Modifications of *Bordetella bronchiseptica* core lipopolysaccharide influence immune response without affecting protective activity

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*Bordetella bronchiseptica* produces respiratory disease primarily in mammals including humans. Although a considerably amount of research has been generated regarding lipopolysaccharide (LPS) role during infection and stimulating innate and adaptive immune response, mechanisms involved in LPS synthesis are still unknown. In this context we searched in *B. bronchiseptica* genome for putative glycosyltransferases. We found possible genes codifying for enzymes involved in sugar substitution of the LPS structure. We decided to analyse BB3394 to BB3400 genes, closed to a previously described LPS biosynthetic locus in *B. pertussis*. Particularly, conservation of BB3394 in sequenced *B. bronchiseptica* genomes suggests the importance of this gene for bacteria normal physiology. Deletion of BB3394 abolished resistance to naive serum as described for other LPS mutants. When purified LPS was analyzed, differences in the LPS core structure were found. Particularly, a GalNA branched sugar substitution in the core was absent in the LPS obtained from BB3394 deletion mutant. Absence of GalNA in core LPS alters immune response in vivo but is able to induce protective response against *B. bronchiseptica* infection.

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The Bordetella LPS core presents one KDO molecule and two heptoses linked to lipid A. This composition is quite different from other LPSs where up to three heptoses are present. Only three genes, \(\text{waaA}, \text{waaC}, \text{and waaF}\) are known to participate in the deep core synthesis.\(^{11,12}\) Gueret and co-workers described in \(B. \) pertussis four glycosyltransferases that participate in sugar core substitutions. However, no effects in \(B. \) pertussis pathogenesis has been described for those mutants.\(^{13}\) We have previously reported that a deep rough LPS mutant is unable to establish an infection in wild type mice.\(^{14}\) Interestingly, we suggested that early elimination may be the strong response to a deep rough LPS compared to smooth LPS. Particularly an enhanced IL-12 and TNF-alpha response was observed in mice treated with a deep rough LPS suggesting that distal portions of LPS are involved in host immune response regulation.\(^{12}\)

We hypothesized that enzymes that catalyse the addition of heptoses to the growing core of \(B. \) bronchiseptica LPS would be similar to known glycosyltransferases from other bacteria. Therefore, we found genes dispersed around all the \(B. \) bronchiseptica RB50 genome as shown in Supplemental Table S1. Pan-genome analysis indicates that genes associated with diverse phenotypes, antibiotic resistance or that confer selective advantages are often found within the accessory genome rather than the core genome.\(^{14}\) Hester and co-workers suggested that horizontally acquired divergent O-antigen contributes to escape from cross-immunity in the classical \(B. \) pertussis.\(^{15}\) Dispersal position of these genes may suggest a possible source of LPS structure diversity between different \(B. \) pertussis circulating strains. However, when other available genomes were analyzed, those genes were in the same relative position suggesting that glycosyltransferase genes are part of the core rather than accessory genome. Some of those genes are arranged in groups of putative glycosyltransferases. One of those groups, involving \(BB3396\) to \(BB3400\) are homologous to \(BP2328\) to \(BP2331\) previously described by Geurtsen and co-workers as glycosyltransferases involved in \(B. \) pertussis Tohama I LPS core synthesis.\(^{13}\) Moreover, a similar group of genes in \(Burkholderia cenocepacia\) were previously described.\(^{16}\) Interestingly, one of \(B. \) cenocepacia genes, \(BCAL2407\), a glycosyltransferase presents homology to \(BB3394\).

In order to confirm the role of this cluster of genes in LPS synthesis, we constructed mutants in \(B. \) bronchiseptica 9.73H\(^+\) with the individual genes \(BB3394\) and \(BB3398\) interrupted by an antibiotic resistance cassette. Wild type LPS presented a full length LPS with defined bands A and B and a diffuse low moving band corresponding to lipid A-core-O antigen structure. As expected, \(Bb\) \(BB3398\) did not grow properly in naive serum in contrast to \(Bb\) 9.73H\(^+\) wild-type, \(BbABB3394\) mutant and \(BbABB3398\) mutant strains. Both mutants showed different susceptibility to naive sera (B). \(~1000\) CFU were exposed to naive sera at different proportions to SS media. Appropriate dilutions were plated in \(BG\) after 2 h at 37°C. Results are expressed as percentage of CFU recovered when exposed to SS media. \(^*\) indicates \(P < 0.05; Bb9.73H+\) versus mutant.

On the other hand, \(BbABB3394\) LPS profile was indistinguishable from wild type LPS suggesting that deletion of genes involved in GlcN, GlcA or GalNA transfer to the core would not alter the length of the LPS (Fig. 1A). As a first approach to evaluate possible LPS modifications, sensitivity to naive serum were evaluated and compared to wild type strain. Interestingly, \(BbABB3394\) and \(BbABB3398\) did not grow properly in naive serum in contrast to \(Bb9.73H+\) strain. Resistance to both agents was restored when \(BbABB3394\) mutant was complemented with the corresponding gene. Results are shown in Fig. 1B.

To get deeper into the LPSs structures, LPSs from each mutant strain were further purified. The corresponding oligosaccharides were released by acid hydrolysis and analyzed by mass spectrometry. The MALDI-MS spectrum of the \(B. \) bronchiseptica wild type 9.73H\(^+\) oligosaccharide in the reflectron positive ion mode using GA as matrix is presented in Fig. 2A and supplemental Fig. S1. In the high molecular weight range a signal at \(m/z\) 1637.0 (calc. \(m/z\) 1637.5537, \(C_{38}H_{67}N_3O_{31}\) Na) consistent with a core fragment containing (Hep)GlcN-GalNAc-GlcA-GlcNHep-Hep bearing a FucN unit from the distal trisaccharide is present. Ion at \(m/z\) 1594.5 (calc. \(m/z\) 1594.3535) would correspond to the loss of an acetyl group from the latter. In accordance, ion at \(m/z\) 1456.1 (calc. \(m/z\) 1456.4319, \(C_{50}H_{44}N_2O_{27}\) Na) would correspond to the core octasaccharide fragment consistent with (Hep)GlcN-GalNAc-GlcA-GlcNHep-Hep as an oxonium ion. In addition loss of the GalNAc unit from the latter gives rise to \(m/z\) 1259.7 (calc. \(m/z\) 1259.4019, \(C_{40}H_{28}N_2O_{17}\) Na), and signal at \(m/z\) 1085.3 (calc. \(m/z\) 1084.3656, \(C_{32}H_{26}N_2O_{12}\) Na) corresponds to the loss of GlcA and a Heptose unit from \(m/z\) 1456.1. In addition, species at \(m/z\) 1191.1 (calc. \(m/z\) 1190.4059, \(C_{44}H_{30}N_2O_{29}\) ) is consistent with the distal trisaccharide.
ride (GlcNAc-Man2,3NacA-FucN) linked to the core fragment GlcN-(GalNA)Glc.

The MALDI-MS spectrum of the mutant BB3394 strain oligosaccharide in the positive ion mode (Fig. 2B and supplemental Fig. S2) showed the highest mass species at m/z 1259.6 (calc. m/z 1260.4103, C₇₀H₀₂N₂Na₂O₃₂) and m/z 1281.1 (ΔNa) consistent with the core fragment (Hep)GlcNC-(GlcNChep)Hep-KdoP. The lack of ion at m/z 1153.2 (calc. m/z 1153.3997, C₇₀H₀₂N₂Na₂O₃₂) is consistent with a FucN-(GlcNChep)Hep-ahKdoPP fragment. Interestingly no signals corresponding to fragments bearing the GalNA unit would be detected. Conservation of BB3394 in sequenced B. bronchiseptica genomes suggests the importance of this substitution for bacteria normal physiology. Resistance to naive sera is a phenotype usually modified when LPS alterations are present. Long LPS structures avoid complement components to reach bacterial surface. However in our hands, a small modification like the absence of the branching GalNA was sufficient to alter resistance to sera.

In several Bordetella LPS, a pentasaccharide fragment that links the O-antigen to the core region has been identified. Previous reports indicated that the O-chain of these bacteria LPS was a linear homopolymer of 1,4-linked 2,3-dideoxy-2,3-diacetamido-L-galacturonic acid (L-Gal2,3NAcA). However, differences between the end groups on B. bronchiseptica O antigens were described such as the presence of a terminal residue (Lac-type, 328.3 Da, and Ala-type, 327.3 Da). Further studies revealed that a proportion of the O-poly saccharide repeating units are present as uronamide (L-Gal2,3NacAN), the number of which varies between strains. Also, the presence in the O-antigen of blocks of amides and blocks of acids, whereas less abundant mixed amide/amid acids ions were evidenced.

In accordance, signals related to the O-antigen oligosaccharide were detected in the MALDI-MS spectrum of the mutant BB3394 strain. Thus, ion peak at m/z 2848.1 (calc. m/z 2848.0675, C₁₁₁H₁₇₅N₂₉O₉₃) may be attributed to the O-antigen fragment built up of a terminal “Lac type” residue, three uronamide units (L-Gal2,3NacAN), four 2,3-dideoxy-2,3-diacetamido-L-galacturonic acids (L-Gal2,3NacA) and three monosaccharides from the linking pentasaccharide (Man2,3NacAN-Glc2,3NacAN-GlcNac) with the concomitant loss of water. Ion at m/z 2583.1 (calc. m/z 2582.0044, C₁₁₂H₁₇₄N₂₉O₉₃) was assigned to a fragment built up by two 2,3-dideoxy-2,3-diacetamido-L-galacturonic acids (L-Gal2,3NacA) and one uronamide unit (L-Gal2,3NacAN) linked to the pentasaccharide plus the Band A trisaccharide unit. Ion at m/z 2418.4 (calc. m/z 2418.9663, C₁₀₂H₁₅₁N₂₈O₅₃) is consistent with one uronamide unit linked to the pentasaccharide plus Band A trisaccharide unit plus Hep-GlcN. In addition, ion at m/z 1418.3 (calc. m/z 1418.5691, C₂₀₆H₃₅N₂₃O₈₃) is attributed to one uronamide unit linked to the pentasaccharide and ion at m/z 1178.9 (calc. m/z 1178.4706, C₈₀H₁₂₈N₁₄O₅₃) corresponds to the pentasaccharide unit.

In contrast, regarding mutant BB3398, the MALDI-MS spectrum of the released oligosaccharide, in the negative mode (Fig. 3B and supplemental Fig. S3) showed the highest mass ion at m/z 2287.9 (calc. m/z 2288.6883, C₁₃₀H₁₇₂N₃O₃₉P₂) as expected for the lack of the O-antigen structure suggested by SDS-PAGE. This ion corresponds to a GlcNac-Man2,3NacA-FucN-GlcN-(GalNA)Glc-(GlcNChep)Hep-ahKdoPP using the O-antigen structure. Thus, ion at m/z 2130.8 (calc. m/z 2131.6508, C₇₂H₁₂₂N₃O₃₉P₂) would correspond to the trisaccharide unit plus GlcN-Glc-(GlcAGlchep)Hep-Kdo-PPEA. In the low MW range, main ion at m/z 842.9 (calc. m/z 843.2051, C₃₅H₆₄N₂O₉P) may be attributed to the GlcN-Glc-ahKdoP fragment. Interestingly a signal at m/z 1021.0 (Δ44) correspond to FucN-GlcN-Glc-ahKdoP fragment.

The MALDI-MS spectrum of the mutant BB3394 strain, performed in the negative ion mode using nor-harmane as matrix (Fig. 3A and supplemental Fig. S3) showed a complex pattern of signals. Ion at m/z 2281.2 (calc. m/z 2280.6719, C₇₉H₁₃₂N₂O₆₃P₂) corresponds to the core region consistent with (Hep)GlcN=Glc-(GlcNGlchep)Hep-KdoP linked to the Band A trisaccharide GlcNac-Man2,3NacA-FucN. Ion at m/z 1706.9 (calc. m/z 1707.5860, C₆₈H₁₃₂N₂O₆₃) corresponds to Man2,3NacA-FucN-GlcN-Glc-(GalNA)Glc-(GlcAGlchep)Hep-KdoPP after the loss of water. Ion at m/z 1840.1 (calc. m/z 1839.6312, C₆₈H₁₁₂N₈O₄₈P) would correspond to the branching oligosaccharide constituted by (GlcN-Glc)Hep-and harmane as matrix component to reach bacterial surface. However in our hands, a small modification like the absence of the branching GalNA was sufficient to alter resistance to sera.

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Interestingly, absence of GalNA in BB3394 mutant is not detrimental for distal structures synthesis. However, absence of lateral Heptose in BB3398 LPS generated a rough LPS indicating that Heptose needs to be present to be recognized as a substrate for enzymes
involved in LPS synthesis. Similar observation was previously reported with core second heptose.\textsuperscript{11} Deletion of waaf, an heptosyl-transferase involved in second heptose addition generated a deep rough phenotype.

Once confirmed we have \textit{B. bronchiseptica} LPS with deep modifications (deep rough LPS from BbLP39 and BbAB3398) or mildly modifications like BbAB3394 we hypothesized that structural differences will hold different immune responses. We have previously shown that a deep rough LPS triggers different interleukine responses in bone marrow derived dendritic cells.\textsuperscript{18}

Dendritic cell response to smooth or complete \textit{B. bronchiseptica} LPS is characterized by high TNF-alpha levels. \textit{Bb} and \textit{E. coli} LPS are short-chain LPS, commonly named heptosyl oligosaccharide. Interestingly, deep rough LPS derived from \textit{B. bronchiseptica} BB3398 or E. coli DH5α were intranasally instdilled in mice, differences were observed (Table 1).

Interestingly, deep rough LPS derived from BbAB3398 elicited non significantly differences to wild type LPS. However, we showed that GalNA substitution is important to induce TNF-alpha response. Absence of this lateral sugar in \textit{B. bronchiseptica} LPS inhibits TNF-alpha response (Table 1). Interestingly, TNF-alpha is an important factor secreted during first steps of infection and its absence is detrimental to limitation of \textit{B. bronchiseptica} infection.\textsuperscript{19}

It has been suggested that LPS immune activity may be responsible for directing an appropriated protective response.\textsuperscript{20,21} Therefore, a previously tested vaccination schedule was used to evaluate protection capacity of the different LPSs here in described.\textsuperscript{22}

Female three weeks old BALB/c mice were i.p. immunized with 5 μg of purified wild type LPS. As a control, whole cell formaldehyde inactivated vaccine was used to immunize mice. After 2 immunizations, mice were challenged intranasally with sublethal doses of wild type \textit{B. bronchiseptica}. Five days after challenge, animals were sacrificed and the number of bacteria in lungs was determined. As expected not immunized mice presented a high number in CFU/lung, while whole cell immunized mice were protected against infection progress (Fig. 4).

One of main components of whole cell vaccine is LPS. When mice were immunized with \textit{B. bronchiseptica} wild type LPS no significantly differences were observed with mice immunized by whole cell (Fig. 4).

Protection induced by \textit{B. bronchiseptica} LPS was genera and species specific. If we immunized with either \textit{B. pertussis} or \textit{E. coli} DH5α LPS, no protection was observed (Fig. 4). Both \textit{E. coli} DH5α and \textit{B. pertussis} LPS are short-chain LPSs, commonly named lipooligosaccharide. Interestingly \textit{B. pertussis} and \textit{B. bronchiseptica} core structure showed no differences.

When mice were immunized with 5 μg of a deep rough LPS from BbLP39 mutant no protection was observed supporting that distal LPS portions are necessary to induce protection (Fig. 4).

Using the same vaccination and challenge schedule, purified LPS from BbAB3398 and BbAB3394 with confirmed structure changes were evaluated. As shown in Fig. 4, BbAB3398 deep rough LPS was not able to elicit a protective response in mice as the other deep rough LPS from BbLP39. Interestingly, LPS from BbAB3394 mutant, lacking the GalNA unit in the core, protects mice against infection as the wild type LPS. This result is in agree-

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
LPS & TNF-alpha expression & Std. Error & P(H1) Result vs Bb 9.73H⁺
\hline
BbAB3398 & 0.556 & 0.271–1.180 & 0.138 Down

BbAB3394 & 0.242 & 0.119–0.527 & 0.001
\hline
\end{tabular}
\caption{Relative expression report generated by REST2009 after 2000 iterations.\textsuperscript{24} Values are obtained after being normalized to alpha-actin levels and compared to levels observed in Bb 9.73H⁺ LPS stimulated mice. P(H1): Probability of alternate hypothesis that difference between sample and control groups is due only to chance.}
\end{table}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{Effect of systemic (i.p.) immunization with different LPS structures and challenged with wild type Bb 9.73H⁺ in C3H/HeN (black columns) or C3H/HeJ mice (grey columns). Statistical analysis was performed by ANOVA and Tukey test. * indicate significant differences with p < 0.001 respect to C3H/HeN mice.}
\end{figure}

\textbf{Conflict of interest}

The authors declare that they have no conflicts of interest with the contents of this article.

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A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.12.049.

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