/z* 717.5, a $^{0,2}A_2$ fragment from *m/z* 1585.6 after the loss of a secondary C14:0 (3-OH) as ketene, evidence that the pyrophosphate group is located in the GlcN I. It must be noted that in this lipid A, no palmitoyl substitution was detected. Instead, main loss of C10:0 (3-OH) as free fatty acid or its ketene from *m/z* 1756.0 gave rise to ions *m/z* 1568.0 (calc. *m/z* 1568.00) and *m/z* 1585.6 (calc. *m/z* 1586.01), respectively, indicating the presence of a decanoyl fatty acid substituting the C14:0 (3-OH) at C-3′ position.

Regarding ion *m/z* 1505.2 (calc. *m/z* 1505.79) from the MS spectrum, it suggests a diglucosamine backbone bearing C14:0 (3-OH) primary N-linked at C-2 and C-2′ and O-linked at C-3′ position, a secondary linked C14:0 (3-OH), a phosphate, and a sodium pyrophosphate group. Absence of fragments corresponding to the loss of the whole C14:0 (3-O-C14 (3-OH)) moieties in the LID-MS/MS of this ion and the presence of ion *m/z* 1261.2 due to the loss of C14:0 (3-OH) evidence that a C14:0 (3-OH) is secondary bound to the C14:0 (3-OH) amide linked to C-2′. Ions *m/z* 79.0 and *m/z* 97.0 assured the phosphate group and *m/z* 158.9 and *m/z* 1322.1 $[M\text{-}NaH_2PO_4]^-$ confirmed the presence of the pyrophosphate group. In addition, the location of the pyrophosphate group at C-1 and the presence of three fatty acids substituting GlcN II was indicated by ions *m/z* 1022.0 corresponding to $^{2,5}A_2$ fragment from *m/z* 1505.2 and *m/z* 795.0, a $^{0,2}A_2$ fragment from *m/z* 1261.2 (Suppl. Fig. S2). In agreement to the above-described structures, MALDI-TOF mass spectrum of lipid A from *Bb*LP39 LPS in the positive reflectron mode (Suppl. Fig. S3) showed signals at *m/z* 1802.3 (calc. *m/z* 1802.12) and *m/z* 1712.4 (calc. *m/z* 1711.95) corresponding to the disodiated adducts of ions *m/z* 1756.1 and *m/z* 1666.4, respectively, from the negative ion mode, as well as *m/z* 1530.5 (calc. *m/z* 1529.78) to the sodiated adduct of *m/z* 1505.2 from the negative ion mode.

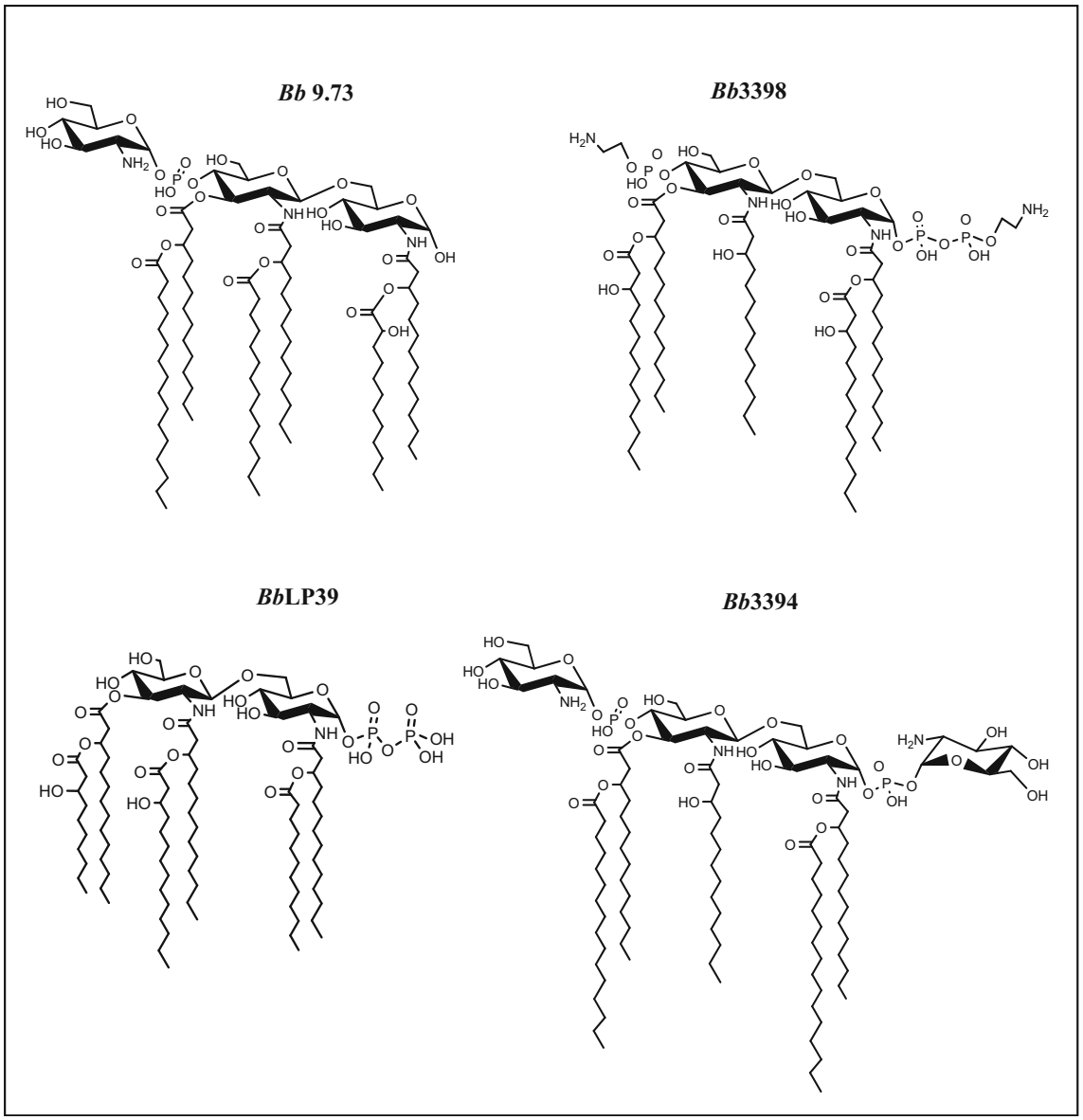


Figure 5. Comparison of representative lipid A structures present in the LPSs obtained from the different mutants of *B. bronchiseptica*

Further on, when the MALDI-TOF MS analysis of the lipid A obtained from *Bb*3394 LPS was performed in the negative ion mode (Fig. 3a), the highest molecular weight ion detected was *m/z* 1976.5 (calc. *m/z* 1976.25). Its structure correlates with ion *m/z* 1667.1 from the wild-type lipid A differing in the presence of a secondary linked C16:0 located at C-2 and an additional GlcNP unit at C-1 (Table 1). Other signals at *m/z* 1880.8 (calc. *m/z* 1880.12) and *m/z* 1694.6 (calc. *m/z* 1693.99) correspond to a penta- and a tetra-acylated diglucosamine backbone, respectively, also carrying the two additional GlcNP units. In agreement, main ion at *m/z* 1403.2 (calc. *m/z* 1403.85) corresponds to the tetra-acylated diglucosamine backbone bearing two phosphate groups at C-1 and C-4′. LID-MS/MS spectrum of the latter ion (Fig. 3b) showed a main fragment ion at *m/z* 835.0 (calc. *m/z* 835.47) that corresponds to the loss of the labile 3′ substituent [21] C14 (3-*O*-C14(3-OH)) and a phosphate group. Fragment ion at *m/z* 1322.6 (calc. *m/z* 1322.87) indicates the loss of one phosphate group from *m/z* 1403.2.

In accordance, when the MALDI-TOF mass spectrum of *Bb*3394 lipid A was performed in the positive ion mode (Suppl. Fig. S4), sodiated species carrying additional GlcNP units [16] were detected. Thus, signal at *m/z* 1745.6 (calc. *m/z* 1746.02) corresponds to the diglucosamine backbone acylated with two C14:0 (3-*O*-C16:0) probably at C-3′ and C-2; major signal at *m/z* 1169.8 (calc. *m/z* 1170.54), probably due to in-source fragmentation, was ascribed to a diglucosamine backbone acylated with C14:0 (3-*O*-C16:0) at C-2 [17] carrying one GlcNP and one sodium phosphate group.

MALDI-TOF MS in the reflectron positive ion mode was used to analyze lipid A obtained from *Bb*3398-LPS (Fig. 4a, Table 2). The highest mass signal at *m/z* 2082.1 (calc. *m/z* 2082.39) is consistent with a hexa-acylated lipid A backbone

with C14:0(3-*O*-C16:0) N-linked at C-2 and C-2′ and O-linked at C-3′ carrying one phosphoethanolamine and one pyrophosphoethanolamine group. Ion at *m/z* 2040.0 (calc. *m/z* 2039.35) corresponds to the latter devoid an ethylamine group confirming the EtNP modification. In source B_1, fragment from *m/z* 2082.1, with concomitant loss of C16:0, gives rise to *m/z* 1213.6 (calc. *m/z* 1213.93) indicating that the phosphoethanolamine group is located at C-4′. Further loss of the EtN group yield *m/z* 932.4 (calc. *m/z* 932.66). In accordance, signal at *m/z* 1838.7 (calc. *m/z* 1838.11) corresponds to a penta-acylated lipid A backbone substituted with C14:0 (3-OH) amide linked at C-2, C-2′, and O-linked at C-3′, a secondary linked C14:0 at C-2′, C16:0 at C-3′, modified with one PPEtN at C-1 and one PEtN at C-4′, as a disodiated ion. Signals at *m/z* 1794.5 (calc. *m/z* 1795.07) (Table 1) and *m/z* 1751.9 (calc. *m/z* 1752.03) differ from *m/z* 1838.7 in one or two EtN groups respectively.

When the lipid A obtained from *Bb*3398 LPS was analyzed in the reflectron negative ion mode (Fig. 4b, Table 2), ion at *m/z* 1795.7 (calc. *m/z* 1796.10) was consistent with a penta-acylated lipid A backbone modified with one pyrophosphoethanolamine and one phosphoethanolamine group. In addition, signal at *m/z* 1749.3 (calc. *m/z* 1749.00) bears one pyrophosphoethanolamine and one phosphate group. Ions detected below *m/z* 1600 correspond to partially deacylated species probably due to in-source fragmentation. A comparison of a representative lipid A structure from each strain is highlighted in Fig. 5.

Conclusions

Some bacterial pathogens modify their lipid A structures and consequently they change their immunological interactions. Hence, *B. bronchiseptica*, in order to adapt to different hosts, modifies its lipid A structure. Interestingly, differences in the heterogeneity of lipid A isolated from non-virulent and virulent strains have been determined [27].

In the present work, getting deeper in the structural analysis of these LPSs, we demonstrate that although gene deletions were not directed to the lipid A moiety, each mutant presented different modifications. The use of MALDI-TOF MS was essential to evidence heterogeneities.

At first, we confirmed that *Bb*9.73 modifies its lipid A adding a GlcN in either 1 or 4′-phosphate groups. This result is in agreement with the described for *B. pertussis* lipooligosaccharide [27] with both phosphates substituted with GlcN. Both, *B. pertussis* virulent and non-virulent isolates, presented GlcN substitutions in 1 and 4′-phosphate groups. The presence of a glucosamine substitution was also described for *B. bronchiseptica* clinical isolates [18]. Moreover, all the sequenced *Bordetella* strains have a gene encoding an ortholog of ArnT [28] that uses GlcN as a substrate [19]. However, we show herein that relevant LPS structural changes, like the premature stop in the core synthesis in *Bb*LP39 and *Bb*3398 mutants, prevent GlcN addition. On the contrary, small core structural modifications like the one present in *Bb*3394 presumably enable ArnT to decorate both 1 and 4′-phosphates.

In addition, deep rough *Bb*LP39 and *Bb*3398 LPSs presented a higher content of secondary hydroxy fatty acids than the smooth LPSs. It has been shown that LpxO is an oxygenase that specifically hydroxylates acyl chains and is required for the presence of C12:0 (2-OH) in *B. bronchiseptica* lipid A. LpxO is predicted to be located in the inner membrane [16]. Hence, modifications introduced by this enzyme occur previously to the transport of core-lipid A to the periplasm. The presence of hydroxylated fatty acids of different lengths in deep rough *Bordetella bronchiseptica* mutants might suggest that transport of the defective core-lipid A structures to the periplasm is impaired or slowed down allowing other enzymes to modify lipid A one or more times.

Notably, in core truncated LPSs, *Bb*LP39 and *Bb*3398, lipid A heterogeneity increased and different glucosamine substitutions with PP at C-1 or PEtN at C-1 and C-4′, respectively, were determined. These kind of modifications were also found in other bacteria [1]. LpxT is a phosphotransferase that phosphorylates lipid A at the 1 position and EptA is a transferase that adds PEtN in either C1 or C4′. Interestingly, we were not able to find homologues of these genes in published *B. bronchiseptica* RB50 genome, suggesting that other enzymes are involved in the decorations observed. As these substitutions were present only when the core synthesis is impaired, the enzymes involved might have low activity in the wild-type strain.

In summary, we showed that inactivation of genes codifying core glycosyltransferases affect the transit of the nascent LPS allowing modifying enzymes to act on the lipid A. Therefore, the mature structures will differ from the wild-type LPS not only in the core region but also in the lipid A moiety. This fact should be especially taken into account when mutant strains are used to evaluate bacterial biological properties.

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