Structural Studies of Lipopolysaccharide-defective Mutants from *Brucella melitensis* Identify a Core Oligosaccharide Critical in Virulence*

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The structures of the lipopolysaccharides from *Brucella melitensis* mutants affected in the WbkD and ManB core proteins have been fully characterized using NMR spectroscopy. The results revealed that disruption of *wbkD* gives rise to a rough lipopolysaccharide (R-LPS) with a complete core structure ([β-\(d\)-GlcP-(1→4)-α-Kdo-(2→4)]{β-\(d\)-GlcP-(1→6)-β-\(d\)-GlcP-(1→4)[β-\(d\)-GlcP-(1→6)-β-\(d\)-GlcP-(1→3)-α-\(d\)-ManP-(1→5)]-α-Kdo-(2→6)-β-\(d\)-GlcP-N3N4P-(1→6)-α-\(d\)-GlcP-N3N1P}, in addition to components lacking one of the terminal β-\(d\)-GlcP and/or the β-\(d\)-GlcP residues (48 and 17%, respectively). These structures are identical to those of the R-LPS from *B. melitensis* EP, a strain simultaneously expressing both smooth and R-LPS, also studied herein. In contrast, disruption of *manB* gives rise to a deep-rough pentasaccharide core ([β-\(d\)-GlcP-(1→4)-α-Kdo-(2→4)-α-Kdo-(2→6)-β-\(d\)-GlcP-N3N4P-(1→6)-α-\(d\)-GlcP-N3N1P] as the major component (63%), as well as a minor tetrasaccharide component lacking the terminal β-\(d\)-GlcP residue (37%). These results are in agreement with the predicted functions of the WbkD (glycosyltransferase involved in the biosynthesis of the O-antigen) and ManB core proteins (phosphomannomutase involved in the biosynthesis of a mannosyl precursor needed for the biosynthesis of the core and O-antigen). We also report that deletion of *B. melitensis* *wadC* removes the core oligosaccharide branch not linked to the O-antigen causing an increase in overall negative charge of the remaining LPS inner section. This is in agreement with the mannosyltransferase role predicted for WadC and the lack of GlcP residues in the defective core oligosaccharide. Despite carrying the O-antigen essential in *B. melitensis* virulence, the core deficiency in the *wadC* mutant structure resulted in a more efficient detection by innate immunity and attenuation, proving the role of the β-\(d\)-GlcP-(1→6)-β-\(d\)-GlcP-(1→4)[β-\(d\)-GlcP-(1→6)-β-\(d\)-GlcP-(1→3)-α-\(d\)-ManP-(1→5)]-structure in virulence.

*Brucella* is a genus of Gram-negative facultative intracellular coccobacilli that causes brucellosis in humans and animals. Although its true extent is not known and the disease is largely unreported (1), it has been estimated that there are half a million new cases every year, most of them located in the poorest rural areas of the world (2). Humans can acquire brucellosis by ingestion of unpasteurized milk from infected animals or by contact with their secretions, but generally they are not themselves a source of contagion. The species within this genus were originally differentiated on the basis of their primary host preferences, with *Brucella melitensis* (sheep and goat), *Brucella suis* (pig), and *Brucella abortus* (cattle) being the most common in domestic livestock. Epidemiological evidence shows that, among them, *B. melitensis* is the most virulent species for humans (3). The lipopolysaccharides (LPS) of Gram-negative bacteria are exposed on the cell surface, and three different regions with different chemical and biological properties can be identified as follows: the lipid A, the core oligosaccharides, and the polysaccharide, which in most cases represents the O-specific polysaccharide (O-PS,2 O-antigen) (4, 5). The LPS of *Brucella* shows very low endotoxicity, which illustrates poor detection by innate immunity. Thus, it is considered one of the virulence factors that allow the pathogen to escape early detection by the host immune system (6, 7). Whereas this characteristic is related to the structure of the lipid A and core oligosaccharide of LPS, the O-PS (8, 9) also plays a major role in virulence, because it has been repeatedly observed that mutants lacking the O-PS (i.e., producing a rough (R) type LPS, also termed lipopolysaccharide (LOS)) are attenuated (10). *Brucella* strains with R-LPS are often caused by spontaneous mutations (11–13).

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and can also be due to mutation in genes encoding proteins involved in the biosynthesis of the monosaccharide components of the O-PS, its polymerization or transport, or the core oligosaccharide. In a previous report, the genes involved in the biosynthesis of the LPS of \textit{B. melitensis} were screened, and several mutants affected in the biosynthesis of both the core oligosaccharide or O-PS were obtained (10). To assign mutations to the biosynthetic pathways, the mutants were classified as R1 (complete core), R2 (defective core), and R3 (deep R core), respectively, according to the decrease in their LPS apparent molecular mass (10). For instance, mutations in \textit{wbkD}, \textit{wadA}, and \textit{manB\textsubscript{core}} gave rise to R1, R2, and R3 LPS core glycoforms, respectively (10). Based on sequence homology comparisons, \textit{wbkD} was proposed to code for a putative epimerase/dehydratase involved in the biosynthesis of quinovosamine, the monosaccharide located at the reducing end of the O-PS (10, 14, 15). In contrast, \textit{wadA} was proposed to encode a glycosyltransferase involved in the biosynthesis of the core, whereas \textit{manB\textsubscript{core}} was proposed to encode a phosphomannomutase involved in the biosynthesis of GDP-mannose, used as a precursor in the synthesis of both the core oligosaccharide and the perosamine residues found in the O-PS (10, 16). Moreover, it was shown in later works that deletion of genes encoding glycosyltransferases \textit{WadB} and \textit{WadC} creates severe but uncharacterized defects in the core without affecting the section linked to the O-PS. Remarkably, \textit{B. abortus} \textit{wadC} mutants are attenuated despite carrying an intact O-PS (17).

In this study, we describe the structural elucidation of the LOS from \textit{B. melitensis} strain EP, a spontaneous mutant producing O-PS and increased amounts of LOS (18), \textit{Bm\_wbkD} and \textit{Bm\_manB\textsubscript{core}}, two mutants in \textit{wbkD} and \textit{manB\textsubscript{core}} disrupted in the corresponding biosynthetic steps that produce R1 and R3 LOS, respectively (Table 1 and Fig. 1). We also report that deletion of \textit{B. melitensis wadC} removes the core oligosaccharide section not linked to the O-PS. We furthermore show that, despite carrying the O-PS essential in virulence, the lack of this core section results in a marked change in bacterial surface physicochemical properties, a more efficient detection by innate immunity, and attenuation in both cellular and animal models.

**Experimental Procedures**

**Bacterial Strains—**\textit{B. melitensis} 16M (biovar 1 reference strain) and \textit{B. melitensis} H38 are wild type virulent strains com-
monly used in virulence studies that are identical with regard to LPS genes (10). *B. melitensis* EP (henceforth BmEPR) is a strain that, although virulent and able to synthesize O-polysaccharides, produces comparatively large proportions of R-LPS (18) and is thus suitable to obtain R-LPS in large amounts for chemical analyses. Mutants with defects in LPS genes (Table 1 and Fig. 1) were obtained, characterized, and stored according to the procedures described previously (10, 17).

**Growth of Bacteria and Isolation of the LOS**—Bacteria were propagated in tryptic soy broth either in a Biostat fermentor (BmEPR, Bm_wbkD, and Bm_manBcore) or in 2-liter flasks (Bm_wadC and Bm_wadC_per) on an orbital shaker in a BSL3 facility. After 36 h of incubation, bacteria were inactivated with phenol, harvested by tangential flow filtration, and washed twice with saline (19). For LOS extraction (BmEPR, Bm_wbkD, Bm_manBcore, and Bm_wadC_per), bacteria were first acetone-dried and then extracted following the phenol/chloroform/organic bound phosphate, and fatty acids were performed as described previously (9, 17).

**Compositional Analyses**—Quantitative analyses of sugars, organic bound phosphate, and fatty acids were performed as described previously (22).

**Preparation of the Decacylated Oligosaccharides**—The LOS from different B. melitensis mutants were O-deacylated using hydrazine, followed by N-deacylation with hot KOH (23) and purified by high performance anion-exchange chromatography.

**Mass Spectrometry**—Electrospray ionization high resolution mass spectra (ESI-HR-MS) were recorded in negative ion mode using a MicrOTOF™ mass spectrometer (Bruker Daltonics). Nitrogen was used as the collision gas.

**NMR Spectroscopy**—The deacylated LOSs of BmEPR (3.8 mg), Bm_wbkD (2.4 mg), and Bm_manBcore (3.4 mg) were deuterium-exchanged by freeze-drying three times from 99.9% D₂O and examined as a solution of 99.96% D₂O (0.55 mL) at pH 8.5 and 25 °C. Chemical shifts are reported in parts/million using internal sodium 3-trimethylsilyl-(2,2,3,3-²H₄)-propanoate (δ₄ = 0.00), external 1,4-dioxane in D₂O (δc = 67.40), or external 2% phosphoric acid in D₂O (δc = 0.00) as references.

The diffusion-filtered ¹H NMR spectrum of the deacylated LOS from Bm_manBcore was recorded on a Bruker Avance 500 MHz spectrometer equipped with a 5-mm Z-gradient (53.0 G/cm) spectrometer equipped with a 5-mm Z-gradient (53.0 G/cm) or external 2% phosphoric acid in D₂O (δc = 0.00) as references. The diffusion-filtered ¹H NMR spectrum of the deacylated LOS from BmEPR and Bm_wbkD were recorded on a Bruker Avance III 700 MHz spectrometer equipped with a 5-mm Z-gradient (53.0 G/cm) or external 2% phosphoric acid in D₂O (δc = 0.00) as references. The1H,13C-H2BC experiments (30) were recorded with a mixing time of 65 ms. For assignments of inter-residue correlations, ¹H,¹H NOESY and ¹H,¹3C heteronuclear multiple-bond correlation (HMBC) experiments were utilized. The gradient-selected ¹H,¹H NOESY experiments (31) were recorded with a mixing time of 100 ms, whereas the gradient-selected ¹H,¹3C HMBC experiments (32) were carried out with an evolution time of 65 ms. Band-selective constant-time ¹H,¹3C HMBC experiments (33) with 2-fold low pass J-filters (hmbccetetgl2nd) were also employed to improve spectral resolution in the anomeric region. The experiments were recorded over a spectral region of 5.4 × 9.0 ppm with 2048 × 256 data points, using an 80-ms delay for evolution of the long range couplings. A selective ¹3C excitation pulse (Q5 gaussian cascade) of 2.5 ms was applied at the center of the anomeric region.

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**TABLE 1**

*B. melitensis* LPS mutants used in the present work

| Name          | Parental strain | Gene (ORF)          | Role (annotation)                        | LPS phenotype | Refs. |
|---------------|-----------------|---------------------|------------------------------------------|---------------|-------|
| Bm_manBcore   | H38             | manBcore (BMEI0899) | Core synthesis (phosphomannomutase)      | R3            | 10    |
| Bm_wbkD      | 16M             | wbkD (BMEI1427)     | O-PS synthesis (epimerase/dehydratase)    | R1            | 10    |
| Bm_per       | 16M             | per (BMEI1414)      | O-PS synthesis (perosamine synthetase)    | R1            | 10, 46, 47 |
| Bm_wadC      | 16M             | wadC (BMEI0509)     | Core synthesis (glycosyltransferase)      | S             | This work |
| Bm_wadC_per  | 16M             | wadC (BMEI0509)/per (BMEI1414) | Core and O-PS synthesis | R3 | This work |

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**Brucella melitensis Rough LPS**

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**Brucella melitensis Rough LPS**

$^{31}$P-based NMR experiments were obtained on a Bruker Avance III 600 MHz spectrometer equipped with a 5-mm Z-gradient ($55.7 \text{ G cm}^{-1}$) inverse TXI ($^{1}H/^{13}C/^{31}P$) probe. The one-dimensional $^{1}H$-decoupled $^{31}$P NMR spectra were recorded with a spectral width of 396 ppm, and the $^{31}$P chemical shifts assignments were obtained from gradient selected $^{1}H/^{31}P$ HMBC (32, 34) and $^{1}H/^{31}P$-hetero TOCSY experiments (35). The $^{1}H/^{31}P$ HMBC spectra were recorded with an evolution time of 100 ms. The $^{1}H/^{31}P$-hetero TOCSY experiments were carried out with mixing times of 23 and 46 ms, using a DIPSI2 mixing sequence set at 2.5 kHz.

Additionally, NMR experiments selected from those described above were carried out on deacylated tetra- and pentasaccharides from Bm$_{wadC,per}$ on 0.28 and 0.26 mg, respectively, in D$_2$O (0.55 ml) at pH 7, a temperature of 15 °C, and a $^{1}H$ frequency of 500 MHz.

### Results

#### Compositional Analyses

The analysis of sugars, fatty acids, and organic bound phosphate (P) of the LOS from mutants Bm$_{manB,core}$, Bm$_{wbkD}$, and BmEPR revealed the presence (in mmol mg$^{-1}$ LOS) of Glc (164/176/110, respectively), Kdo (714/536/572), GlcN (53/637/680), P (1017/782/659), 12:0 (349/286/188), 18:0 (100/89/102), cyclo19:0 (55/154/105), 16:0 (349/286/188), and 28:0 (27-OH/27-oxo) (161/99/90). GlcN3N was not quantified. Mannose was present in trace amounts in the LOS from BmEPR but was lacking in the other two LOSs, because it was not present at all in Bm$_{manB,core}$ and was not released under the acidic hydrolysis conditions used due to its substitution by GlcN (compare structures below).

#### Mass Spectrometry

**HRMS of the LOS from Bm$_{manB,core}$**—The two sets of single negatively charged pseudo-molecular ions observed in the high resolution mass spectrum of the deacylated LOS from Bm$_{manB,core}$ (Fig. 2A) are consistent with the presence of two different oligosaccharides. The ions at m/z 1099.3 and 937.2 correspond to species formed through the loss of a proton. The former ion is in agreement with the presence of a pentasaccharide composed of one hexose (Hex), two Kdo, two diaminohexoses (HexNN), and two phosphate groups (P), whereas the latter indicate the presence of a tetrasaccharide composed of two Kdo residues, two diaminohexoses, and two phosphate groups (see Table 2). Different singly negatively charged sodium adducts of the pentasaccharide are observed at m/z 1121.3, 1143.2, and 1165.2, whereas those of the tetrasaccharide can be found at m/z 959.2, 981.2, and 1003.2.

**HRMS of the LOS from Bm$_{wbkD}$**—The mass spectrum of the deacylated core oligosaccharide from Bm$_{wbkD}$ (Fig. 2B) is also consistent with the presence of two major oligosaccharides, which produce the doubly negatively charged ions at m/z 952.3 and 871.8 through the loss of two protons each. The ion with higher m/z value corresponds to a decasaccharide composed of two Hex, four hexosamines (HexN), two Kdo, two HexNN, and two phosphate groups (see Table 2); the different doubly charged sodium adducts of this oligosaccharide appear.
at \(m/z\) 963.3, 974.3, and 985.3. The ion at \(m/z\) 871.8 corresponds to a nonasaccharide composed of two Hex, three HexN, two Kdo, two HexNN, and two phosphate groups; ions from three different sodium adducts of this compound are present at \(m/z\) 882.8, 893.7, and 904.7.

**NMR Spectroscopy**

**LOS from Bm\_manB\_core**—In the \(^1\)H NMR spectrum, three resonances corresponding to anomic protons were identified as follows: two doublets at 4.577 and 4.632 ppm (\(J_{H_1,H_2} = 8.1\) and 8.0 Hz, respectively) and one doublet of doublets at 5.430 ppm (\(J_{H_1,H_2} = 3.2\) Hz and \(J_{H_1,H_2} = 8.2\) Hz). The residues were named \(\text{A, E, and B, in order of decreasing } ^1\text{H chemical shifts (Fig. 3A). Two major resonances were identified in the } ^31\text{P NMR spectrum at } 2.6\) and 3.2 ppm, suggesting the presence of two phosphomonomer groups, one of which is consistent with the splitting of the H1 resonance of residue \(\text{A. All the protons from H1 to H6 could be traced using } ^1\text{H,}^3\text{H TOCSY experi-}"

**TABLE 2**

**HR-MS data (negative ion mode) and proposed compositions of the LPS from Bm\_manB\_core and Bm\_wbkD**

Summary of the diagnostic pseudo-molecular ions observed in the high resolution mass spectra of the deacetylated LPS of Bm\_manB\_core and Bm\_wbkD and the proposed composition of the two main components present in each sample. The components are abbreviated as follows: Hex (hexoses), HexN (hexosamine), HexNN (diaminohexose), and P (phosphate group).

| Mutant name | Observed | Calculated | Annotation | Molecular formula | Proposed composition (exact molecular mass) |
|-------------|----------|------------|------------|-------------------|--------------------------------------------|
| Bm\_manB\_core (Fig. 2A) | 1099.2680 | 1099.2750 | \([M - H]^−\) | C\(_{28}\)H\(_{50}\)N\(_4\)O\(_{27}\)P\(_2\)Na | Hex\_Kdo\_HexNN\_P\(_2\) (1100.2822) |
| | 1121.2520 | 1121.2569 | \([M - 2H + Na]^−\) | C\(_{34}\)H\(_{58}\)N\(_4\)O\(_{32}\)P\(_2\)Na\(_3\) | |
| | 1143.2344 | 1143.2389 | \([M - 3H + 2Na]^−\) | C\(_{58}\)H\(_{100}\)N\(_7\)O\(_{49}\)P\(_2\)Na\(_3\) | |
| | 1165.2195 | 1165.2208 | \([M - 4H + 3Na]^−\) | C\(_{64}\)H\(_{112}\)N\(_8\)O\(_{53}\)P\(_2\)Na\(_2\) | |
| | 937.2173 | 937.2221 | \([M - H]^−\) | C\(_{28}\)H\(_{50}\)N\(_4\)O\(_{27}\)P\(_2\)Na | Kdo\_HexNN\_P\(_2\) (938.2294) |
| | 959.1989 | 959.2041 | \([M - 2H + Na]^−\) | C\(_{34}\)H\(_{58}\)N\(_4\)O\(_{32}\)P\(_2\)Na\(_2\) | |
| | 981.1896 | 981.1860 | \([M - 3H + 2Na]^−\) | C\(_{58}\)H\(_{100}\)N\(_7\)O\(_{49}\)P\(_2\)Na\(_2\) | |
| | 1003.1579 | 1003.1680 | \([M - 4H + 3Na]^−\) | C\(_{64}\)H\(_{112}\)N\(_8\)O\(_{53}\)P\(_2\)Na\(_2\) | |
| | 952.2962 | 952.2978 | \([M - 2H]^2−\) | C\(_{28}\)H\(_{51}\)N\(_4\)O\(_{27}\)P\(_2\)Na | |
| | 963.2803 | 963.2888 | \([M - 3H + 3Na]^−\) | C\(_{34}\)H\(_{58}\)N\(_4\)O\(_{32}\)P\(_2\)Na\(_3\) | |
| | 974.2724 | 974.2798 | \([M - 4H + 2Na]^2−\) | C\(_{58}\)H\(_{100}\)N\(_7\)O\(_{49}\)P\(_2\)Na\(_2\) | |
| | 985.2674 | 985.2708 | \([M - 5H + 3Na]^−\) | C\(_{64}\)H\(_{113}\)N\(_8\)O\(_{53}\)P\(_2\)Na\(_3\) | |
| | 871.7581 | 871.7635 | \([M - 2H]^2−\) | C\(_{28}\)H\(_{51}\)N\(_4\)O\(_{27}\)P\(_2\)Na | Hex\_HexN\_Kdo\_HexNN\_P\(_2\) (1745.5415) |
| | 882.7532 | 882.7544 | \([M - 3H + 3Na]^−\) | C\(_{34}\)H\(_{58}\)N\(_4\)O\(_{32}\)P\(_2\)Na\(_3\) | |
| | 893.7469 | 893.7454 | \([M - 4H + 2Na]^2−\) | C\(_{58}\)H\(_{100}\)N\(_7\)O\(_{49}\)P\(_2\)Na\(_2\) | |
| | 904.7361 | 904.7364 | \([M - 5H + 3Na]^−\) | C\(_{64}\)H\(_{113}\)N\(_8\)O\(_{53}\)P\(_2\)Na\(_3\) | |

**FIGURE 2.** Mass spectra of deacylated LOS. Selected regions of the high resolution mass spectra of the deacylated core oligosaccharides of Bm\_manB\_core (A) and Bm\_wbkD (B) recorded in negative ion mode. The clusters of pseudo-molecular ions originating from the two major components of each sample are annotated (see Table 2). Note that the peaks observed in A and B correspond to singly and doubly negatively charged ion species, respectively.
Brucella melitensis Rough LPS

![NMR spectra of the deacylated LOS from Bm\_manB\_res. A, selected region of the diffusion-filtered $^1$H NMR spectrum. B, selected regions of the multiplicity-edited $^1$H,$^{13}$C HSQC spectrum showing the anemic region (right bottom), the region for the nitrogen-bearing carbons (right middle), and the region for the ring carbons and those from hydroxymethyl groups (left) in which the cross-peaks from the latter appear in red. C, selected region of the $^1$H,$^{13}$C HMBC spectrum showing intra- and inter-residue correlations from anemic carbons. D, selected region of the $^1$H,$^{31}$P-hetero TOCSY spectrum ($r_m = 92$ ms) showing correlations from the phosphate groups in residues A and B. Signals from impurities of lower molecular mass than the LOS oligosaccharides of B. melitensis are indicated by the hash symbol.](https://example.com/figure3.png)

Mons, indicating that these residues have the gluco-configuration. $^{13}$C NMR chemical shifts were assigned using multiplicity-edited $^1$H,$^{13}$C HSQC (Fig. 3B) and $^1$H,$^{13}$C H2BC experiments. Both C2 and C3 resonances of residues A and B were found in the region between 54 and 59 ppm (Fig. 3B, right middle), which indicates that these are nitrogen-bearing carbons. $^1$H,$^1$H couplings constants were extracted from a coupled $^1$H,$^{13}$C HSQC spectrum and revealed that residues B and E are $\beta$-linked ($J_{C1,H1} = 164$ and 163 Hz, respectively), whereas residue A is $\alpha$-linked ($J_{C1,H1} = 174$ Hz); thus A is $\alpha$-D-GlcpN3N, B is $\beta$-D-GlcpN3N, and residue E is $\beta$-D-Glcp. In the $^{13}$C NMR spectrum, three anomeric carbons were identified at 100.10, 100.15, and 100.46 ppm and attributed to C2 resonances in three different populations of Kdo residues (D*, D, and C, respectively). In the $^1$H,$^{13}$C HMBC spectrum, the C2 carbon of each Kdo residue could be correlated to its respective H3 proton via two-bond heteronuclear correlations (Fig. 3C), and the H3 resonances were used as starting points for the assignments of the respective spin systems. The multiplicity-edited $^1$H,$^{13}$C HSQC spectrum (Fig. 3B, right top) showed cross-peaks of different relative intensities for each of the Kdo residues, which was confirmed by integration of the H3a resonances in the $^1$H NMR spectrum and revealed that residues B and A core were determined by integration of the $^1$H,31P HMBC and $^1$H,31P-hetero TOCSY spectra.

The substitution positions of the phosphomonoester groups were determined using $^1$H,$^{31}$P HMBC and $^1$H,$^{31}$P-hetero TOCSY experiments and were found at C1 in residue A ($J_{P,CH_3} = 8.2$ Hz, $J_{P,C1} = 5.4$ Hz, and $J_{P,C2} = 6.1$ Hz) and C4 in residues B (Fig. 3D). Three additional resonances of minor intensities were found in the $^{31}$P NMR spectrum at $\delta_P = 4.0$ (t, $J_{P,CH_3} = 7.0$ Hz), 4.1 (d, $J_{P,CH_3} = 8.2$ Hz), and 4.4 (t, $J_{P,CH_3} = 6.8$ Hz) and were attributed to free phosphoethanolamine, glycerol 2-phosphate, and glycerol 3-phosphate, respectively. The $^1$H resonances of these three components could readily be identified in the $^1$H,$^{31}$P HMBC and $^1$H,$^{31}$P-hetero TOCSY spectra and correlated to their respective carbons in the multiplicity-edited $^1$H,$^{13}$C HSQC (denoted with the hash symbol in Fig. 3B, left). The chemical shifts assignments of these three components (phosphoethanolamine, 3.975/61.05 and 3.210/41.48 ppm; glycerol 2-phosphate, 4.155/75.64 and 3.682/62.24 ppm;
TABLE 3
NMR chemical shift assignments of the LOS from Brum_manBcore and Brum_wadC_per and inter-residue correlations from 1H, 13C HMBC NMR spectra

The 1H, 13C, and 31P NMR chemical shifts (ppm) of the LOS from Brum_manBcore were determined at 25 °C and pD 8, whereas the 1H and 13C NMR chemical shifts of the tetra- and penta-saccharide from Brum_wadC_per (OS1 and OS2, respectively) were obtained at 15 °C and pD 7. The H,C,134 values are given in Hz in parentheses and 1J,C,134 values in Hz. For residue A, 3J,C,132 = 8.2 Hz, 3J,C,131 = 5.4 Hz, and 3J,C,133 = 6.1 Hz. ND means not determined.

| Sample | sugar residues | 1H/13C | 31P HMBC correlations |
|--------|----------------|--------|-----------------------|
| Brum_manBcore |                 |  |                |
| (→)−α–d–GlcNP1 | A | 5.430 (3.2) | 2.955 | 3.171 | 3.508 | 4.128 | 3.742 | 4.263 | 2.6 |
| (→)−α–d–GlcNP4−(1→) | B | 94.08 (174) | 54.59 | 54.97 | 69.27 | 72.43 | 70.23 | 3.2 | C6, A |
| (→)−α–d–Kdo−(2→) | C | 57.77 (8.1) | 2.786 | 3.172 | 3.797 | 3.730 | 3.481 | 3.384 | H6a, H6b, A |
| (→)−α–d–Kdo−(2→) | D | 103.87 (164) | 55.47 | 58.60 | 71.05 | 76.41 | 63.40 | H6a, B |
| α–Kdo−(2→) (without residue E) | E | 177.40 | 100.15 | 33.13 | 75.63 | 66.60 | 73.25 | 70.55 | 64.16 |
| β–d–Glcp−(1→) | E | 3.462 (8.0) | 3.296 | 3.521 | 3.418 | 3.485 | 3.737 | 3.918 | C4, D |
| Brum_wadC_per OS1 |                 |  |                |
| (→)−α–d–GlcNP1 | A | 5.445 | 3.031 | 3.242 | 3.562 | 4.143 | 3.752 | 4.268 | H4, C |
| (→)−α–d–GlcNP4−(1→) | B | 94.05 | 54.22 | 55.14 | 68.88 | 72.55 | 70.25 | H4, D |
| (→)−α–d–Kdo−(2→) | C | 104.00 | 55.40 | 58.66 | 70.82 | 76.47 | 63.46 | H4, D |
| α–Kdo−(2→) | D | 176.66 | 100.15 | 35.22 | 66.71 | 66.95 | 73.44 | 70.27 | 64.22 |

| Brum_wadC_per OS2 |                 |  |                |
| (→)−α–d–GlcNP1 | A | 5.448 | 3.038 | 3.249 | 3.572 | 4.143 | 3.751 | 4.267 | C4, D |
| (→)−α–d–GlcNP4−(1→) | B | 93.95 | 54.18 | 55.02 | 68.66 | 72.43 | 70.15 | H4, D |
| (→)−α–d–Kdo−(2→) | C | 103.82 | 55.32 | 58.54 | 70.69 | 76.41 | 63.34 | H4, D |
| α–Kdo−(2→) | D | 1.848 | 2.290 | 4.241 | 4.277 | 3.635 | 3.987 | 3.757 | 4.018 |
| β–d–Glcp−(1→) | E | 4.636 | 3.292 | 3.515 | 3.408 | 3.485 | 3.746 | 3.921 | H4, C |

*δH18 of residue C* = 4.156.

glycerol 3-phosphate, 3.858/72.19, 3.822/65.52, 3.787/65.52, 3.686/63.11, and 3.616/63.11 ppm) were in agreement with data previously reported in the literature (39, 40). Diffusion-filtered experiments (1H NMR and 1H,1H TOCSY spectra) were employed to confirm that these compounds had lower masses than the deacylated LOS material of Brum_manBcore and thus were not linked to LPS. The genome of *B. melitensis* contains an LptA homologue putatively involved in the transference of ethanolamine to an unknown position of the core-lipid A, which could account for the 2-aminoethyl phosphate group (16). This may have been substituting the O4 position of residue B (either as phosphodiester or phosphodiesthether), but under the basic conditions used to prepare the oligosaccharides, this was probably lost (41–43). Additional spin systems of lower intensity, similar to A and B but with slightly different chemical shifts, were also identified in the 1H,1H TOCSY spectra (A*: 5.406, 2.848, 3.106, 3.480, and 4.093, and B*: 4.516, 2.762, 3.152, 3.830, and 3.737, from H1 to H5, respectively), as well as resonances in the 1H NMR at 1.286 (CH2) and 0.869 (CH3) ppm, attributed to residual acyl groups. The structure of the penta- and penta-saccharide components of the LOS from Brum_manBcore, shown in Fig. 4A and consistent with the structure of the lipid A reported previously for *B. abortus* (44).

**LOS from BrumEPR**—The 1H NMR spectrum of the deacylated LOS (Fig. 5A, top) revealed a complex material, with several signals of different intensities in the anomeric region (between 5.42 and 4.43 ppm). Eight distinctive spin systems originating from the anomeric protons were identified in the 1H,1H TOCSY spectra, and the different sugar residues were denoted A, F, G, E, B, J, H, and I, in order of decreasing 1H chemical shifts. In addition, a minor spin system similar to residue F but with slightly different chemical shifts was also identified and denoted F*. For residues A, G, E, B, J, H, and I, all protons from H1 to H6 could be identified in the 1H,1H TOCSY spectrum recorded with the longest mixing time (Fig. 5B), indicating that these monosaccharide components have the glaco-configuration. The distinctive downfield chemical shift of H2 in residues F and F* suggested that these are Man residues. The anomeric proton of residue A is a doublet of doublets (3J,C,131H2 = 3.2 Hz and 3J,C,131H1 = 8.3 Hz) indicating that this could be the α–d–GlcN31H1 residue at the reducing end of the lipid A moiety.

13C chemical shifts were assigned using multiplicity-edited 1H,13C HSQC (Fig. 5C, left), 1H,13C-H2BC, 1H,13C HMBC, and 1H,13C HSQC TOCSY experiments. 1H and 13C chemical shifts assignments are compiled in Table 4. Both C2 and C3 resonances of residues A and B were found in the region between 54 and 59 ppm, confirming that these are the two GlcN3 residues of the lipid A. The C2 resonances of residues G, J, H, and I were also found in the region of the nitrogen-bearing carbons, indicating that these are GlcN residues (Fig. 5C, left top); thus, residue E is Glc. 1J,C,134 couplings constants were extracted from a coupled 1H,13C HSQC spectrum and revealed that residues A and F are α–linked (1J,C,131H1 = 173–174 Hz), whereas residues B, E, and G–J are β–linked (1J,C,131H1 = 162–165 Hz). The spin systems of the Kdo residues were analyzed as described before, and four distinctive populations were found and denoted D and C.
Los (major decasaccharide and nonasaccharide components of the LOS in the 1H,13C HSQC spectrum) have the following sequence of residues (in the case of the major component that extends from a Man residue). The same two basic structures as above were identified, with the only difference being a branched oligosaccharide moiety composed of GlcN residues (I-H-[J]G), in the case of the major component that extends from a Man residue (F) linked to position 5 of the Kdo residue C. Thus, the major decasaccharide and nonasaccharide components of the LOS (∼66 and ∼34%, respectively, determined by integration of the characteristic C3/H3b cross-peaks of residues D and D* in the 1H,13C HSQC spectrum) have the following sequence of sugar residues: E-D-[I-H-[J]G-F]C-B-A and D*-[I-H-[J]G*-F*]C*-B-A. The substitution positions of the phosphonoester groups were as described above. The two major resonances in the 31P NMR spectrum (2.5 and 3.1 ppm) were attributed to α-d-GlcP3N1P (residue A) and α-d-GlcP3N4P (residue B); likewise, the minor resonances found at 8, 4.0, 4.1, and 4.4 were attributed to free ethanolamine phosphate, glycerol 2-phosphate, and glycerol 3-phosphate. Additional cross-peaks of lower intensity (∼19%) were also found in the multiplicity edited 1H,13C HSQC spectrum and attributed to an oligosaccharide similar to the decasaccharide described above, but without the GlcN residue I linked to residue H. In this case, 1H and 13C chemical shift assignments were carried out by comparison with the chemical shifts of the same oligosaccharide found in the LOS from Bm_wbkD (∼48%) (see below), and the corresponding sugar residues were denoted with a double asterisk. The C4/H4 cross-peaks of residues G and G** in the 1H,13C HSQC spectrum were used to estimate the percentage of these oligosaccharides in the sample. The structures of the decaca- and nonasaccharide components of the LOS from BmEPR are shown in Fig. 4B.

Los from Bm_wbkD—The 1H NMR spectrum of the decacylated LOS from Bm_wbkD (Fig. 5A, bottom) appears quite similar to that of BmEPR (Fig. 5A, top), with the only difference being the relative intensities displayed by some of the resonances. 1H and 13C chemical shifts assignments were carried out as described previously; the same oligosaccharide structures as above were identified, E-D-[I-H-[J]G-F]C-B-A and D*-[I-H-[J]G*-F*]C*-B-A, and particularly the nonasaccharide E-D-[H**-[J]G**-F**]C-B-A, that was fully characterized due to the higher relative concentration of the component in the sample (∼48%, instead of 19% observed in the case of BmEPR). Comparison of the multiplicity-edited 1H,13C HSQC spectrum of the decacylated LOS from BmEPR (Fig. 5C, bottom left) and
that of Bm\_wbkD (Fig. 5C, bottom right) allowed for the identification of a conspicuous resonance at δ\_H / δ\_C 4.440/103.48 (H1/C1 in residue H***) that is noticeably stronger in the latter spectrum. The ¹H and ¹³C resonances of residue H** were assigned employing ¹H,¹H TOCSY, ¹H,¹³C HSQC TOCSY, and ¹H,¹³C HMBC experiments. The H6a and H6b resonances (3.752 and 3.934 ppm, respectively) were identified in the ¹H,¹H TOCSY spectrum (τ\_m = 100 ms) due to their significant chemical shifts differences with respect to those of H6a and H6b in residue H and were correlated to the C6 carbon in the multiplicity-edited ¹H,¹³C HSQC spectrum. The differences in the chemical shifts of C6 in residues H** and H (61.46 and 69.66 ppm, respectively) indicated that the former is not 6-substituted and thus is a terminal residue. Another conspicuous signal was observed in the multiplicity-edited ¹H,¹³C HSQC spectrum at δ\_H / δ\_C 3.736/79.12 (H4/C4 in residue G**); the ¹H resonances in that spin system were identified using correlations from the carbon at 79.12 ppm in the ¹H,¹³C HSQC TOCSY spectrum (τ\_m = 100 ms), and the corresponding ¹³C resonances were assigned using a multiplicity-edited ¹H,¹³C HSQC spectrum. Furthermore, an inter-residue correlation was observed in the band-selective constant-time ¹H,¹³C HMBC spectrum (recorded with enhanced resolution in the carbon anomeric region) between the anomeric carbon of residue H** and the proton at 3.736 ppm (H4 in residue G**). In addition, the regular ¹H,¹³C HMBC spectrum showed a correlation between the anomeric proton of residue H** and the C4 carbon in residue G**(Fig. 5D), thus confirming that the G** residue is 4-substituted with residue...
Brucella melitensis Rough LPS

**TABLE 4**
NMR chemical shifts assignments of the resonances of the LOS from BmEPR and Bm_wbkD

| Sugar residues | 1H/13C | HMBC correlations
|---------------|--------|------------------|
|               | 1 2 3a 3b 4 5 6a 6b 7 8a 8b 31P |

| →(1→3)-α-D-GlcpN3N1P | A 5.419 (3.2) 2.898 3.126 3.503 4.127 3.738 4.290 2.5 |
| →(1→6)-β-D-GlcpN3N4P (1→) | B 4.535 (8.0) 2.753 3.112 3.764 3.724 3.472 3.782 3.1 C a, A |
| →(2→4)-α-Kdop (→) | C 175.68* 100.45 35.25 70.54 74.36 72.80* |
| →(2→) (without residue E) | C* 1.996 2.099 4.177 2.425 ND ND ND ND |
| →(2→) (without residue E) | D 175.34* 101.18 33.25 76.22 66.68 72.83 70.76 63.95 H4, C |
| →(2→) (without residue E) | D* 1.802 2.161 4.042 4.022 3.702 3.994 3.782 3.983 |
| β-→Glc-p (→) | E 4.609 (7.9) 3.299 3.520 3.426 3.480 3.747 3.915 C4, D |
| →(3→)-α-D-Man-p (→) | F 5.268 (NR) 4.312 4.129 3.847 4.130 ~3.887 C5, C |
| →(3→)-α-D-Manp (→) (without residue E) | F* 5.275 4.310 4.089 3.874 4.089 ~3.887 H5, C |
| →(4→)-β-→GlcN-p (→) | G 6.427 (8.2) 2.758 3.584 3.709 3.844 3.891 4.272 C3, F |
| →(4→)-β-→GlcN-p (→) (without residue I) | G** 6.426 (8.30) 2.756 3.595 3.736 3.844 3.891 4.272 |
| →(6→)-β-→GlcN-p (→) | H 4.442 (8.1) 2.748 3.405 3.867 3.668 3.851 4.267 C4, G |
| →(6→)-β-→GlcN-p (→) (without residue I) | H** 4.440 2.727 3.405 3.480 3.489 3.752 3.934 C4, G** |
| β-→Glc-p (→) | I 4.426 (8.1) 2.682 3.384 3.464 3.460 3.752 3.934 C6, H |
| β-→Glc-p (→) | J 4.465 (8.2) 2.723 3.373 3.409 3.460 3.748 3.913 C6, G |
| β-→Glc-p (→) | K 101.74 35.29 66.77 67.37 72.96 71.03 64.85 |

a Tentative assignments are shown.
b Assignments may be interchanged.

**H**. Furthermore, the anomeric carbon of residue G** showed an inter-residue correlation in the band-selective constant-time 1H,13C HMBC spectrum to a proton at 4.129 ppm, attributed to H3 of residue F** (overlapping with H3 of residue F). 1H and 13C chemical shifts assignments of the deca- and nonascaric acid components of the LOS from Bm_wbkD are compiled in Table 4; the corresponding structures and relative percentages are shown in Fig. 4B.

**LOS from Bm_wadC_per**—Two oligosaccharides were isolated from the deacylated LOS of the double mutant, viz. a tetra- and a pentasaccharide (OS1 and OS2, respectively), the structures of which correspond to those described for the oligosaccharide mixture from Bm_manBcore. Their 1H and 13C NMR chemical shifts (Table 3) were assigned by two-dimensional NMR experiments (cf. 1H,13C HSQC NMR spectra in Fig. 6, A and B, respectively), in good agreement with those from the Bm_manBcore oligosaccharides. In particular, an interglycosidic heteronuclear correlation was present in the 1H,13C HMBC spectrum of the pentasaccharide from the anomeric proton (H1) of the glucosyl residue to the glycosyloxyxylcarbon atom (C4) of the second Kdo residue (Fig. 6C), thus establishing and confirming the structural element E-D (Fig. 4).

In a recent investigation of oligo- polysaccharides obtained by mild acid hydrolysis of LOS from different Brucella serotypes, structural elements consistent with core oligosaccharides presented herein were present (15). Notably, it was shown that D-QuNAc, which is the primer for the O-chain polysaccharide, is β-(1→4)-linked to the glucose residue of the core (B. suis data), thereby defining the attachment site of the O-antigen to the core region of Brucella serotypes, a finding anticipated to be valid in the Brucella serotypes investigated herein.

**Role of Brucella Melitensis Core in Virulence**

For an unambiguous analysis of the role of the LOS core in the virulence of B. melitensis, the use of core-defective O-PS-bearing bacteria is necessary. To this end, a mutant in wadC was constructed. As shown in Fig. 7, SDS-PAGE and Western blot analyses show that this wadC mutant carries a core defect but keeps the O-PS, a result in agreement with previous work in B. abortus (17). This phenotype is consistent with the putative role of WadC as a mannosyltransferase because this enzymatic activity would be necessary to create the mannosyl-Kdo linkage in the structure shown in Fig. 4B. To verify this, a double Bm_wadC_per mutant was constructed and its LOS analyzed. Consistent with the role of Per in O-PS synthesis (Fig. 1) and the need of the O-PS for export to the periplasm and subsequent linkage to the core oligosaccharide (5), the LOS of this double mutant lacked the full O-PS section (quinoxivosamine, mannosine, and N-formyl perosamine polymer). Moreover, this LOS also completely lacked the mannosyl/glucosamine-containing oligosaccharide linked to Kdo (residue C), being similar to that of the manBcore mutant (blocked in mannose synthesis). The lack of aminosugars should increase the negative charge of the inner sections of LPS, and this was shown to occur in [sion] potential measurements of the Bm_wadC_per mutants and Bm_per (Fig. 8).

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To assess the biological effect of the core, the ability of Bm\textsubscript{wadC} to multiply in BMDCs was studied in comparison with wild type B. melitensis. Mutant bacteria displayed a comparatively reduced ability to multiply in these cells suggestive of attenuation (Fig. 9A). When the virulence was assessed \textit{in vivo} using the mouse model of brucellosis, Bm\textsubscript{wadC} and the parental strain yielded similar cfu at post-infection week 2 (Fig. 9B). At post-infection week 8, however, the mutant was present in comparatively reduced numbers in the spleens (Fig. 9C).

To determine whether the reduced virulence of Bm\textsubscript{wadC} was associated with an alteration in the immunogenicity of its LOS structure, further experiments were performed to assess its potential to induce pro-inflammatory responses in BMDCs. Unlike the Bm\textsubscript{wt} LPS, which induced no secretion of inflammatory cytokines, Bm\textsubscript{wadC} LPS induced the release of the pro-inflammatory cytokines IFN-\gamma, IL-12p40, IL-6, and TNF-\alpha at high levels that were comparable with those obtained with LPS from \textit{E. coli} (Fig. 10A). Similarly, with \textit{E. coli} LPS, Bm\textsubscript{wadC}-stimulated BMDCs underwent maturation as judged by the surface expression of MHC-II and the co-stimulatory markers CD86, CD80, and CD40. By contrast, BMDCs treated with Bm\textsubscript{wt} LPS maintained an immature phenotype with no evident up-regulation of these surface receptors (Fig. 10B). Taken together, these results demonstrate that an intact LPS core is not only required for full virulence of \textit{B. melitensis}, but it also contributes to limiting the activation and maturation of dendritic cells while undergoing replication in these target cells. An alteration in the LPS core, as demonstrated here with Bm\textsubscript{wadC}, would appear to confer a more endotoxigenic phenotype rendering the pathogen more visible to host target cells, attenuating its intracellular replicative capacity and virulence in mice.

**Discussion**

Rough mutants of \textit{B. melitensis} have been reported to show attenuated responses with respect to strains having a smooth LPS. In addition, phenotypes with different core defect structures have been observed to give rise to different attenuation...
patterns, and this has been associated with changes on the bacterial surface (10, 17). Therefore, knowledge of the primary structure of the LOS may help not only to have a better understanding of the genetics involved in biosynthetic processes but also to comprehend the interaction of the bacteria with the host immune system. Herein, we present a comprehensive study of the structures of different LOSs extracted from four *B. melitensis* strains. These include the native R-LPS (LOS) obtained from a strain producing increased proportions of R-LPS (18) (BmEPR), a mutant in which the *wbkD* gene was disrupted (Bm_\*wbkD\*) and a mutant affected in the ManBcore protein (Bm_\*manBcore\*) as well as the double mutant strain Bm_\*wadC\_*per\*.

The structural elucidation of the deacylated LOSs from BmEPR and Bm_\*wbkD\*, carried out using NMR spectroscopy, revealed that both mutants are capable of producing a complete core structure (Fig. 4B) as well as minor components lacking the terminal residue I (19 and 48%, respectively) and/or residue E (34 and 17%, respectively). Furthermore, the pseudo-molecular ions observed in the mass spectra of the deacylated LOS from Bm_\*wbkD\* (Fig. 2B and Table 2) were in agreement with the two major components determined by NMR spectroscopy as follows: (i) a complete core decasaccharide with a molecular formula of C_{64}H_{116}N_{8}O_{53}P_{2} that is consistent with a structure containing two Hex (Glc and Man), four HexN (GlcN), two Kdo, and two HexNN residues bearing phosphomonoester substituents (GlcN_{3N1}P and GlcN_{3N4}P), and (ii) a nonasaccharide with a molecular formula of C_{58}H_{104}N_{7}O_{49}P_{2} that is consistent with the same components as above but having three GlcN residues instead of four. It needs to be stressed that the full structures obtained for BmEPR and Bm_\*wbkD\* are identical.

*B. melitensis* EP was used in this work because it keeps the ability to synthesize S-LPS while producing increased amounts of R-LPS, which makes possible to obtain larger amounts of a native R-LPS for chemical analysis. Because a complete core structure was present in the LOS from Bm_\*wbkD\*, the *wbkD* gene can be anticipated to encode for a protein involved in the biosynthesis of the O-PS. This is in agreement with the predicted function of WbkD as involved in the biosynthesis of QuiNAc, the undecaprenol-priming residue and therefore the first sugar of the O-PS chain (10). In a smooth LPS the O-PS chain, having a 3-substituted QuiNAc residue located at its reducing end, would be extended from the O4 position of the Glc residue (15). Consequently, the pentasaccharide moiety...
composed of GlcN residues linked to a mannosyl residue constitutes a core branch.

The mutant in the manBcore gene showed a deeply truncated core (Fig. 4A) in which the lateral branch is absent. This is in agreement with previous observations that such a kind of mutants (Bm_manBcore) have a lower molecular mass LOS (R3 phenotype) than those of BmEPR and Bm_wbkD (R1 phenotype) and fail to react with monoclonal antibodies specific for the outer core (10, 16). The studies carried out using NMR spectroscopy showed that the LOS from Bm_manBcore is com-
posed of two major oligosaccharide components as follows: a pentasaccharide and a tetrasaccharide (63 and 37%, respectively), with the only difference being the presence or absence of the terminal Glc residue, respectively (Fig. 4A). These structures are in agreement with the two sets of pseudo-molecular ions observed in the mass spectrum that correspond to structures of general formula $C_{34}H_{62}N_4O_{32}P_2$ and $C_{28}H_{52}N_4O_{27}P_2$ (Fig 2A and Table 2), respectively. The ManB$_{core}$ has been previously predicted to be the phosphomutase involved in the biosynthesis of GDP-mannose (Fig. 1), which is used in the biosynthesis of monosaccharide components of the core and O-PS. Indeed, it is shown here that when the $\text{manB}_{core}$ gene is blocked the LOS oligosaccharide lacks the mannosyl residue and the branched structure that extends from it.

From a biological point of view, the structure described here is not a trivial one as it explains a number of characteristics related to the virulence of an important pathogen. The brucellae are resistant to components of the innate immune system such as bactericidal peptides and complement while prompting a weak pro-inflammatory response during infection (7), characteristics that are in part related to the biological and physicochemical properties of its LPS (6, 7, 17). Many of these properties have been attributed to the poor cytokine-inducing characteristics of $Brucella$ lipid A, on account of its very long chain fatty acids that may prevent effective recognition by TLR4-MD2 (6). However, it has been predicted that the core should play a complementary role whereby its structure allows it to conceal the inner sections of lipid A, making them less accessible for binding by the TLR4-MD2 complex or for binding bactericidal peptides or complement (15, 17). The loss in monoclonal antibody reactivity observed with a $B. \text{abortus}$ $\text{wadC}$ mutant suggested that the core of smooth brucellae may atypically be composed of two separate oligosaccharide branches, one linking the lipid A to the O-PS and another that protrudes laterally thereby concealing the negatively charged groups essential for interaction with the TLR4-MD2 complex (17). The characterization presented here confirms these predictions as the GlcN-rich oligosaccharide stemming from the first Kdo residue (C) also creates a positively charged structure that can neutralize the negatively charged groups of Kdo and lipid A, the primary targets of innate immunity receptors and effector molecules. This is further supported by the attenuated intracellular replicative/virulence profile and change in surface charge exhibited by the Bm$_{wadC}$ mutant strain, as well as the enhanced ability of its LPS to stimulate maturation and pro-inflammatory cytokine secretion (including Th1-type cytokines IL-12 and IFN-γ) when compared with its WT counterpart.

In addition to reaffirming the above hypothesis, these observations provide new understanding of the important role of $\text{Brucella core-LPS}$ in influencing key host-pathogen interactions during the early stages of infection and perhaps when chronic disease develops. Moreover, our findings have important implications as they identify $\text{Brucella LPS}$ as a key target/protagonist for strategies aimed toward the development of safer attenuated brucellosis vaccines that can promote more efficient protective Th1 responses (45), thought to be critical for the control of intracellular brucellae.

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