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A dozen species of human and animal pathogens have been described to date in the *Bordetella* genus, with the majority being respiratory tract pathogens. *Bordetella avium* lipopolysaccharides have been shown to be important virulence factors for this bird pathogen. *B. hinzii* is closely related to the *B. avium* species, but has also been isolated from humans. *B. trematum* is associated to ear and blood infections in humans. Its lipid A structure, the biological active moiety of LPS, was found to be closely related to those of *B. avium* and *B. hinzii*. It is important to unveil the subtle structural modifications orchestrated during the LPS biosynthetic pathway to better understand host adaptation. The present data are also important in the context of deciphering the virulence pathways of this important genus containing the major pathogens *B. pertussis* and *B. parapertussis*, responsible for whooping cough. We recently reported the isolated lipid A structures of the three presented species, following the previously identified O-chain structures. In the present study, we provide details on the free and O-chain-linked core oligosaccharide which was required to characterize the complete LPS structures. Data are presented here in relation to relevant biosynthesis genes. The present characterization of the three species is well illustrated by Matrix Assisted Laser Desorption Mass Spectrometry experiments, and data were obtained mainly on native LPS molecules for the first time.

A comparative study of the complete lipopolysaccharide structures and biosynthesis loci of
Bordetella avium, *B. hinzii*, and *B. trematum*

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Highlights

- Complete *Bordetella avium*, *B. hinzii* and *B. trematum* LPS structures are characterized by MALDI-MS and compared
- Three *Bordetella* LPS structures are presented in relation to genomic data
- MALDI Mass-spectrometry was applied to long-chain LPS leading to spectra of native LPS, presented for the first time without use of chemical modifications or fragmentation

Abstract

A dozen species of human and animal pathogens have been described to date in the *Bordetella* genus, with the majority being respiratory tract pathogens. *Bordetella avium* lipopolysaccharides have been shown to be important virulence factors for this bird pathogen. *B. hinzii* is closely related to the *B. avium* species, but has also been isolated from humans. *B. trematum* is associated to ear and blood infections in humans. Its lipid A structure, the biological active moiety of LPS, was found to be closely related to those of *B. avium* and *B. hinzii*. It is important to unveil the subtle structural modifications orchestrated during the LPS biosynthetic pathway to better understand host adaptation. The present data are also important in the context of deciphering the virulence pathways of this important genus containing the major pathogens *B. pertussis* and *B. parapertussis*, responsible for whooping cough. We recently reported the isolated lipid A structures of the three presented species, following the previously identified O-chain structures. In the present study, we provide details on the free and O-chain-linked core oligosaccharide which was required to characterize the complete LPS structures. Data are presented here in relation to relevant biosynthesis genes. The present characterization of the three species is well illustrated by Matrix Assisted Laser Desorption Mass Spectrometry experiments, and data were obtained mainly on native LPS molecules for the first time.

Keywords: *Bordetella avium*, *B. hinzii*, *B. trematum*, endotoxin, genomics, lipopolysaccharide MALDI MS

Abbreviations: LOS, lipooligosaccharide; LPS, lipopolysaccharide; MALDI MS, matrix-assisted laser-desorption ionization mass spectrometry; OS, Oligosaccharide; PEA, Phosphoethanolamine; PPEA, pyrophosphoethanolamine; PS, polysaccharide.

1. Introduction

A dozen species have already been described in the proteobacterial genus *Bordetella* [1, 2]. *Bordetella pertussis* and *B. parapertussis* being the well-known agents of whooping cough. These two important human pathogens evolved separately from a *B. bronchiseptica*-like progenitor [3, 4]. *B. bronchiseptica* infects many mammalian species, including humans, causing respiratory diseases [5]. *B. avium*, as its name indicates, is a pathogen for birds [6][4]. *B. hinzii*, *B. trematum* [7], *B. holmesii* [8], *B. petrii* [9] and *B. ansorpi* [10] were more recently described, some of them being isolated from immunosuppressed patients [11]. An additional group of *Bordetella hinzii*-like species, recently isolated from human respiratory samples, were proposed for classification according to various traits. These species include *B. bronchialis*, *B. flabilis* and *B. sputigena* [2]. At the same time, three new environmental *Bordetella* species were described, *B. muralis*, *B. tumulicola*, and *B. tumbae*, isolated from a plaster wall surface of 1,300-year-old mural paintings in Japan [12, 13].

Endotoxins made of lipopolysaccharides (LPS) heterogeneous structures, are the main components of the outer membrane of Gram-negative bacteria. They are usually made up of three distinct moieties, the very important lipid moiety called lipid A, responsible for the majority of LPS biological activities. A core of about 10 sugars is linked through the proximal 2-keto-3-deoxyoctulosonic acid, known as Kdo, to this lipid A moiety, which in turn is anchored in the bacterial outer membrane. The third moiety, substituting the core in the so-called Smooth-type strains, is extending outside of the bacterium. This large structure, made of oligosaccharide repetitive units, can reach a total of 100 sugars. It is called O-chain, or O-antigen, as it carries the antigenic properties of the molecules and represents the main antigen of Gram-negative bacteria, being as unique as a human finger print can be [14].

Involvement of lipopolysaccharide (LPS) in *Bordetella* pathogenesis was first illustrated with *B. pertussis*, its LPS damaging ciliated trachea cells by induction of NO in synergy with the tracheal cytotoxin [15]. *B. avium* binds to ciliated tracheal epithelial cells, damaging them and favouring lethal superinfection by different pathogens [6]. This is an important pathogen in poultry production, and a better knowledge of its LPS structure is important to understand its pathogenesis. Orndoff and collaborators have described different aspects of bacterial virulence concerning this species, and *B. avium* LPS has also been described as an important virulence factor [16]. Mutants were found with altered LPS SDS-PAGE gel profiles and concomitant decreased capacities for binding to tracheal cells [17].

Our long-lasting interest in the *Bordetella* LPS structures allows interesting comparisons between members of the genus. We describe here the detailed structures of *B. avium*, *B. hinzii* and *B. trematum* free and O-chain-substituted heterogeneous cores, in order to complete the already described LPS elements represented by the lipid A and O-chain structures for characterizing the complete native LPSs [18-23]. The major lipid A molecular species from *B. avium*, *B. trematum* and *B. hinzii* are presented in Figure 1A, B [20] in comparison to those from *B. pertussis* (Figure 1 C) [24]. Structures of the O-antigen subunits of the three bacterial species under study are presented in Figure 2.

It is important to note that most of the time, the core structures of an LPS molecule present in Smooth-type bacteria are only defined by similarity to free core structures. These short-LPS molecular species are almost always present in the heterogeneous LPS mixture of any given Gram-negative Smooth-type bacterial strains. Interestingly, we demonstrated earlier that in some cases, such free-core moieties could be structurally different from the cores of higher molecular weight LPS species linked to lipid A, and being substituted by the O-chains in the same bacterium [23]. Duda *et al.* also showed in 2011 that free and O-chain substituted cores could differ by two sugars in an *E. coli* strain responsible for mastitis [25].

In *B. pertussis*, the LOS molecular species containing a free core structure has been termed Band B, based on its SDS-PAGE profile, and corresponds to a nona-saccharide. Most of the time, it can be substituted by a distal tri-saccharide and in this case the resulting dodecassaccharide LOS is named Band A [26-28]. In Smooth-type LPS, the O-chain can be added to either one of these structures, thereby contributing to additional heterogeneity of the LPS molecular species expressed by Gram-negative bacterial species. It was therefore important to complete the entire LPS molecular structure, with the present data, thereby adding the 'missing link' to the O-chain and lipid A structures we had already characterized and reported previously. Our different recently developed analytical methods using MALDI-MS were used to reach this goal [29].

2. Materials and Methods

2.1 Bacterial strains and cultures. *B. avium* smooth-type strain ATCC 5086 [19], *B. hinzii* ATCC 51730 [18] and *B. trematum* CCUG 13902 [29] were grown at NRC, Ottawa, Canada as described [22]. The cells were killed in 2% phenol before harvesting.

LPS. The LPS were extracted by the modified enzyme-phenol-water method [30], or alternatively by the Isobutyric acid-Ammoniac method for *B. pertussis*1414 [31] and purified

as described previously [32]. LOS from two *B. pertussis* strains, BP1414 and A100 (Institut Mérieux), were used as references for the SDS PAGE analysis.

SDS-PAGE LPS analysis. Freshly prepared (4%/15%) polyacrylamide gels were loaded with samples of 0.2 to 0.5µg of the LPS preparation in a sample buffer, electrophoresed as previously described [33] and then silver-stained after periodic acid oxidation [34].

2.2 LPS cleavage used to release lipid A and oligo- or polysaccharide regions for analysis.

When necessary, LPS cleavage was performed by the triethylamine citrate hydrolysis [35]. A concentration of 0.01M TEA-citrate (1:1 molar ratio, pH 3.6) was used. LPS samples were dispersed in Eppendorf ® tubes in the above-mentioned reagent at concentrations of 5 or 10 µg/µl, respectively, for Rough- or Smooth-type LPS and depending on their solubility. After agitation and homogenization in an ultrasonic bath, the tubes were incubated for 1.5 h in an Eppendorf Thermomixer system at 100°C under stirring at 1000 RPM.

The severed lipid A was sedimented from hydrolysates by centrifugation for 10 min at 4°C and 20000 RPM. After centrifugation, the hydrolysed supernatants containing the cores oligosaccharides and/or long-chain polysaccharides (PS=core+O-chains) were lyophilized, additionally washed with a chloroform / methanol / water mixture to remove lipid A traces, dried and taken in water for further MALDI-MS analysis.

2.3 LPS de-O-acylation.

In order to liberate all ester-linked fatty acids, 5.0 mg of LPS were suspended in 500 µl of 41% methylamine solution and stirred for 5 h at 37°C in an Eppendorf thermomixer system [36]. After hydrolysis, the samples were dried first under a stream of nitrogen, then in a speed-vac system. The dried samples were taken in water for further MALDI-MS analysis.

2.4 MALDI/MS was carried out on a Shimadzu Axima Performance time-of-flight mass spectrometer system in the negative-ion mode. Gentisic acid (2,5-dihydroxybenzoic acid, DHB) was purchased from Sigma Chemical Co. (St. Louis) and used as a matrix. It was dispersed at 10 mg/ml in 0.1M citric acid, either in water for analysis of LPS, core oligosaccharides and long-chain polysaccharides, or in a mixture of chloroform: methanol: water (3:2:0.25, vol:vol:vol) for lipid A analyses [37]. A few microliters of each type of samples dissolved in the same appropriate solvent at 1 µg/µl were desalted with a few grains of ion-exchange resin (Dowex 50W-X8) (H⁺) in an Eppendorf tube. An aliquot of 0.5 or 1 µl

of the sample solution was spotted on the target, covered with the same volume of the matrix solution and let dry [37]. Different ratios between the samples and DHB were tested. *B. pertussis* LPS was used as an external standard. Analyte ions were desorbed from the matrix with pulses from a 337 nm nitrogen laser. Spectra were recorded in the linear negative-ion mode at -20 kV acceleration and delayed extraction, with an average of laser 1000 pulses.

2.5 Gene organization analysis and sequence comparisons. NCBI's Basic Local Alignment Search Tools (BLAST) (available at: <https://blast.ncbi.nlm.nih.gov>), its graphical outputs and available gene annotations were used to identify orthologous genes and regions of similarity between DNA and protein sequences. To test for sequence similarities at the protein level (if not already described in the available gene annotations), TBLASTN 2.8.0+ was used to search translated nucleotide sequences of selected organisms, including *Bordetella hinzii* ATCC 51730 (taxid:1392838), *Bordetella avium* 197N (taxid:360910) and *Bordetella trematum* (taxid:123899) using the protein sequence of selected LPS biosynthesis genes of *Bordetella bronchiseptica* RB50 (taxid:257310) or *Bordetella pertussis* Tohama I (taxid:257313) as query. For cross-species comparisons of selected gene loci at the nucleotide level, the discontinuous megablast algorithm was used. Clustal Omega (available at <https://www.ebi.ac.uk/Tools/msa/clustalo/>) was used for multiple sequence alignment of 3 or more protein sequences and to generate phylogenetic trees.

3. Results and discussion

3.1. SDS-PAGE analysis.

LPS or LOS Electrophoretic profiles from the three *Bordetella* species are presented in Figure 3. The LPS from *B. trematum*, *B. hinzii* and *B. avium* are compared to *Bordetella pertussis* LOS. These LOS originate from two strains, namely BP1414 containing mostly the dodecasaccharide core (band A) and BP A100, containing only the nonasaccharide core (band B).

B. trematum displayed an interesting LPS profile consisting of three well-separated bands: the first one, weak, but fairly visible, is migrating at the level of band B, the second band is migrating at the level of band A, and the third and major one, is displaying longer molecular species than the *B. pertussis* LOS.

B. hinzii LPS also displayed a peculiar profile with short LPS molecular species migrating slightly lower than *B. pertussis* band B and a major higher-mass band with discrete satellite molecular species at plus and minus one O-chain unit.

Like the *B. trematum* and *B. hinzii* LPS, that of *B. avium* comprises both fast and slow migrating molecular species. Interestingly, the fast migrating band is a broad distribution of non-resolved molecular species entirely covering the range limited by the *B. pertussis* A and B bands, thus suggesting a great heterogeneity of the core oligosaccharide structures for this LPS. The slower migrating region of the *B. avium* LPS indicates that it has relatively short O-chains compared to other LPS species with long O-chain repetitive units. Also, no ladder-like pattern of LPS bands was observed, but rather a smear, which was consistent with the monosaccharide repeating unit described previously [19], similar to what has been observed with *B. bronchiseptica* and *B. parapertussis* LPSs [38, 39].

3.2. MALDI mass spectrometry analyses.

Native chemically non-modified LPS isolated from the three species were analysed by MALDI-MS in the linear negative-ion mode. Their PS regions isolated by a mild acid hydrolysis were also analysed and compared. The mass-spectra of the native LPS from *B. trematum* and *B. hinzii* are presented in Figures 4A and 5A respectively. In these spectra, signals were observed in three different m/z regions corresponding to: (1) molecular $[M-H]^-$ ions of intact LPS molecules in the higher masses region, and in-source-decay generated fragment ions corresponding to the (2) lipid A and (3) PS regions, in the lower masses region. Therefore, MS fragmentation gives precious data originating from a single spectrum, since native LPS molecular species, as well as their derived fragments, can be analysed. When no elimination of peculiarly labile constituents is observed, LPS molecular species correspond to the addition of lipid A and PS molecular species masses. If a difference is observed between this sum and the LPS molecular species, the “missing” element, whose mass corresponds to the calculated difference, has to be characterized by other means. Such discrepancies are more often observed with PS and lipid A molecular species liberated by LPS mild acid hydrolysis, fragmentation in the mass spectrometer being more gentle. Therefore, we always recommend to analyse and compare native LPS mass spectra to those of their separated hydrolysed components for a complete and more reliable analysis [35]. Analysis of *B. avium* native LPS was difficult because of high heterogeneity of this sample, as discussed below. In this particular case, we present a spectrum of the isolated PS region (see Figure 6A), which was compared to a spectrum obtained after de-O-acylation of the native LPS, in order to demonstrate additional structural features such as Kdo phosphorylation.

3.2.1 *B. trematum* 13902 native LPS mass spectrum.

The negative-ion MALDI mass spectrum of the native *B. trematum* LPS is presented in Figure 4A. Lipid A associated fragment ions were observed at m/z : 1376.8, 1603.2 and 1841.6; they correspond to the tetra-, penta- and hexa-acyl molecular species described earlier [20] and presented in Figure 1B. In agreement to previous analyses, some micro-heterogeneity was also observed for each acylation level, at -16u and +28u, due to the modifications in hydroxylation and length of the secondary fatty acids (see Figure 1B).

Two clusters of peaks corresponding to PS-associated fragment ions were observed between 2100u and 3300u. These patterns are distanced by 701.7u corresponding to one of the O-chain *B. trematum* LPS subunits described earlier [22] and presented in Figure 2A.

As shown previously [22], the *B. trematum* O-antigen contains only two O-chain elements. Both elements differed in the anomeric bond of their first sugar. In order to characterize the often unreachable hinge between core and O-chain, the nitrous deamination technique was applied to the whole native LPS. This technique was first described with success with *B. pertussis* [40]. With *B. trematum*, it led to the release of the following heptasaccharide corresponding to two trisaccharide subunits linked to a 2,5-anhydromannitol residue resulting from the deamination of the core glucosamine, thus highlighting the O-chain to core linkage position. The structure corresponded to: β -ManNAc3NAmA-(1-4)- β -ManNAc3NAmA-(1-3)- **α -FucNAc**-(1-4)- β -ManNAc3NAmA-(1-4)- β -ManNAc3NAmA-(1-3)- **β -Fuc-NAc**-(1-6)-2,5-anhManol. The calculated mass of the repeating unit corresponded to 701.7u. The FucNAc is engaged in two different anomeric bounds in each unit. Such peculiarity has already been reported in at least two other examples [41, 42].

In the native LPS spectrum, the PS-associated fragments, observed at m/z : 2424.3 and 3126.2, correspond to the complete PS structures containing one and two O-chain units, respectively, attached to the anhydrous core octa-saccharide defined as the free core type. The *B. trematum* free core structure is presented in Figure 4B. The Kdo residue is substituted with PPEA and Hep II is phosphorylated. As mentioned above, the major advantage of the analysis of native unmodified LPS resides in preserving labile substituents, or like here the Kdo substitution, which is not liberated by MS, while it would be beta-eliminated during acid hydrolysis. The theoretical molecular weight of such anhydrous core oligosaccharide substituted by 1 PPEA and 1 phosphate group is 1723.2. When one or two O-chain units are present, the theoretical molecular weight of the resulting polysaccharides are 2424.9u and 3126.6u, respectively.

Interestingly, the terminal Hep is absent from the *B. trematum* LPS core oligosaccharide, when compared to similar structures from other *Bordetella* species. The absence of the terminal Hep in the cores of the *Bordetella* genus has already been evocated and demonstrated as being partial in the *B. bronchiseptica* LPS structures [43] or total in *B. hinzii* [18]. This absence is also corroborated by the fact that this Hep residue is absent from the distal part of the *B. trematum* LPS that we liberated after nitrous deamination [22].

In the upper mass region, from 3300u to 4900u, LPS molecular-ion peaks appear, reflecting the same differences due to the molecular heterogeneity of lipid A (different acylation levels) and PS regions (number of O-chain units). Some other features are easier to observe from the entire LPS molecular pattern. In this particular case, a series of peaks are observed, shifted at -80u (Phosphate) and -123u (PEA) relatively to the major ones, e.g. at 4649.4u and 4605.8u, relatively to 4729.4u. This is due to the additional heterogeneity of the Kdo and Hep II substitutions. In the molecular species observed at 4649.4u, Hep II is not phosphorylated and in the one observed at 4605.8u, Kdo is substituted with phosphate, instead of PPEA.

As expected from the SDS PAGE analysis, neither molecular nor fragment ions were observed that corresponded to molecular species containing the free core. However, although fairly minor, such species are also present and can be observed in the *B. trematum* PS spectrum obtained after mild acidic hydrolysis which confirm that this core is a octasaccharide molecular species, in this case identical to the O-chain substituted core (data not shown).

The complete structure of the *B. trematum* LPS is thus described and characterized here for the first time.

3.3. *B. hinzii* native LPS mass spectrum.

The negative-ion MALDI mass spectrum of *B. hinzii* native LPSs is presented in Figure 5A. In agreement with earlier data, the lipid A-associated fragment ions are observed at m/z : 1376.4, 1602.8 and 1841.7 for tetra-, penta- and hexa-acyl molecular species, respectively (see Figure 1B). They are corresponding to the same structures as those observed for the *B. trematum* LPS.

Two types of LPS molecules are clearly observed in the spectrum related to the structures of their PS region: short-chain molecules, composed of a lipid A moiety plus a free core oligosaccharide, and long-chain LPS molecules, composed by a lipid A moiety plus a so-

called substituted core oligosaccharide, carrying a tetrasaccharide linker plus the O-antigen chain [44].

The free core oligosaccharide corresponds to the same structure as the one already described for *B. trematum*, (see Figure 5B), with a theoretical molecular weight at 1723.23. The corresponding free-core OS associated fragment-ion is observed at m/z 1723.6. This core moiety appears to be associated with tetra-, penta-, hexa-acyl lipid A moieties, corresponding to LPS molecular species observed at m/z 3099.9, 3336.14 and 3564.9. Satellite peaks are observed relatively to these molecular ions at minus 123u for PEA, corresponding to the structures where the Kdo residue is substituted by a phosphate group, instead of a PPEA group. A small peak observed at m/z : 3018.8, i.e. 3099.9-80u, suggests that the Hep II residue is non-stoichiometrically substituted with phosphate.

The structure of the substituted core oligosaccharide in the LPS molecules containing O-chains is different (see Figure 5C). To the short octasaccharide, there is a trisaccharide added, differing from the classical *B. pertussis* distal trisaccharide by the absence of the methyl group on the di-amino-fucosamine, as well as by replacement of the distal GlcNAc by a GalNAc residue [45]. Another important modification, compared to the classical schematic core-O-chain structures, is the addition of a tetrasaccharide as an intermediate link between the undecasaccharide and the O-chain. This tetrasaccharide linker, as shown in Figure 4C, is composed of 4 units of Di-aminoNACA sugars which are amidated on their acid groups. This element corresponds to 1028.98u. The *B. hinzii* LPS is particularly rich in uronamides.

PS-associated fragment-ions and molecular-ions corresponding to the long-chain molecules, are observed in the upper m/z regions, i.e. 5500-6000 and 7000-8000. The *B. hinzii* O-chain polysaccharide structure was characterized earlier simultaneously by NMR [46] and mass spectrometry [18]. The $-4-\beta\text{-GlcNAc}3\text{NAcAN}-4-\beta\text{-GlcNAc}3\text{NAcAN}-4-\alpha\text{-GalNAc}-4-\beta\text{-trisaccharide}$ repeating unit, with a molecular weight corresponding to 771.73u, was described (see Figure 2B). Long PS-associated fragment ions are observed at m/z 5985.4 and 5861.7. Both contain the bound-core undecasaccharide plus the linker tetrasaccharide in addition to an O-chain containing 3 repeating units and one terminal methylated residue. In the first one, Kdo is substituted with PPEA; the second one only with Phosphate.

In the higher-masses region corresponding to the long-chain LPS molecular ions, the peaks observed at m/z : 7239.9, 7466.6 and 7827.2 correspond to the LPS molecular species in which the complete polysaccharide structure is linked to the tetra-, penta- and hexa-acyl lipids A, respectively. Some molecular heterogeneity is also observed with molecular species at -80u, -

123u, and -203 u, corresponding to the non-substitution of phosphate on Hep II and to the substitution of the Kdo with phosphate, instead of PPEA, or both.

Again, this is the first time that we describe, and confirm, fragmented published elements of the *B. hinzii* LPS, as found in their complete native structure.

3.4. *B. avium* LPS mass spectra

B. avium LPS possesses the highest molecular weight as compared to other LPS examined in this study. Although we were able to get masses as high as 6kDa with the long-chain polysaccharide moieties, weak signals were obtained with native molecular species due to the fact that each LPS molecule got increased heterogeneity with twelve major lipid A molecular species (see Figure 1A and B). As shown below, we also identified at least six free-core oligosaccharide structures and four substituted-core oligosaccharide structures linked to O-antigen chains of variable lengths. This heterogeneity resulted in a redistribution of the overall MS signal over a very high number of co-existing molecular species and consequently, in a very poor signal-to-noise ratio. In this case, we first de-O-acylated an LPS sample in order to reduce this heterogeneity. The analysis of the de-O-acylated LPS allowed us to obtain clear peaks corresponding to short-chain LPS consisting of de-O-acylated lipid A and free-core oligosaccharide molecular species corresponding to long-chain LPS being poorly resolved. Finally, the analysis of separated lipid A and PS moieties was performed and gave satisfactory complementary results for the identification of major different structures present in the natural mixture.

The isolated-lipid A analysis confirmed our earlier published data [20]. Similar to *B. trematum* and *B. hinzii*, three major acylation states were observed, i.e. tetra-, penta-, and hexa-acyl, at m/z : 1376.5, 1602.8 and 1841.9 with micro-heterogeneity due to the modifications of the hydroxylation and length of the secondary fatty acids (-16u and +28u). The major difference observed for the *B. avium* lipid A consisted in the presence of molecular species with one or both phosphate groups being substituted with GlcN residues. These molecular species were observed at +161u and +322u relatively to the non-substituted molecular species common for the three LPS under study. The *B. avium* lipid A structures are presented in Figure 1 A and B. All in all, they corresponded to at least 36 molecular species, the ones with GlcN-substituted phosphate groups being highly susceptible to fragmentation, thus giving an additional contribution to the chemical background.

A negative-ion MALDI mass-spectrum of the *B. avium* polysaccharide isolated by mild acid hydrolysis is presented on Figure 6A. Two major groups of signals are identified and attributed to (1) free-core oligosaccharide molecular species and to (2) substituted-core oligosaccharide molecular species carrying a variable number of O-chain subunits. Both groups are highly heterogeneous. All observed peaks corresponded to anhydrous forms of

molecular species, resulting from beta-elimination of the phosphate group, originally substituting the Kdo residue at C-4 [47] .

The free-core oligosaccharide is represented by six major peaks. The most complete structure, corresponding to $m/z=2278.8$, were attributed to the anhydrous dodecasaccharide presented in Figure 6B. This structure differs only from the *B. pertussis* core by the absence of the Me group on the FucN4NAc. From this complete structure, the following structural elements can be absent: lateral Hep, terminal GlcNAc or terminal trisaccharide (Figure 6B), thus giving rise to the six molecular species observed at m/z : 2278.8, 2086.7, 2073.0, 1883.5, 1632.0 and 1439.9 (theoretical values, $m/z= 2279.02, 2086.86, 2075.83, 1883.66, 1631.39$ and 1439.22). Some minor peaks are also observed at +80u, (e.g. m/z : 1711 relatively to m/z : 1631), suggesting the presence of the same structures but with phosphorylated Hep II, thereby doubling the total number of molecular species.

The general structure of the long-chain PS consists of a bound-core oligosaccharide substituted with an O-chain of a variable number of subunits. Compared to the previous data obtained for the short-core structures, it is obvious that the cores carrying the O-chain repetitive units have longer structures. They correspond to the *B.pertussis* anhydrous dodecasaccharide [26] with different heterogeneities described above. Two main different bound-core structures are suggested (see figure 6B). The most complete structure (Structure 1) represents a dodecasaccharide with the terminal trisaccharide containing a FucNAcNMe residue like in *B. pertussis* (to compare with FucNAcN of the *B. avium* free core). From this structure, the lateral Hep can be absent, as well as the acetate group in FucNAcNMe.

The *B. avium* O-chain unit structure as characterized by NMR and mass spectrometry is a polymer composed of 1,4-linked 2-acetamido-3-[3-hydroxybutanamido]- 2,3-dideoxy-L-D-glucopyranosyluronic acid residues, with a mass corresponding to 301.3u per element. The different cores were substituted by side-chains of variable numbers of O-antigens subunits, from 5 to 13. For example, peaks observed at m/z 3867.5 and 4058.6 were attributed to polysaccharide structures containing core oligosaccharide with non-acetylated FucNNMe with or without lateral Hep, and both carrying six O-chain subunits, (theoretical m/z values are 3866.59 and 4058.75). Peaks observed at m/z 4512.3 and 4703.9 were attributed to polysaccharide structures containing core oligosaccharide with acetylated FucNAcNMe, either with or without lateral Hep and both carrying eight O-chain subunits (theoretical m/z values are 4511.2 and 4703.37). As mentioned above, some structural information can be lost, when the analysis of the PS region is performed on PS samples

isolated after acid hydrolysis, even if the hydrolysis conditions are very mild. For example, the substitution of Kdo with PPEA or phosphate can be lost via β -elimination from position C-4, thus giving rise to non-phosphorylated anhydrous forms of the corresponding PS or OS, displaying a double bond at C3-C4.

To obtain additional information on the Kdo substitution of *B. avium* LPS, we carried out MALDI-MS analyses after LPS de-O-acylation with methylamine. In the resulting mass-spectrum, we observed similar molecular species of free core as found in the spectrum of the acid-cleaved OS, but shifted at plus 80u. We also observed molecular ions of the short de-O-acylated LPS, corresponding to the free core structures described above in their Kdo phosphorylated form and linked to the di-acyl lipid A at +953u. These complementary data show that the Kdo of the *B. avium* LPS is at least phosphorylated. Fragments or molecular ions corresponding to the Kdo substitution with PPEA (+203u) were however observed in some native LPS partial spectra (not shown), which confirms the presence of this decoration present in the other *Bordetella* species.

3.5. Overview on LPS structural and genetic heterogeneity among *Bordetella* spp.

LPS from the *Bordetella* genus have always been complex and in-depth studies of each species led to peculiar traits, together highlighting the high degree of heterogeneity of LPS molecular species expressed by members of this genus.

One of the peculiarities of the *Bordetella* LPS is the presence of a trisaccharide element added to the distal part of the *B. pertussis* core structure [27]. This element is added due to the presence of the *wlb* gene cluster (also referred to as *bpl* genes) [48]. The distal trisaccharide was shown to be present in *B. pertussis*, *B. bronchiseptica* and *B. avium*. In the present study, we demonstrated that the trisaccharide is absent from both short and long *B. trematum* LPS. The trisaccharide is present in *B. hinzii* long LPS, and absent from the short molecular species. Finally, in *B. avium* the trisaccharide is present in long-chain LPS and some, but not all, short-chain structures. *B. pertussis*, which lacks O-chains altogether, displays such a trisaccharide for enlarging the nonasaccharide core; this addition is important for resistance to pulmonary surfactant protein A [49]. In addition to the presence of the distal trisaccharide, alternative strategies have evolved in this pathogen to inhibit complement activation at an early step of the cascade [50, 51] and therefore, an O antigen appears to be dispensable in *B. pertussis* for mediating resistance to the formation of membrane attack complexes—the terminal step of the complement pathways. In contrary, for other bordetellae which display either O-chains or

Semi-rough type structures, the presence of a distal trisaccharide does not appear to be crucial. Interestingly, the distal trisaccharide found in bordetellae bears structural similarity to the trisaccharide repeating units of the Enterobacterial common antigen (ECA) [52].

Some differences were also observed in the presence or absence of the terminal heptose. This element is completely absent from *B. trematum* and *B. hinzii* LPS and non-stoichiometrically distributed in the LPS molecular species of the *B. avium* strain analyzed in this study. An orthologous gene encoding the putative glycosyltransferase for the terminal heptose in *B. pertussis* (locus tag BP2487 in strain Tohama I), *B. bronchiseptica* (locus tag BB3923 in strain RB50) and *B. parapertussis* (locus tag BPP3474 in strain 12822) appears to be absent in the *B. hinzii*, *B. trematum*, *B. avium* strains sequenced thus far (not shown). The presence of the terminal heptose in some molecular species of *B. avium* LPS, as described above, may be explained by strain-specific differences, since we analyzed the LPS structure of a different *B. avium* strain than 197N. Remarkable strain-specific differences have also been found in *B. bronchiseptica*. In this species, strains with at least two distinct O antigen gene clusters have been identified, resulting in the expression of antigenically distinct O antigens [53].

Most structural differences in the LPS molecular species are consistent with the heterogeneity of the *wlb* genes and corresponding gene products in the different *Bordetella* spp. Whereas the gene products of *wlbA*, *wlbB*, *wlbC* and *wlbL* are conserved among all *Bordetella* spp at the amino acid level, there are species and strain-specific differences with regard to other *wlb* genes. In the *B. avium* 197N genome for example, *wlbD* (a UDP-N-acetylglucosamine 2-epimerase gene) is absent. However, this strain harbors the *wlbJK* gene encoding a putative membrane protein that is in turn absent in the genome of *B. pertussis* Tohama I and is encoded by two separate CDS in *B. bronchiseptica* strain RB50, namely *bplI* and *bplJ*. In the genome of *B. trematum* strain H044680328, the *wbl* locus constitutes of *wlbA*, *wlbB*, *wlbC*, *wlbD* and *wlbL*. In contrary, the *wlb* locus in *B. hinzii* strain NCTC13200 also harbors *wlbE* (encoding a glycosyl transferase family protein), *wlbF* (probable aminotransferase gene) and *wlbG* (encoding a probable sugar transferase). A schematic overview of the genomic organization and diversity of the *wbl* loci in the different bordetellae is shown in Figure 7A. An even greater degree of genetic diversity is found in the *wbm* loci, which encode gene products responsible for the LPS O-antigen synthesis. In the genomes of *B. bronchiseptica* RB50 and *B. parapertussis* 12822, *wbm* genes are located immediately downstream of the *wlb* genes. In *B. pertussis*, the entire *wbm* locus has been replaced by an insertion element [54], thereby leading to a lack of O-chains in this species. Although *B. hinzii*, *B. trematum* and *B.*

avium have retained the ability to express LPS species with O-chains, the genomic organization of the corresponding biosynthesis genes diverges considerably from that in *B. bronchiseptica* RB50 (Figure 7B) and *B. parapertussis* (not shown). Notably, a *wbmE* gene, whose gene product has been shown to catalyze the deamidation of uronamide-rich O chains in *B. bronchiseptica* [55], is found in *B. parapertussis* (not shown) but appears to be absent in the other bordetellae studied here (Figure 7B). This may explain why the O polysaccharide of *B. hinzii* is unusually rich in uronamides, because the genome of latter species, like *B. bronchiseptica* and *B. parapertussis*, also harbors a *wbmC* gene (encoding a glutamine-dependent amidotransferase family protein), which in turn is absent in the genomes of *B. avium* and *B. trematum*. This needs further investigation. Another noteworthy difference is the O-antigen ligase and polymerase genes in the different *Bordetella* species. Unfortunately, only very limited detailed biochemical investigations focused on the O antigen biosynthesis gene clusters in bordetellae have been conducted [55]. Therefore, it should be noted here that individual enzymatic mechanisms which are based on sequence similarities are speculative. Multiple pathways have been described in other Gram-negative bacteria for the biosynthesis of O polysaccharides [56]. The best described pathway is the so-called Wzy-dependent pathway, which requires the integral inner membrane proteins Wzx (O-antigen flippase), Wzy (O-antigen polymerase), and WaaL (O-antigen ligase) that participate the biosynthesis of well-described LPS structures such as the B-band lipopolysaccharide in *Pseudomonas aeruginosa* [57]. In *B. bronchiseptica* RB50 and *B. parapertussis* 12822, a putative O-antigen ligase is encoded by BB3922 and BPP3473 respectively; the gene products are nearly identical at the amino acid level [45] and show similarity to WaaL in other bacterial species. There is very low amino acid sequence similarity (29% sequence identity) of BB3922 with a putative O-antigen polymerase in *B. avium* N197 (encoded by BAV0122, GenBank ID of protein: CAJ47728.1) but no significant amino acid sequence similarity to any of the gene products of *B. hinzii* or *B. trematum*, nor with *B. bronchiseptica* strain 1289 that expresses an antigenically distinct O-antigen than RB50 [53], was found by basic local alignment search (BLAST) against the genomes of these species. Multiple sequence alignment (Clustal Omega) of the putative O-antigen polymerases in *B. avium*, *B. trematum*, *B. hinzii* and the gene product of BB3922 revealed that the O-antigen polymerase protein sequences in *B. trematum* are phylogenetically most distinct and all proteins bear some degree of sequence similarity to the putative O antigen ligase in *B. bronchiseptica* RB50 (Figure 8). This is consistent with our observations that *B. trematum* only displays 2 O-chain elements, in contrast to the other

Smooth-type bordetellae. More detailed biochemical investigations and targeted gene knock-out and complementation studies are warranted to elucidate the biosynthesis pathway(s) for the O-chains in bordetellae. Taken together, this remarkable genetic diversity of the *wlb* loci and genes responsible for the biosynthesis of O-chains in the different *Bordetella* spp. is consistent with the vast structural heterogeneity of LPS molecular species described above and suggests that *wlb* and *wbm* genes have been under enormous selection pressure leading to loss of many of these genes as a consequence of host adaptation. In contrary to the *wlb* and *wbm* genes, the core OS biosynthesis loci are highly conserved at the gene and protein levels among all bordetellae studies here (Figure 7C). This is consistent with the high degree of structural similarity of the core OS between *B. avium*, *B. trematum* and *B. hinzii* as well as with that of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* as demonstrated in this study. It was shown that some *Bordetella* species, like *B. bronchiseptica*, which is known to having the more heterogeneous and variable LPS molecular species, can be substituted with other intermediary structures like tetrasaccharides between their cores and O-chains. The later have a strong influence on bacterial antigenicity and on the capacity of the pathogen to escape the host-immune system [58]. This tetrasaccharide was shown to be present in *B. hinzii* LPS on different fragments obtained after chemical modifications in acidic or basic conditions [44]. We confirmed here its presence on unmodified LPS molecular species and demonstrated that no extra structural element was lost. Another interesting trait is a “stop-signal” like observed with *B. hinzii* [59] displaying a methyl group defining its last unit for regulating the exact length of the O-chain. The genetic basis for this remains unknown. LPS are known to help bacteria in their survival or host adaptation. The O-chain length probably favors virulence and protection of the bacteria from the complement system as described for *Shigella*, in which LPS glucosylation promotes bacterial invasion and evasion of innate immunity, which may have contributed to the emergence of serotype diversity in this genus [60]. It is well-known that the presence of O-chains help bacteria evading the complement system and it was described for *B. parapertussis* as well [61]. The importance of the O-chain was also described for its role in serum resistance [62]. However, no work was performed, to our knowledge, for establishing the reason and role of the characteristic relatively short O-chains (LPS size from 4kDa to 8kDa) present in all bordetellae, compared to LPS from other genera easily reaching 20kDa. A schematic representation of the different LPS structures in *B. trematum*, *B. hinzii*, *B. avium* and *B. pertussis* is shown in the Graphical abstract.

4. Conclusions

The structural originality of *Bordetella* LPSs has already been reported and attributed to species specificity. In this genus, for which LPS structures represent so many peculiarities, one of them is that the long-chain LPS molecular species are relatively short compared to the classical *Escherichia* LPS [43] and that of the majority of other commonly described genera, as shown by SDS-PAGE analysis. We showed in the present work that *B.avium* LPS displayed its highest molecular species at about 8000u, followed by *B.hinzii* at about 6000u and *B.trematum* LPSs at 5000u.

The *B. avium* free core molecular species are more related to the *B. bronchiseptica* core type. This is illustrated with different masses, and a real diversity due to incompleteness, compared to the *B. pertussis* core. These differences can be explained by the lack of different terminal sugars like Hep and GalNA. In addition, a main trait in these structures is also the partial absence of the methyl substituent of one of the amino group of the dia-amino-Fucosamine [43].

Most of the important details highlighted in these LPS structures were only accessible thanks to the capacity of mass spectrometry to reach molecular species directly without having to split or modify the LPS. Such chemical modifications were often applied for neutralizing charges and removing fatty acids from these molecules. These treatments might have facilitated mass spectrometry analyses in the past, but often led to other discrete unexpected modifications like release of amino acids [35], O-acyl groups or cleavage of pyrophosphate groups, as well as very labile sugars [29, 36]. These decorations are very important regarding the antigenicity and biological activities of LPS. This is why experiments directly performed on native molecules, or use of very specific or mild hydrolyses unveiled precious data, as demonstrated here and earlier [35]. It is interesting to note that it was with this aim that we worked at getting the first non-modified LPS spectra decades ago [40, 63] also showing the *B. pertussis* PPEA decoration of Kdo lost during acidic treatment (24). Improvement of the methods [37] evolved together with physicochemical treatments of native LPS, and mass spectrometry technology [64]. It would not be surprising to find that many structures described in the past on modified LPS, in different genera, present similar peculiarities, or other traits having escaped the analysis. In the present work, obtaining spectra from non-modified LPS allowed to clarify the level of substitution of PPEA and phosphate groups on different species. It also gave an idea of the degree of heterogeneity of the molecules, and allowed us to determine the exact level of substitution of the O-chains to the cores moieties

and the nature of the latter. Comparison of genes involved in the different osidic elements explains how selection pressure led to such heterogeneity and diversity rendering the pathogens more likely to escape the host innate immune system. Our findings also provide a rich foundation for future hypothesis-driven and detailed biochemical investigations to gain more insight into the biosynthesis of LPS structures in bordetellae.

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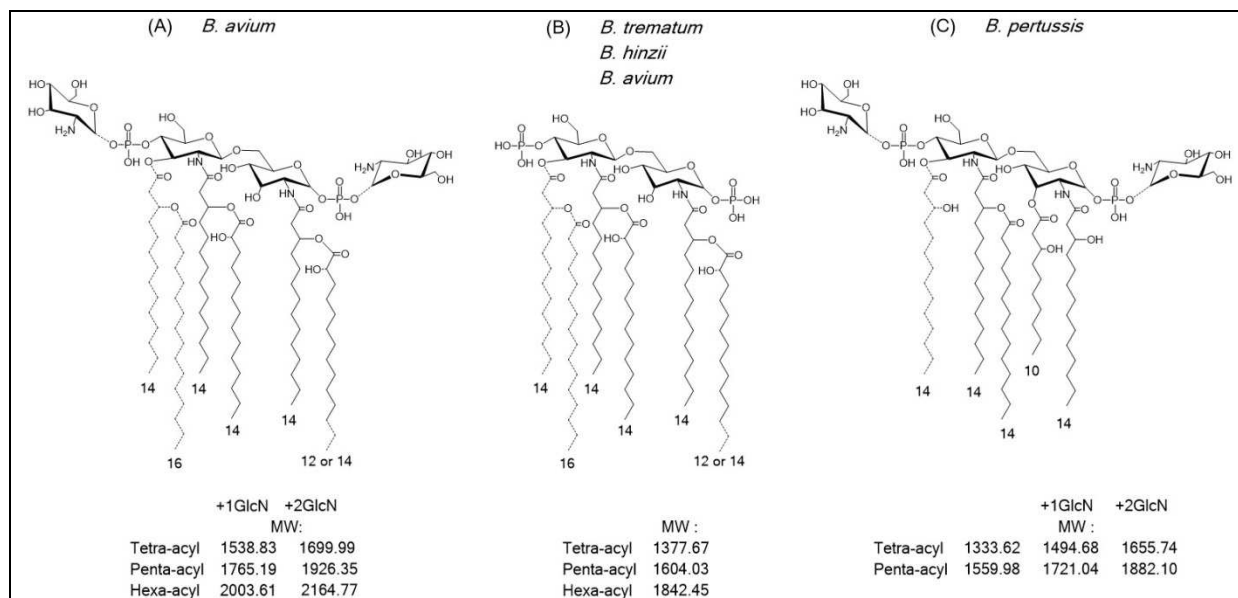
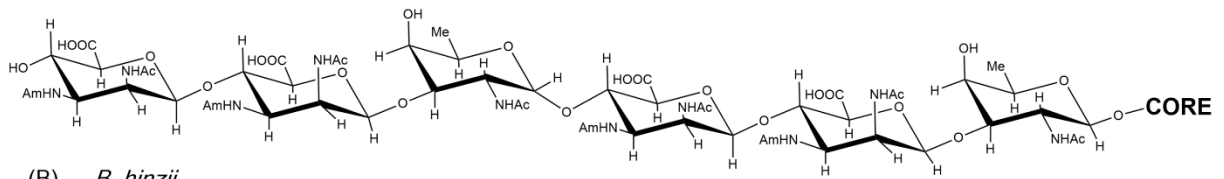
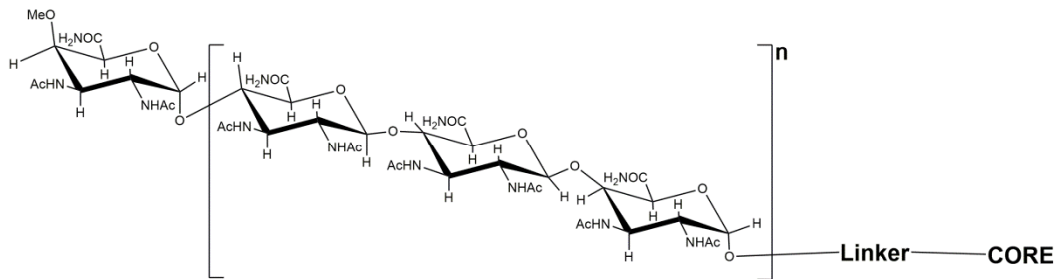


Figure 1: Structures and molecular weights of the major lipid A molecular species present in *B. avium* (A, B), *B. trematum* (B) and *B. hinzii* (B) [20] compared to *B. pertussis* (C) lipid A structure.

(A) *B. trematum*



(B) *B. hinzii*



(C) *B. avium*

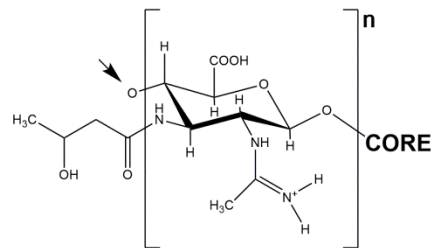


Figure 2: Structures of the O-chains present in *B. trematum* (A) [22], *B. hinzii* (B) [58, 59], and *B. avium* (C) [19].

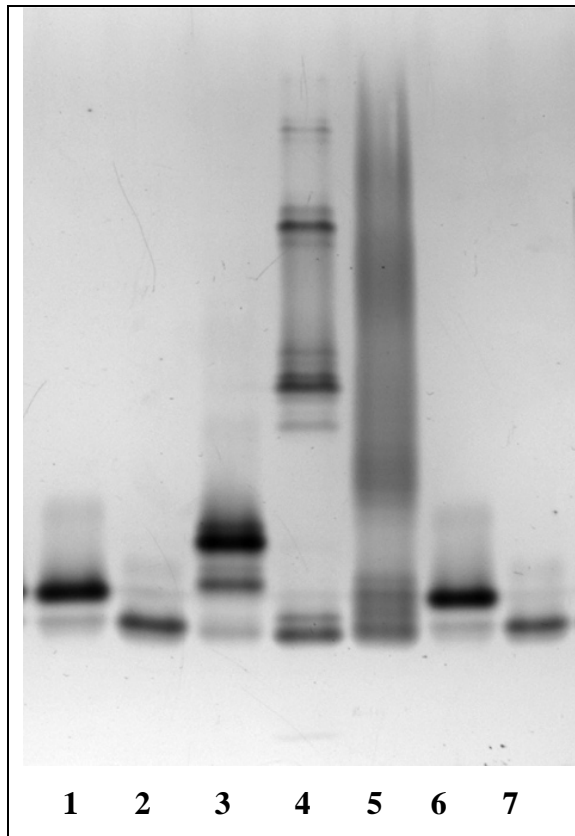


Figure 3: SDS-PAGE profile of LPS from *B. trematum* (3), *B. hinzii* (4) and *B. avium* (5), compared to LOS from *B. pertussis* BP1414 (1,6, Band A) and BPA100 (2,7, Band B).

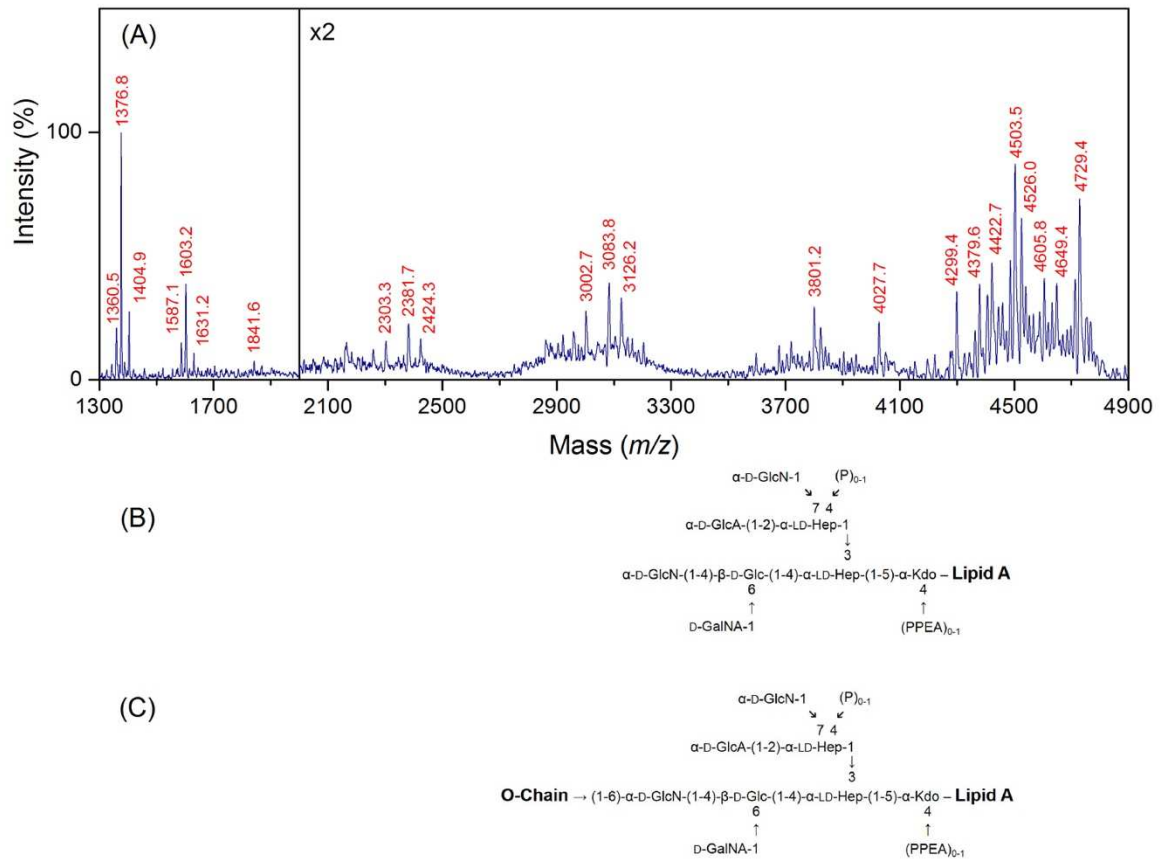


Figure 4: (A) *B. trematum* native LPS negative ion-mode MALDI mass spectrum, (B) chemical structure of the *B. trematum* free-core LOS, (C) structure of the *B. trematum* long-chain LPS. The O-chain elements have been described in [22].

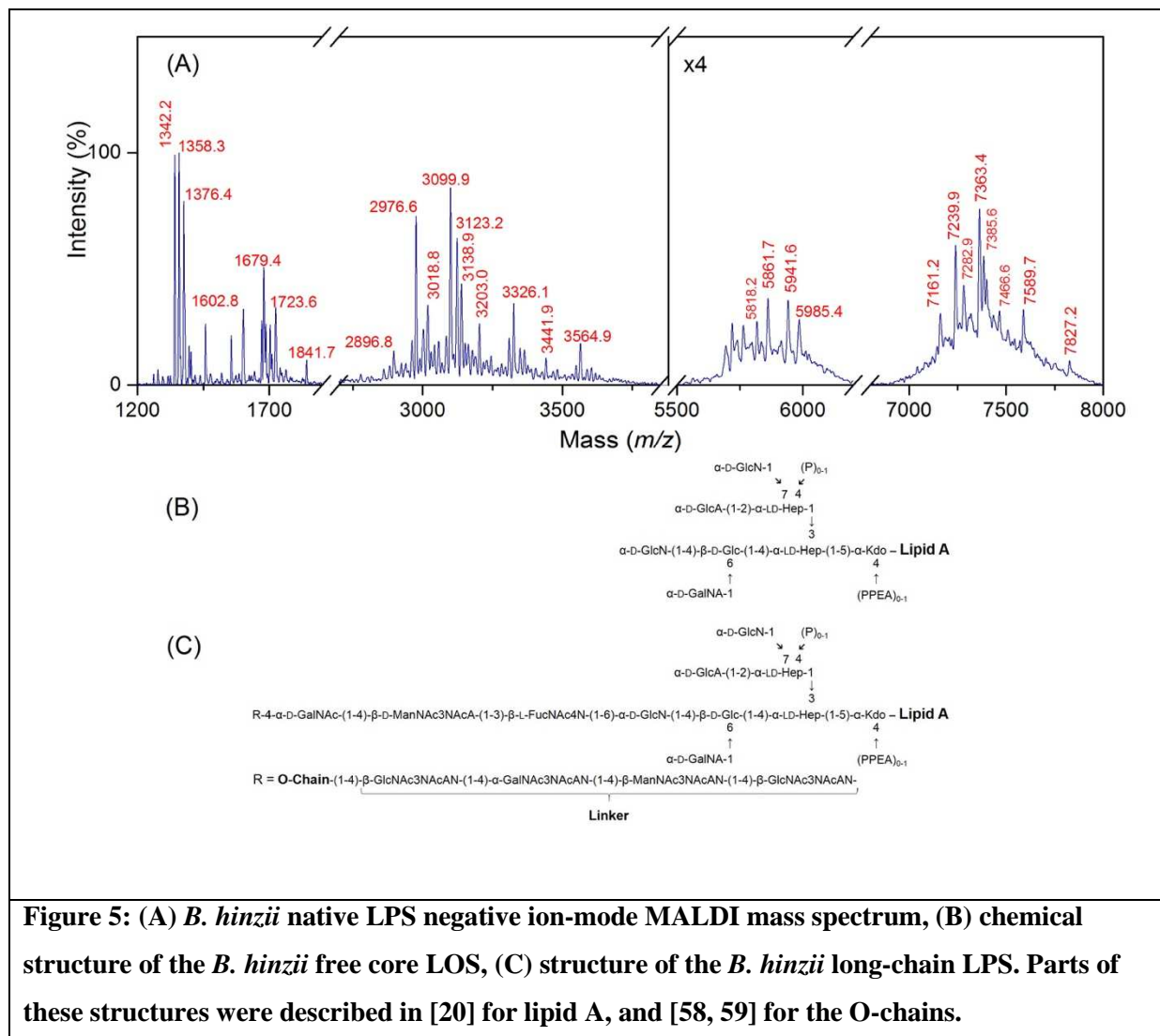


Figure 5: (A) *B. hinzii* native LPS negative ion-mode MALDI mass spectrum, (B) chemical structure of the *B. hinzii* free core LOS, (C) structure of the *B. hinzii* long-chain LPS. Parts of these structures were described in [20] for lipid A, and [58, 59] for the O-chains.

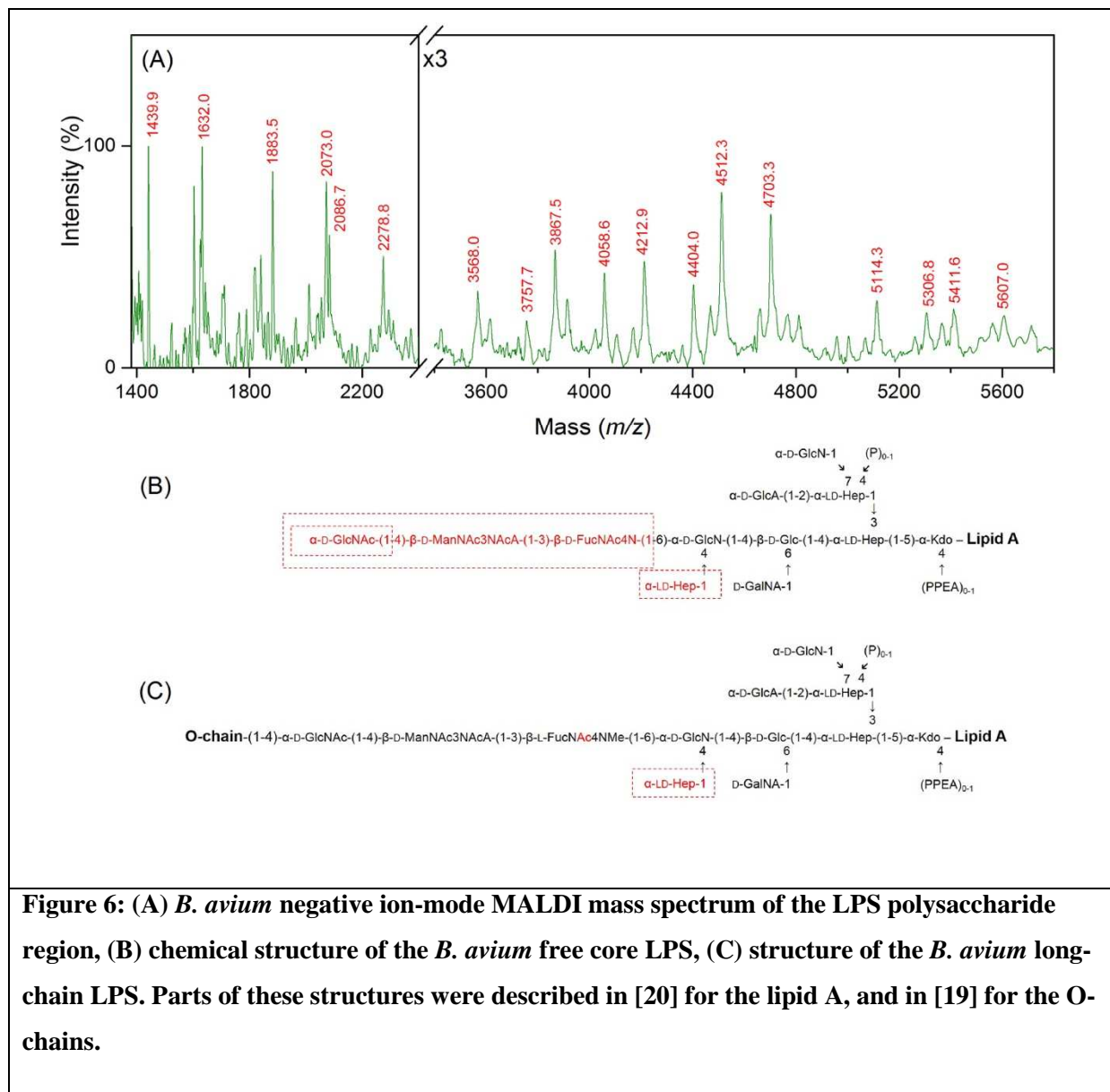
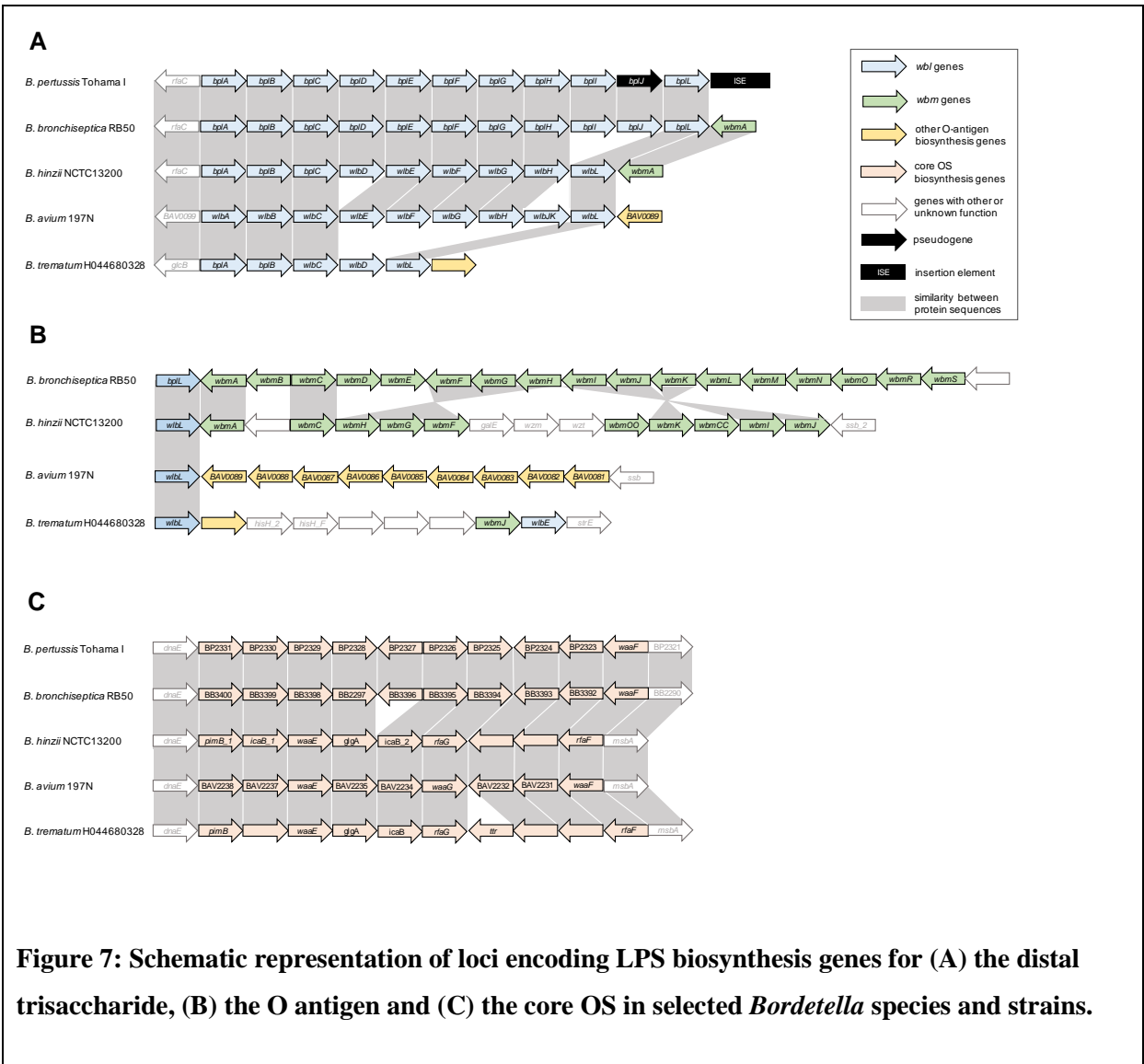


Figure 6: (A) *B. avium* negative ion-mode MALDI mass spectrum of the LPS polysaccharide region, (B) chemical structure of the *B. avium* free core LPS, (C) structure of the *B. avium* long-chain LPS. Parts of these structures were described in [20] for the lipid A, and in [19] for the O-chains.



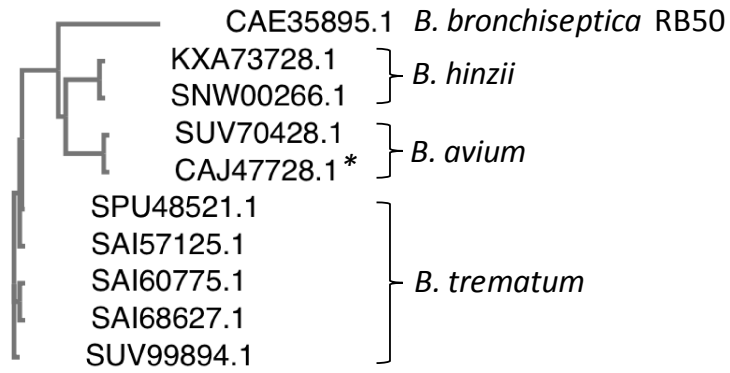
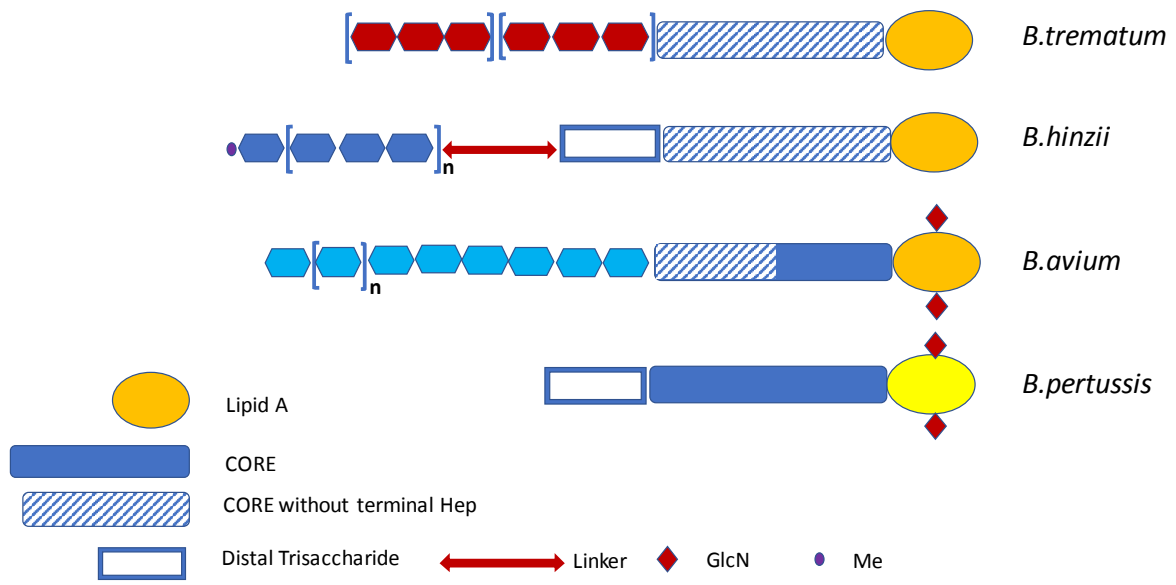


Figure 8: Phylogenetic tree representing a multiple sequence alignment of the putative O antigen polymerases in *B. avium*, *B. hinzii* and *B. trematum* with the gene product of BB3922 in *B. bronchiseptica* RB50. *, Putative O antigen polymerase in *B. avium* 197N.



Graphical Abstract: Schematic representation of the different LPS structures in *B. trematum*, *B. hinzii*, *B. avium* and *B. pertussis*.

Table 1: Genes shown in figure 7 with the predicted function of their gene products

GENE SYMBOL / LOCUS TAG	ANNONATION / FUNCTION OF GENE PRODUCT
<i>bplA, wlbA</i>	probable oxidoreductase
<i>bplB, wlbB</i>	probable acetyltransferase
<i>bplC, wlbC</i>	lipopolysaccharide biosynthesis protein
<i>bplD, wlbD</i>	UDP-N-acetylglucosamine 2-epimerase
<i>bplE, wlbE</i>	probable glycosyl transferase
<i>bplF, wlbF</i>	lipopolysaccharide biosynthesis protein
<i>bplG, wlbG</i>	probable sugar transferase
<i>bplH, wlbH</i>	putative glycosyl transferase
<i>bplI, wlbI</i>	lipopolysaccharide biosynthesis protein
<i>wblJ, wblJK</i>	putative membrane protein
<i>bplL, wblL</i>	lipopolysaccharide biosynthesis protein
<i>wbmA</i>	putative glycosyl transferase
<i>wbmB</i>	hypothetical protein
<i>wbmC</i>	putative glutamine amidotransferase
<i>wbmD</i>	putative membrane protein
<i>wbmE</i>	putative exported protein
<i>wbmF</i>	nucleotide sugar epimerase/dehydratase
<i>wbmG</i>	nucleotide sugar epimerase/dehydratase
<i>wbmH</i>	nucleotide sugar epimerase/dehydratase
<i>wbml</i>	putative asparagine synthetase
<i>wbmJ</i>	hypothetical protein
<i>wbmK</i>	hypothetical protein
<i>wbmL</i>	ABC transporter integral membrane protein
<i>wbmM</i>	ABC transporter ATP-binding protein
<i>wbmN</i>	ABC transporter ATP-binding protein
<i>wbmO</i>	hypothetical protein
<i>wbmR</i>	putative formyl transferase
<i>wbmS</i>	hypothetical protein
<i>wbmCC</i>	glutamine amidotransferase
<i>wbmOO</i>	O PS chain-termination methyltransferase
BAV0081	putative O-antigen translocase
BAV0082	putative lipopolysaccharide biosynthesis protein
BAV0083	putative lipopolysaccharide biosynthesis protein
BAV0084	putative lipopolysaccharide biosynthesis protein
BAV0085	putative lipopolysaccharide biosynthesis protein
BAV0086	putative glycosyl transferase
BAV0087	putative lipopolysaccharide biosynthesis protein
BAV0088	putative lipopolysaccharide biosynthesis protein
BAV0089	putative lipid A biosynthesis protein
BP2331, BB3400, BAV2238, <i>pimB, pimB_1</i>	lipopolysaccharide core biosynthesis glycosyl transferase
BP2330, BB3399, BAV2237	lipopolysaccharide core oligosaccharide biosynthesis protein
BP2329, BB3398, <i>waaE</i>	lipopolysaccharide core biosynthesis glycosyl transferase
BP2328, BB2297, BAV2235, <i>glgA</i>	lipopolysaccharide core biosynthesis glycosyl transferase
BP2327, BB3396	putative outer membrane protein
BP2326, BB3395, BAV2234, <i>icaB, icaB_1, icaB_2</i>	polysaccharide deacetylase
BP2325, BB3394, <i>waaG, rfaG</i>	lipopolysaccharide core biosynthesis glycosyl protein
<i>ttr</i>	acetyltransferase
BP2324, BB3393, BAV2232	uncharacterized protein
BP2323, BB3392, BAV2231	membrane protein
<i>waaF, rfaF</i>	ADP-heptose--LPS heptosyltransferase II
<i>msbA</i>	lipid A export ABC transporter ATP-binding/permease

Highlights

- Complete *Bordetella avium*, *B. hinzii* and *B. trematum* LPS structures are characterized by MALDI-MS and compared
- Three *Bordetella* LPS structures are presented in relation to genomic data
- MALDI Mass-spectrometry was applied to long-chain LPS leading to spectra of native LPS, presented for the first time without use of chemical modifications or fragmentation