The identification of wadB, a new glycosyltransferase gene, confirms the branched structure and the role in virulence of the lipopolysaccharide core of Brucella abortus


a Departamento de Microbiología y Parasitología e Instituto de Salud Tropical, Universidad de Navarra, 31008 Pamplona (Spain)
b Instituto de Agrobiotecnología (CSIC-Universidad Pública de Navarra-Gobierno de Navarra), 31006 Pamplona (Spain)
c Centre d'Immunologie de Marseille-Luminy, Aix-Marseille University, Centre d'Immunologie de Marseille-Luminy, 13288 Marseille, France
d INSERM U1104, 13288 Marseille, France
e CNRS UMR7280, 13288 Marseille, France

1 Present address: Centre for Infection and Immunity, Queen's University Belfast Health Sciences Building, 97 Lisburn Rd. Belfast, UK BT9 7AE, UK.

¥ Corresponding author
Instituto de Salud Tropical y Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad de Navarra, Edificio de Investigación, Universidad de Navarra, c/Irunlarrea 1, 31008 Pamplona, Spain.
email address: miriart@unavarra.es
Phone number: +34948425600; ext. 6524

* These authors contributed equally to this work.

Yolanda Gil-Ramírez: ygilrami@alumni.unav.es
Raquel Conde-Álvarez: rconde@unav.es
Leyre Palacios-Chaves: lpalacios@alumni.unav.es
Amaia Zúñiga-Ripa: azuniga@unav.es
María-Jesús Grilló: mariajesus.grillo@unavarra.es
Vilma Arce-Gorvel: arce@ciml.univ-mrs.fr
Sean Hannify: hannify@ciml.univ-mrs.fr
Ignacio Moriyón: imoriyon@unav.es
Maite Iriarte: miriart@unav.es
Abstract
Brucellosis is a worldwide extended zoonosis caused by Brucella spp. These gram-negative bacteria are not readily detected by innate immunity, a virulence-related property largely linked to their surface lipopolysaccharide (LPS). The role of the LPS lipid A and O-polysaccharide in virulence is well known. Moreover, mutation of the glycosyltransferase gene wadC of B. abortus, although not affecting O-polysaccharide assembly onto the lipid-A core section causes a core oligosaccharide defect that increases recognition by innate immunity. Here, we report on a second gene (wadB) encoding a LPS core glycosyltransferase not involved in the assembly of the O-polysaccharide-linked core section. As compared to wild-type B. abortus, a wadB mutant was sensitive to bactericidal peptides and non-immune serum, and was attenuated in mice and dendritic cells. These observations show that as WadC, WadB is also involved in the assembly of a branch of Brucella LPS core and support the concept that this LPS section is a virulence-related structure.
1. Introduction

The \( \alpha-2 \) Proteobacteria of the genus Brucella cause brucellosis, a disease that is considered one of the most common global zoonoses [1]. The genus includes several closely related species among which B. abortus preferentially infects cattle, B. suis swine and wildlife and B. melitensis goats and sheep, and in all these animals brucellosis is a major cause of abortions and infertility. Humans become infected via direct contact with affected livestock and through consumption of unpasteurized dairy products. Human brucellosis is a grave and debilitating disease that may lead to permanent sequelae, requires prolonged and combined antibiotherapy and is fatal in 1-5% of untreated cases [2].

The brucellae behave as facultative intracellular parasites, and their virulence results from a complex and not fully elucidated set of mechanisms that include the ability to escape prompt detection by innate immunity during the initial stages of infection [3]. Studies carried out mostly with B. abortus have shown that the outer membrane lipopolysaccharide (LPS) [1,3] lipoproteins [3] and ornithine lipids [4] induce a low proinflammatory response indicative of the absence of the marked pathogen-associated molecular patterns that are detected by innate immunity. These outer membrane components structurally depart from counterparts molecules in most gram-negative bacteria, and the differences are particularly accentuated in the LPS. In B. abortus, B. melitensis and B. suis, LPS is of the smooth (S) type and is thus made of an O-polysaccharide bridged to lipid A by an intermediate core oligosaccharide. Although it has been known for decades that Brucella lipid A and O-polysaccharide are involved in virulence [5], the role of the core oligosaccharide has only been uncovered recently. We identified a core glycosyltransferase gene (wadC) whose disruption increases recognition of the LPS by complement, bactericidal peptides and the receptor complex MD2-TLR4, which leads to an increase in the proinflammatory response and the subsequent attenuation of the mutated bacteria [6]. Mutants in wadC remain S (i.e. they carry the O-polysaccharide), which is in contrast with the lack of a S-LPS of mutants in wadA, the only other glycosyltransferase known to be involved in the synthesis of the Brucella LPS core [7]. On these bases, we proposed that the B. abortus LPS core has a branched structure [6] and this was confirmed in a more recent structural study [8]. Here, we report that wadB, a hitherto unidentified LPS gene, encodes a glycosyltransferase involved in the synthesis of the LPS core branch, and show that its disruption also leads to attenuation.

2. Experimental procedures

2.1 Bacterial strains and growth conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were routinely grown in standard tryptic soy broth (TSB; Biomerieux; http://www.biomerieux.com) or agar (TSA; Conda-Pronadisa; http://www.condalab.com/es) either plain or supplemented with kanamycin (Km) at 50 μg/mL, chloramphenicol (Cm) at 20 μg/mL, nalidixic acid (Nal) at 25 μ
g/mL, and/or 5% sucrose. All strains were stored in skim milk (Scharlau) at -80°C.

2.2 DNA manipulations.

Plasmid and chromosomal DNA were extracted with Qiaprep spin Miniprep (Qiagen GmbH, Hilden, Germany) and Ultraclean Microbial DNA Isolation Kit (Mo Bio Laboratories) respectively. When needed, DNA was purified from agarose gels using a Qiagroup Gel extraction kit (Qiagen). DNA sequencing was performed by “Servicio de Secuenciación del CIMA” (“Centro de Investigación Médica Aplicada”, Pamplona, Spain). Primers were synthesized by Sigma-Genosys Ltd. (Haverhill, United Kingdom). Searches for DNA and protein homologies were carried out using the NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov) and the KEGG database (http://www.genome.jp/kegg/) servers. Analysis of glycosyltransferases families and domains was performed using CAZy database (www.cazy.org).

An in-frame deletion mutant in BAB1_0351 (henceforth BaΔwadB) was constructed by PCR overlap using genomic DNA of B. abortus 2308 (Baparental) as DNA template. Primers were designed based on the sequence of B. abortus 2308 (http://www.ncbi.nlm.nih.gov/). First, two PCR fragments were generated: oligonucleotides wadB-F1 (5’-GCATGATTACCCCGCTGAT-3’) and wadB-R2 (5’-CGCAATCTCGTCTTTGTTGAG-3’) were used to amplify a 296 bp fragment including codons 1 to 48 of BAB1_0351 as well as 152 bp upstream of the BAB1_0351 start codon; and oligonucleotides wadB-F3 (5’-CTCAACAAAGACGAGATTGCAGGTGGCGTGAAGGAAATCT-3’) and wadB-R4 (5’-TGATAGCCCGAGCCTTCTCAG-3’) were used to amplify a 274 bp fragment including codons 196 to 239 of the ORF and 139 bp downstream of the stop codon. Both fragments were ligated by overlapping PCR using oligonucleotides wadB-F1 and wadB-R4 for amplification and the complementary regions between wadB-R2 and wadB-F3 for overlapping. The resulting fragment, containing the BAB1_0351 deletion allele, was cloned into pCR2.1 (Invitrogen), to generate plasmid pYRI-1, sequenced to ensure the maintenance of the reading frame, and subsequently subcloned into the BamHI and the XbaI sites of the suicide plasmid pJQKm (Table 1). The resulting mutator plasmid (pYRI-2) was introduced in Ba-parental by conjugation. The first recombination (integration of the suicide vector in the chromosome) was selected by Nal and Km resistance, and the second one (excision of the mutator plasmid producing the mutant by allelic exchange) by Nal and sucrose resistance and Km sensitivity. The resulting colonies were screened by PCR with primers wadB-F1 and wadB-R4, which amplify a fragment of 570 bp in the
mutant and a fragment of 1011 bp in Ba-parental. The mutation resulted in the loss of 60% of the ORF (88% of the glycosyltransferase domain).

For complementation, a plasmid harboring ORF BAB1_0351 (p\textit{wadB}) was constructed as described previously [9] using genomic DNA of \textit{B. abortus} 2308 as the DNA template. Briefly, PCR-amplified ORF BAB1_0351 was inserted into the Gateway-compatible vector pDONR221 by site-specific recombination (pYRI-3). The product was then introduced into One Shot® OmniMAX™ 2-T1R Chemically Competent \textit{E. coli} cells and the bacterial transformants were selected on TSA-Km. The clone carrying \textit{B. abortus} BAB1_0351 was extracted, and the DNA containing this ORF was subcloned in pRH001 [10] to produce plasmid \textit{pwadB}. To complement the \textit{wadB} mutation, plasmid \textit{pwadB} was introduced into Ba\textDelta\textit{wadB} by mating with \textit{E. coli} S17-1\textlambda pir and the conjugates harboring \textit{pwadB} were selected by plating onto TSA-Nal-Cm plates, which were incubated at 37°C for 3 days.

For detection of bacteria in the cell infection experiments (see below), plasmid pBBR1MCS-2 GFP was introduced by conjugation in the different \textit{Brucella} strains (Table 1).

### 2.3 Sensitivity to brucellaphages, dyes, antibiotics and polymyxin.

The sensitivity to the S (Tb, Wb, Iz) and R (R/C) specific brucellaphages was measured as described previously [11]. The minimal inhibitory concentration (MIC) of fuchsin, thionin and safranin (dyes used in \textit{Brucella} biovar typing) was determined in Müller-Hinton broth by standard procedures. For polymyxin B, exponentially growing bacteria were adjusted to an optical density equivalent to 1 of the McFarland scale. The MIC was determined by the E-test method (AB Biomérieux, Solna, Sweden) on Müller-Hinton agar.

### 2.4 Sensitivity to non-immune serum.

Exponentially growing bacteria were adjusted to $10^4$ colony forming unit (CFU/mL) in phosphate buffered saline (PBS) and dispensed in triplicate in microtiter plates (45 μL/well) containing fresh normal bovine serum (90 μL/well). After 90 min of incubation at 37 °C, 200 μL of brain heart infusion broth was dispensed into each well, the bacterial suspension mixed, and 100 μL aliquots plated on TSA. The Ba::Tn5-\textit{per} (R-LPS) and Ba::Tn5-\textit{bvrR} (S-LPS) mutants were used as controls. The results were expressed as the percentage of survival with respect to the CFU in the inocula.

### 2.5 LPS extraction and characterization.

S-LPS was obtained by methanol precipitation of the phenol phase of a phenol-water extract, followed by digestion with nucleases (10 mg S-LPS/mL in 175 mM NaCl, 0.05% NaN₃, 0.1M Tris-HCl [pH 7.0]; 50 μg/mL each of DNase-II type V, and RNase [Sigma, St. Louis, Missouri, U.S.A.] 30 min at 37°C) and
proteinase K (three cycles; 50 µg/mL, 3 h at 55°C). LPS was then sedimented by ultracentrifugation, extracted four times with chloroform-methanol (2:1 [vol/vol]) to remove free lipids, and freeze-dried [12]. Alternatively, LPS was extracted from whole bacteria by the sodium dodecylsulfate (SDS) -proteinase K protocol [13]. LPS were electrophoresed in 7 cm 18% polyacrylamide (PAGE) gels in SDS Tris-HCl-glycine [1,14]. Alternatively, LPS were analyzed in 16 cm 18% PAGE gels in Tris-Tricine-HCl-glycine (TSDS-PAGE) [2,15]. For Western blots, gels were electro transferred onto nitrocellulose sheets (Whatman, Dassel, Germany), blocked with 3% skim milk in PBS with 0.05% Tween 20 (PBST) overnight, and washed with the same buffer. Monoclonal antibodies A68/03F03/D05, A68/24D08/G09 and A68/24G12/A08 (all of which recognize R-LPS epitopes [3,16]) were diluted in PBST and blots were developed with peroxidase-conjugated goat anti-mouse immunoglobulin (Nordic immunological laboratories, Tilburg, Netherlands) or peroxidase labeled protein G and 4-chloro-1-naphtol-H2O2.

2.6 Polycationic peptide-LPS interactions.

The affinity of Ba-parental and BaΔwadB LPS for polycationic peptides was measured using peptide 19.8-4 (derived from the Limulus anti-LPS factor) [1,3,17] in comparison with the LPS of the B. abortus wadC mutant (BaΔwadC) described previously [3,6], and E. coli ATCC 35218. For this purpose, ELISA plates were coated with the peptide (10 µg/mL in 20 mM in PBS; 100 µL/well) at 4°C overnight and washed extensively with PBS-Tween 20. Then, the appropriate LPS was dispensed (10 µg/mL in PBS; 100 µL/well) and plates incubated at 37°C for 1 h. Free peptide was detected with biotinylated E. coli LPS (100 µL/well of 1 µg/mL in PBS for 30 min at 37 °C) and a horseradish peroxidase-conjugated Streptavidin (100 µL; 1/2000 in PBS at room temperature for 30 min) - 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)/H2O2 system. Binding was expressed as the ratio ([LPS_{tested} – LPS_0] x 100)/(LPS_{Ec} – LPS_0) where LPS_{tested} is the value obtained with the LPS of each B. abortus strain, LPS_{Ec} is the value obtained with E. coli LPS, and LPS_0 the values obtained for the biotinylated E. coli LPS and no buffer and no LPS in the binding steps.

2.7 Intracellular replication.

Bone marrow cells were isolated from femurs of 7-8-week-old C57BL/6 female mice and differentiated into bone marrow-derived dendritic cells (BMDCs) as described previously [4,9,18,19]. Infections were performed by centrifuging bacteria carrying the plasmid pBBR1-MCS-2 GFP onto BMDCs (400 x g for 10 min at 4°C; multiplicity of infection of 40) followed by incubation at 37°C for 30 min under a 5% CO2 atmosphere. The virB10 GFP mutant was
used as an avirulent control. BMDCs were washed extensively to remove extracellular bacteria and incubated in complete medium supplemented with 50 μg/mL gentamicin for 1 h to kill extracellular bacteria. Thereafter, the antibiotic concentration was decreased to 10 μg/mL.

To determine the number of intracellular bacteria per cell, BMDCs were grown on glass coverslips and inoculated with bacteria as described above. At 2 and 48 h post-inoculation, coverslips were fixed with 3% paraformaldehyde, pH 7.4, at 37°C for 15 min and washed three times with PBS. Coverslips were processed for immunofluorescence staining as previously described [5,20]. BMDCs were labeled using an antibody (rabbit anti mouse Rivoli) against a conserved cytoplasmic domain of MHC-II I-A-β subunits [6,21], followed by the secondary antibody donkey anti-rabbit Cy5. Bacteria were visualized directly since they expressed the green fluorescence protein encoded by the pBBR1MCS-2 GFP plasmid. For each bacterial infection, a minimum of one hundred cells was analyzed under a Zeiss Axioplan 2 Fluorescence Microscope. The number of cells containing more than 10 bacteria/cell was recorded and expressed as a percentage with respect to the number of total infected cells. These experiments were done at least three times.

### 2.8 Virulence assay in mice.

One week before the start of the experiment, 7 week-old female BALB/c mice (Charles River, Elbeuf, France) were accommodated in cages with water and food ad libitum under biosafety containment conditions in the animal building of CITA of Aragón government (ID registration number ES-502970012005). The animal handling and experimental procedures were performed in accordance with the current Spanish and European legislation (RD 53/2013; Directive 14 86/609/EEC, respectively), and were supervised by the Animal Welfare Committee of the Institution (Protocol number R109/2009). Groups of 25 mice each were inoculated intraperitoneally with 5 x 10⁴ CFU of BaΔwadB, BaΔwadC or Ba-parental, and the number of CFU in spleens was determined at 2, 4, 8, 12 and 24 weeks post-inoculation, as described previously [6,7]. An additional group of 10 mice were infected with the BaΔwadB-pwadB complemented strain, and analyzed at 8 and 12 weeks post-infection. The identity of the spleen isolates was confirmed by PCR at each point-time and the individual data (CFU/spleen) were normalized by logarithmic transformation and expressed as the mean and standard deviation (n=5) of log₁₀ CFU/spleen. Statistical comparisons of means were performed by a one-way ANOVA test followed by the Fisher’s Protected Least Significant Differences (PLSD) test.
3. Results

3.1 Identification of wadB, a glycosyltransferase gene involved in the synthesis of the LPS core section not linked to the O-polysaccharide.

To identify Brucella core glycosyltransferase candidates, we first searched the KEGG database (http://www.genome.jp/kegg/) for glycosyltransferases in Brucella spp., ruling out those previously assigned to the O-polysaccharide. Then, we looked for orthologous of these ORFs in Ochrobactrum anthropi (ATCC 49188), a close phylogenetic relative of Brucella that has a core structure close to that of B. abortus [6,8,22]. In addition to the Kdo transferase (BAB2_0209, kdtA or waaA), which is highly conserved in gram-negative bacteria and thus of less interest, we found B. abortus BAB1_1522 and O. anthropi Oant_1661 to be the closest (85.6%) homologues, followed by BAB1_0351 and Oant_0415 (62.0% homology) and BAB1_0639 and Oant_0608 (38.9%). Other matches showed less than 35% homology, the threshold below which protein sequence alignment becomes meaningless [8,23]. Of these three ORFs, B. abortus BAB1_1522 encodes WadC and B. abortus BAB1_0639 encodes WadA, the only two core glycosyltransferases described previously [7,9,24]. Their identification supported the appropriateness of the screening method and, accordingly, the remaining ORF (BAB1_0351) was a strong candidate for an additional core glycosyltransferase. Consistent with this possibility, the BAB1_0351 predicted protein contained the cI01298, cd06532 and PFAM01755 domains of glycosyltransferase family 25, which includes Brucella WadA as well as LPS glycosyltransferases of other bacteria. Following the accepted nomenclature for LPS genes [10,25], we provisionally named this gene wadB.

To investigate whether BAB1_0351 was in fact involved in LPS synthesis, we constructed a non-polar mutant by in frame deletion of the sequence coding for amino acids 49 to 195, which eliminated the three consensus domains of glycosyltransferase family 25. The non-polar mutant (BaΔwadB) did not differ from the parental strain (Ba-parental) in growth rate, sensitivity to fuchsin, thionin and safranin. Moreover, the parental and the mutant strains showed similar sensitivity and resistance, respectively, to S and R brucellaphages [11], and an anti-S-LPS serum agglutinated both strains similarly. We then extracted the LPS from BaΔwadB, the pwadB-complemented and the parental strain, and compared the profiles in standard Tris-glycine SDS-PAGE (Figure 1A). The analysis showed that the high molecular weight S-LPS fraction of both BaΔwadB and Ba-parental had a similar profile. On the other hand, the R (low molecular weight) LPS fraction showed an increased mobility suggestive of a core defect (Figure 1A), and complementation with plasmid pwadB restored the parental mobility profile (Figure 1A). When we probed the BaΔwadB LPS with antibodies specific for the core of Brucella LPS, Moab A68/24G12/A08 (Figure 1B), A68/24D08/G09 and A68/3F03/D05 (not shown) failed to react with either the high (S) or low molecular weight (R) fractions of the LPS of BaΔwadB, and
complementation with plasmid p\textit{wadB} restored the reactivity. We finally compared the LPS of \textit{Ba\textDelta wadB} and \textit{Ba\textDelta wadC} by Tris-tricine-HCl-glycine SDS-PAGE, a high-resolution method that showed a higher molecular weight for R-LPS fraction of the former (Figure 1C). Since these results demonstrate that it encodes a core glycosyltransferase, they confirm the appropriateness of the \textit{wadB} designation. Moreover, they show that WadB is not related to the section linked to the O-polysaccharide, and suggest that the LPS core defect in \textit{Ba\Delta wadB} is less severe than that in \textit{Ba\Delta wadC}.

3.2 Deletion of \textit{wadB} decreases the resistance to the bactericidal action of polycationic peptides and normal serum.

\textit{B. abortus} is characteristically resistant to killing by non immune serum and polycationic bactericidal peptides, two properties linked to the LPS structure. Thus, we first probed the mutants with polymyxin B. The MICs we found were 1.5 \(\mu\)g/mL for \textit{Ba}-parental, 0.75 \(\mu\)g/mL for \textit{Ba\Delta wadC}, 0.30 \(\mu\)g/mL for \textit{Ba\Delta wadB} and 1.5 \(\mu\)g/mL for the \textit{p\textit{wadB}}-complemented \textit{Ba\Delta wadB} strain. To confirm that the defective core LPS is responsible for the increased sensitivity of the \textit{wadB} mutant to polycationic peptides, we also probed the binding of purified LPS to the potent \textit{Limulus} anti-LPS peptide 19.8-4 [12,17]. We found that the LPS of \textit{Ba\Delta wadB} and \textit{Ba\Delta wadC} bound more peptide than the LPS of \textit{Ba}-parental (Figure 2, left panel). Binding of peptide 19.8-4 was much less intense for any \textit{B. abortus} LPS than for \textit{E. coli} LPS indicating that the core accounts only in part for the LPS-mediated resistance of \textit{B. abortus} to polycationic peptides. This is in accordance with previous works that showed that, in contrast to enterobacterial lipid A, polymyxin B failed to bind \textit{B. abortus} lipid A [13,26].

Concerning the resistance to the killing action of normal serum, we observed that \textit{Ba\Delta wadB} and \textit{Ba\Delta wadC} were more sensitive than \textit{Ba}-parental and that the core defect was less deleterious than the upset OM present in a mutant in the master regulator BvrR-S [27,28] (Figure 2, right panel). Interestingly, \textit{Ba\Delta wadB}, \textit{Ba\Delta wadC} and a \textit{Ba::Tn5-per} mutant lacking the O-polysaccharide but carrying an intact core (see Table 1 for reference) showed similar serum sensitivity (Figure 2, right panel). These results demonstrate that an intact core is as important as the O-polysaccharide in protecting \textit{S B. abortus} against killing by bactericidal systems in normal serum.

3.3 Deletion of \textit{wadB} causes attenuation in dendritic cells and mice.

\textit{B. abortus} is characteristically able to multiply in dendritic cells [29]. To study the behavior of \textit{Ba\Delta wadB} in these cells, we infected C57BL/6 BMDC with \textit{Ba\Delta wadB}, \textit{Ba\Delta wadC} and \textit{Ba}-parental, and scored the number of intracellular bacteria in infected MHCII positive cells at 2 and 48 h post-infection. As a control, we used a \textit{virB10} mutant (Table 1) defective in the Type IV secretion system required for normal intracellular trafficking and multiplication. As
expected [20, 29], and regardless of the strain, the vast majority of infected BMDCs contained less than 10 bacteria at 2 h post-infection (Figure 3A). At 48 h post-infection, the proportion of BMDCs containing more than 10 bacteria increased for Ba-parental but not for the virB10 GFP mutant, as expected from their respective virulent and attenuated phenotype [6]. Both BaΔwadC and BaΔ wadB displayed an intermediate phenotype (Figure 3A), as described before for the former mutant [6].

We finally tested BaΔwadB in mice in comparison with BaΔwadC and Ba-parental. Both BaΔwadB and BaΔwadC were attenuated (Figure 3B). Although the differences were not marked, the evolution of the CFU/spleen suggested a less attenuated profile for BaΔwadB, with higher CFU/spleen at weeks 8 and 24 (p<0.05) (Figure 3B). In an independent experiment, the CFU/spleen obtained for the BaΔwadB -pwadB complemented control were similar to those of Ba-parental strains at the post-infection time tested (8 weeks; mean and standard deviation of log CFU 6.14 ±0.39 and 6.63±0.50 respectively; p = 0.442).

4. Discussion

The results of this work extend those of a previous study in which we showed that a B. abortus mutant in wadC displayed an altered LPS core despite carrying the O-polysaccharide [6]. Kubler-Kielb and Vinogradov [8] have shown that, whereas the B. abortus, B. melitensis and B. suis N-formyl-perosamine O-polysaccharide is linked to a few sugars stemming from the distal 3-deoxy-D-manno-octulosonic-acid residue (Kdo2) of the core, the lipid A-linked Kdo1 is bound through mannose to four glucosamine units. Fully consistent with the identification of wadB and the characteristics of the BaΔwadB mutant, synthesis of this oligosaccharide should require more than one glycosyltransferase. Based on the arrangement of sugars in this oligosaccharide, the electrophoretic profiles reported here, and the annotation of wadC as a mannosyltransferase gene, it can be hypothesized that WadC transfers mannose to Kdo1 and that WadB takes part in a later step. Indeed, although the genomic comparisons do not clarify its substrate specificity, it can also be hypothesized that WadB acts as a glucosamine transferase (Figure 4). Consistent with the description of the core branch in the above-mentioned S Brucella species [8], orthologues of wadB and wadC are present in all these bacteria and their disruption does not alter their S phenotype (Mancilla, M., Conde-Álvarez, R., Moriyón, I, and M. Iriarte; unpublished results).

Based on the observation that the core defect in BaΔwadC generates attenuation and that the mutated LPS is more readily recognized by elements of innate immunity, we have proposed that the Brucella LPS core branch is a virulence-related structure accounting in part for the stealthy behavior of these bacteria [3,6]. Since mutation of another glycosyltransferase involved in the synthesis of the same structure also causes attenuation, our hypothesis is
confirmed in the present work. As reported before (and confirmed here) for \textit{wadC}, the disruption of the core caused by mutation of \textit{wadB} diminished the resistance to bactericidal peptides and non-immune serum, increased peptide binding to LPS, and generated strains that were attenuated in dendritic cells and mice. It has to be noted that, although showing an overall similarity, the phenotypes of the \textit{BaΔwadB} and \textit{BaΔwadC} mutants were not identical. \textit{In vitro}, we noted differences in polymyxin B sensitivity, a polycationic lipopeptide that targets the lipid A phosphates and anionic sugars in the inner core (the Kdo residues in the case of \textit{Brucella}). Indeed, disruption of the Kdo1 glucosamine branch easily explains the decreased resistance of both mutants. However, the decrease did not parallel the extent of core damage observed in the electropherograms, and thus other factors may be relevant. They could relate to topological changes in other outer membrane components, to the three dimensional arrangement of the remaining core, or to both. On the other hand, the experiments in mice suggested a less attenuated phenotype for \textit{wadB} than for \textit{wadC} that would be consistent with a less severe damage of the LPS core. A putative model of LPS structure based on the studies of Kubler-Kielb and Vinogradov (8) and our results studying the role of \textit{wadC} and \textit{wadB} is presented in Figure 4. Obviously, a definite interpretation of these results requires structural elucidation of the core structure remaining in the \textit{wadB} and \textit{wadC} mutants or/and in a \textit{wadB-wadC} double mutant.

We have proposed before that disruption of the structure of the \textit{Brucella} LPS core can be exploited to develop vaccines that would elicit the early innate immunity recognition that leads to Th1 protective responses [24]. Although vaccine S19 has been successfully used in developed countries for the control and eradication of cattle brucellosis, the protection achieved is not optimal [30] and success using this vaccine requires a very proficient veterinary infrastructure. Previous experiments in the mouse model suggest that the \textit{B. abortus} \textit{wadC} core mutations could be either introduced in S19 to reduce its residual virulence but bolstering its immunogenic properties or in a wild-type background, in combination with other appropriate mutations, to develop improved vaccines [24]. In this context, the identification of \textit{wadB} adds another target for the development of new generation anti-\textit{Brucella} vaccines less pathogenic and more immunogenic than currently available vaccines A study of the vaccine properties of \textit{wadB} and \textit{wadC} mutants in various \textit{Brucella} backgrounds is in progress.
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Competing interests

RC, MI, VAG, IM and JPG are co-owners of patent N°PCT/EP2010/063921 (WO2011/033129) that covers the use of the Brucella core polysaccharide genes for vaccine development.

Literature cited


Lelouard H, Gatti E, Cappello F, Gresser O, Camosseto V, Pierre P.


FIGURE LEGENDS

Figure 1. Mutant Ba\textsuperscript{wadB} has a defect in the LPS core. (A) SDS-PAGE analysis of phenol-water LPS; and (B) Western blot analysis of phenol-water LPS with anti-LPS-core Moab A68/24G12/A08; and (C) TSDS-PAGE analysis of SDS-proteinase K LPS.

Figure 2. Mutant Ba\textsuperscript{wadB} shows increase sensitivity to polycationic peptides and normal serum. Left panel, LPS binding to the anti-LPS Limulus peptide 19.8.4. The graphic represents the results of one representative experiment. Right panel, bacterial survival after exposure to normal bovine serum for 90 min (bars are the mean ± standard error).

Figure 3. Mutant Ba\textsuperscript{wadB} is attenuated in dendritic cells and mice. (A) Multiplication in BMDC (mean ± standard deviation of bacterial numbers in at least 100 BMDC); (B) infection kinetics in spleen in BALB/c mice (each point represents the mean ± standard deviation [n=5] of the logarithm of CFU).

Figure 4. Schematic representation of Brucella LPS according to the work of Kubler-Kielb (8). The putative sugars transferred by \textit{wadC} and \textit{wadB} are indicated. Kdo (3-deoxy-d-manno-octulosonic acid); Glc (glucose); Man (mannose); GlcN (glucosamine); Quin (quinovosamine)
### Table 1. Bacterial strains and Plasmids

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<td><strong>Brucella</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ba-parental</td>
<td>NaI(^b) spontaneous mutant of the reference strain B. abortus 2308</td>
<td>[31]</td>
</tr>
<tr>
<td>Ba_wadB</td>
<td>Ba-parental with a wadB(^{49,195}) deletion</td>
<td>This work</td>
</tr>
<tr>
<td>Ba_wadB-pwadB</td>
<td>Ba_wadB harboring plasmid pwadB</td>
<td>This work</td>
</tr>
<tr>
<td>Ba_wadC</td>
<td>Ba-parental LPS core mutant</td>
<td>[6]</td>
</tr>
<tr>
<td>Ba_Tn5-per</td>
<td>Ba-parental harboring the Tn5 inserted in per</td>
<td>Mutant 9.49 in ref [32]</td>
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<tr>
<td>Ba_Tn5-bvrR</td>
<td>Ba-parental harboring Tn5 inserted in bvrR</td>
<td>Mutant 65.21 in ref [27]</td>
</tr>
<tr>
<td>(\text{virB10~GFP})</td>
<td>(\text{virB<del>mutant</del>harboring<del>pBBR1MCS-2</del>GFP~Km}^R)</td>
<td>[20]</td>
</tr>
<tr>
<td>(\text{Ba-parental~GFP})</td>
<td>(\text{Ba<del>parental</del>harboring<del>pBBR1MCS-2</del>GFP~Km}^R)</td>
<td>[6]</td>
</tr>
<tr>
<td>Ba_wadB-GFP</td>
<td>Ba_wadB harboring pBBR1MCS-2 GFP Km(^R)</td>
<td>This work</td>
</tr>
<tr>
<td>Ba_wadC-GFP</td>
<td>Ba_wadC harboring pBBR1MCS-2 GFP Km(^R)</td>
<td>[6]</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S17-13,pir Top10F</td>
<td>Mating strain with plasmid RP4 inserted into the chromosome. F' (lacIq, Tn10(TetR)) mcrA (\Delta(mrr-hsdRMS-mcrBC)) (\Phi80\text{lacZAM15}) (\Delta\text{lacX74 recA1 araD139}) (\Delta(\text{ara leu})) (7697\text{galU galK rpsL (StrR)}) (\text{endA1}) Invitrogen</td>
<td></td>
</tr>
<tr>
<td>One shot OMNIMAX™</td>
<td>F' (\text{proAB lacIq}) (\text{lacZAM15}) (\text{Tn10(TetR)}) (\text{Δ(ccdAB)}) mcrA (\Delta(mrr-hsdRMS-mcrBC)) (\Phi80\text{(lacZAM15})) (\Delta(lacZYA-argF))U169 (\text{endA1 supE44 thi-1 gyrA96 relA1 tonA panD}) Invitrogen</td>
<td></td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCR2.1</td>
<td>Cloning vector</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pJQKm</td>
<td>Derivative of pJQ200KS +; Km(^R)</td>
<td>[34]</td>
</tr>
<tr>
<td>pDONR221</td>
<td>Cloning vector containing attP recombination sites for BP reaction (Gateway system)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pRH001</td>
<td>Derivative of pMR10 Km(^R); Cm(^R)</td>
<td>[10]</td>
</tr>
<tr>
<td>pYRI-1</td>
<td>570-bp of Ba-parental chromosomal DNA containing the wadB deletion allele, generated by PCR and cloned into pCR2.1 This work</td>
<td></td>
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<tr>
<td>pYRI-2</td>
<td>(\text{BamH1-XbaI}) fragment from pYRI-1 cloned into the corresponding sites of pJQKm This work</td>
<td></td>
</tr>
<tr>
<td>pYRI-3</td>
<td>(\text{B. abortus<del>2308</del>chromosomal<del>DNA</del>containing<del>the</del>complete<del>wadB</del>gene<del>with</del>attB<del>sites,<del>generated</del>by</del>PCR<del>and</del>cloned<del>into</del>pDONR221}) (Invitrogen) This work</td>
<td></td>
</tr>
<tr>
<td>pwadB</td>
<td>attL1-attL2 fragment of pYRI-3 cloned into the attR1-attR2 sites of pRH001 This work</td>
<td></td>
</tr>
<tr>
<td>pBBR1MCS-2 GFP</td>
<td>pBBR1MCS-2 derivative expressing the gfp-mut3 gene under the control of the lac promoter Dr. J.P. Gorvel, INSERM-CNRS, Marseille, France.</td>
<td></td>
</tr>
</tbody>
</table>