RomA, a Periplasmic Protein Involved in the Synthesis of the Lipopolysaccharide Tunes Down the Inflammatory Response Triggered by *Brucella*

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Runninng title: *Immune response during Brucella infection*

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ABSTRACT

Brucellaceae are stealthy pathogens with the ability to survive and replicate in the host in the context of a strong immune response. This capacity relies on several virulence factors that are able to modulate the immune system, and in their structural components that have low pro-inflammatory activities. Lipopolysaccharide (LPS), the main component of the outer membrane, is a central virulence factor of *Brucella* and it has been well established that induces a low inflammatory response. We describe here the identification and characterization of a novel periplasmic protein (RomA) conserved in alpha-proteobacteria, involved in the homeostasis of the outer membrane. A mutant in this gene showed several phenotypes, such as membrane defects, altered LPS composition, reduced adhesion and increased virulence and inflammation. We show that RomA is involved in the synthesis of LPS, probably coordinating part of the biosynthetic complex in the periplasm. Its absence alters the normal synthesis of this macromolecule and affects the homeostasis of the outer membrane, resulting in a strain with a hyperinflammatory phenotype. Our results suggest that the proper synthesis of LPS is central in order to maximize virulence and minimize inflammation.

Keywords: *Brucella*; lipopolysaccharide; inflammation.

INTRODUCTION

Brucellaceae are wide spread zoonotic intracellular pathogens with the capacity to evade and modulate the immune response of the infected hosts, a hallmark of their infectious processes [1]. Many of these immunomodulatory activities are achieved by a plethora of virulence factors that are able to manipulate the immune system to its benefit, promoting bacterial proliferation and the establishment of the chronic infectious phase [2-7]. *Brucella* is considered a pathogen with a stealthy strategy, meaning that is able to avoid a strong immune response "hiding" its pathogen-associated molecular patterns (PAMPs). This strategy is achieved by either downregulating activation of PAMPs, or though the synthesis of structural components with low pro-inflammatory activities [8]. This last concept raises an interesting question; are the structural cellular components of *Brucella*, like lipopolysaccharide (LPS), silent PAMPs by default or is the bacterium actively modifying them to be non-detected?

Lipopolysaccharide (LPS) is the main component of the outer membrane in all Gram-negative bacteria. It is composed of lipid A (the lipidic portion inserted in the membrane), a core oligosaccharide and the O-antigen, which is the most exposed structure [9]. LPS is necessary for a wide range of functions such as protection against harsh environmental conditions, selective permeability, immune protection and evasion among others. The synthesis of this macromolecule is achieved through a complex biosynthetic pathway that starts in the cytoplasm where the precursors are synthesized, continues in the inner membrane and periplasmic space where the assembly, polymerization and transport take place, and ends when the complete molecules are inserted in the outer membrane [9]. Many of these processes require multiprotein complexes that coordinate their activities in a spatial and temporal way in order to effectively synthesize, transport and insert this complex macromolecule in its final organelle [10]. In *Brucella*, LPS has been shown to be a central virulence factor necessary for intracellular replication and virulence in mice [8]. Additionally, it has been shown that a structurally complete LPS is needed for the efficient immune evasion and that it also acts as a

shield against the innate immune response [1, 11].

We describe the identification in *Brucella abortus* of a gene encoding a protein with no known function but conserved among almost all α-proteobacteria that we propose is involved in the homeostasis of the outer membrane. The 84 amino-acids protein has a periplasmic localization and its absence results in a strain with several phenotypes: membrane alterations, changes in the LPS composition, defects in the intracellular replication capacity of the bacteria and deregulated inflammatory response in mice. We hypothesize that the absence of this protein alters the proper synthesis of the LPS, probably modifying the assembly of the biosynthetic complex in the periplasm, which in turn affects outer membrane homeostasis and the modulation of the immune response during the infectious cycle.

METHODS

A complete description of the Methods has been included in supplementary information.

Periplasmic and cytoplasmic localization assay

For the periplasmic localization assays the *B. abortus* strains were grown in TSB for 16–24 hrs at 37°C and $2.5x10^{10}$ bacterial cells were centrifuged 10 min at 3300xg and the periplasmic and cytoplasmic fractions obtained as we have previously described [12]. For Western-Blot an anti-FLAG M2, anti GroEL (1:2000) and anti OMP-1 (1:2000) kindly provided by Dr. Axel Cloeckaert were used as primary antibodies.

Detergent sensitivity assays

For all *B. abortus* strains, overnight cultures were diluted and seeded onto TSB plates supplemented with detergents as described [13]. Final detergent concentrations were 125 µg/ml Sarkosyl, 25 µg/ml Zwittergent 3-16, 1 g/ml sodium deoxycholate. Sensitivity was calculated determining the viable CFU.

Total lipid extraction

For the extraction of total lipids, the Bligh and Dyer method was used [14] on exponentially grown bacteria.

Cristal violet staining of *B. abortus*

Serial dilutions of *B. abortus* cultures were plated in TSB plates and incubated at 37°C until growth was observed. Plates were stained with a crystal violet solution as described [15].

LPS purification

LPS was extracted from *B. abortus* strains using a modification of the phenol-hot water method [16] from 250 ml of stationary cultures. LPS concentration was determined by the 3-Deoxy-D-manno-2-octulosonic acid method and analyzed on 12% SDS-PAGE gels and stained by silver nitrate.

Gentamicin protection assays

Gentamicin protection assays were performed as previously described [17].

Mice infections

Mice infections were performed as previously described [18].

Analysis of fluorescent fusion proteins

Stationary phase cultures of *B. abortus* strains were diluted in fresh media and grown until exponential phase was reached. A volume of 3 µl of culture was seeded onto the center of a PBS 1% agarose pad as previously described [19]. Images were acquired and processed as described above.

RESULTS

Bab1_1280 encodes a periplasmic protein necessary for the homeostasis of the outer membrane in *Brucella*.

In a genetic screen to identify genes from *Brucella abortus* coding for secreted or surface exposed proteins, we isolated Bab1_1280. This gene encodes an 84 amino-acid hypothetical protein conserved in most α-proteobacteria with no known function to date [20] (Fig. S1). We serendipitously found that an insertion mutant in this gene resulted in a strain with altered membrane properties. Because the gene was identified in a screen for either secreted/periplasmic or surface exposed proteins, we performed subcellular fractionation assays to further determine its localization. We generated a strain expressing FLAG tagged RomA from a genomic allele and performed a periplasmic extraction protocol [12]. Fig. 1A shows that the FLAG-tagged protein product of Bab1_1280 fractioned with the periplasm/outer membrane as the outer membrane protein 1 (OMP1). Presence of the protein due to bacterial lysis was discarded since no cytoplasmic contamination was observed (GroEL). We additionally lysed the strain and determined membrane association by ultracentrifugation, which indicated that the protein interacts with total membranes (Fig. 1B). In order to determine the degree of this association, total membranes were resuspended in different buffers, re-centrifuged and evaluated if the protein remained associated. Fig. 1C shows that only a mild wash with 10 or 50 mM sodium phosphate was enough to partially loosen the membrane association, condition that was completely lost with sodium chloride, or detergent treatments. These results indicate that the product of Bab1_1280 is a periplasmic protein with a weak association to either the inner or the outer membranes.

To determine if the product of Bab1_1280 is involved in maintaining the normal composition of the outer membrane, we measured the resistance of the mutant to the detergents Sarkosyl, Zwittergent 3-16 and Sodium Deoxycholate. Fig. 2A shows that the Bab1_1280 mutant was less resistant to the three detergents. To further determine if this increased sensitivity to

detergents is related to the hydrophobicity of the membrane, we performed an N-phenyl-1-naftilamine (NPN) incorporation assay. As it can be observed in Fig. 2B, the hydrophobic probe was incorporated more efficiently in the mutant indicating that the outer membrane is more permeable to hydrophobic molecules. These alterations in the outer membrane properties of the mutant could be the consequence of differences in the phospholipids or fatty acid profile. One and two-dimensional thin layer chromatography (TLC) using C^{14}-labeled total bacteria or extracted periplasms (phospholipids of the outer membrane) showed no differences between strains (Fig. 2C and Fig. S2). These results indicated that the alterations observed with the mutant in terms of detergent sensitivity as well as membrane hydrophobicity, are not the result of a differential phospholipid composition but are probably due to differences in other components of the outer membrane. The localization of the protein, together with the defects in the membrane properties of the mutant strain, suggested that RomA might be playing a role in maintaining a degree of homeostasis of the periplasm and/or outer membrane. For these reasons we renamed *Bab1_1280* as *romA*, for regulator of outer membrane.

RomA is implicated in the biosynthesis of LPS

The fact that the Δ*romA* mutant showed altered membrane properties but no differences in the phospholipid composition raised the possibility that it might have a defective LPS. To test if this we performed a crystal violet (CV) staining to determine if the strain has a complete LPS (smooth strain, excludes the staining) or if it lacks the assembled O-antigen (rough strain, includes the staining). Surprisingly, the Δ*romA* (Δ*Bab1_1280*) strain showed a higher degree of exclusion of CV (Fig. 2D), strongly suggesting a modified LPS, which was further confirmed by western blot on whole bacteria. Fig. 3A shows that the mutant strain exhibited a LPS pattern that seemed to have, not only a higher antigenic load, but also higher molecular weight forms. To further advance in its characterization we performed a western blot on

whole bacteria but using anti O-antigen (α-S-LPS) and anti-rough (α-R-LPS) monoclonal antibodies. As it can be observed in Fig. 3B and Fig. 3C, the Δ*romA* mutant showed higher levels of smooth LPS and lower levels of the rough LPS indicative that the strain might have an altered equilibrium of the S-LPS:R-LPS ratio in the membrane. To confirm that this alteration is present in the outer membrane and it is not a consequence of an accumulation of the S-LPS in the inner membrane, we performed a periplasmic/outer membrane extraction and analyzed the LPS. As can be observed in Fig. 3C (right panel), the same pattern of S-LPS:R-LPS was observed in the outer membrane with the mutant, confirming that this strain exhibits a higher percentage of S-LPS. An additional characteristic that we observed while analyzing the gels of the Δ*romA* LPS, was that it seemed to have longer O-antigen chains, although an alternative explanation could be that it was the result of a higher concentration of smooth LPS and not a chain length issue. To distinguish between these two possibilities we characterized purified LPS from both strains by Western Blot with the α-S-LPS and □-R-LPS monoclonal antibodies and silver staining. In Fig. 3D it can be seen that purified LPS showed the same pattern observed either with whole cells or periplasmic extractions. These preparations were used to chemically characterize the O-antigen and the core (see Methods). Monosaccharide analysis by High- Performance Anion-Exchange Chromatography coupled with Pulsed Amperometric Detection (HPAEC-PAD) of the oligosaccharides released from the LPSs showed that the Δ*romA* strain LPS presents a significant higher ratio of perosamines (Rha4N, present in the O-antigen) to N-acetyl-glucosamines (GlcN, present in the core), indicating that the mutant has a longer O-antigen. (Fig. 4A and Fig. S3).

Furthermore, when the oligosaccharides released after mild acid hydrolysis of the corresponding LPSs were characterized by MALDI-TOF m.s in the positive mode, significant differences in the high mass range were detected. Thus, the spectrum corresponding to the wild type oligosaccharide presented the highest mass signals at *m/z* 3077.9 and *m/z* 3100.0 (□Na) (calc. *m/z* 3099.2977; $C_{122}H_{210}N_{15}Na_2O_{73}$) corresponding to a structure bearing

KdoGlcQuinMan$_2$Rha4N$_{14}$Fo$_4$Na$_2$. In accordance, signal at *m/z* 2858.2 (calc. *m/z* 2857.2574; $C_{114}H_{199}N_{15}NaO_{66}$) corresponds to the structure GlcQuinMan$_2$Rha4N$_{14}$Fo$_4$Na. On the other side, the spectrum of the mutant strain showed not only the signals described above, but also signals at *m/z* 4349.3 (calc. *m/z* 4349.8865; $C_{173}H_{299}N_{23}NaO_{101}$) attributed to a structure bearing KdoGlcQuinMan$_2$Rha4N$_{22}$Fo$_8$Na and *m/z* 4325.3 (calc. *m/z* 4325.8630; $C_{173}H_{296}N_{23}Na_2O_{99}$) attributed to anhKdoGlcQuinMan$_2$Rha4N$_{22}$Fo$_7$Na$_2$. Furthermore, a signal at *m/z* 4835.5 (calc. *m/z* 4835.0851; $C_{193}H_{331}N_{26}Na_2O_{111}$) corresponds to the latter with three additional Rham4N and two Fo groups (Fig. 4B). The fact that the ratio of Rha4N to GlcN in the mutant is increased five times and the O-antigen is almost two folds longer confirms that the mutant has an altered smooth to rough ratio. Despite that the O-antigen was longer, the core showed no differences (data not shown).

Altogether these results indicate that RomA affects membrane properties and composition, and is required for a proper LPS assembly.

RomA is involved in the virulence process and its inactivation profoundly alters the inflammatory response

The changes in the LPS profile in the Δ*romA* mutant strain led us to evaluate the potential role of this gene in the virulence of *B. abortus*. Fig. 5A shows that the Δ*romA* strain exhibited a significant defect in the intracellular survival capacity in murine bone marrow derived macrophages during the initial stages of infection (4 and 24 hrs post-infection) but was able to replicate and, at 48 hrs post-infection, we did not observe any differences with the wild type parental strain. This intracellular replication pattern was similar when J774 A.1 cells were used (Fig. S4). Due to the altered membrane of the Δ*romA* mutant we further analyzed if these early effects were due to a defect in the adhesion of the bacteria to the cells or in their reduced capacity to exclude the lysosomal marker Lamp-1 during the intracellular trafficking.

As can be seen in Fig. S5 and Fig. S6, the mutant showed a statistically significant defect in the adhesion to J774 A.1 cells as well a reduced capacity to exclude Lamp-1 at 24 hrs post-infection. These results indicate that the altered membrane structure of the Δ*romA* mutant probably impacts on several steps of the interaction of the bacterium with the host cells (adhesion/invasion as well as in the intracellular trafficking).

The results obtained *in vitro* encouraged us to evaluate the role this gene might play during pathogenesis in the mouse model. Surprisingly, the Δ*romA* mutant showed a dramatic increase in the number of bacteria in the spleens of intraperitoneally infected mice at 14 days post-infection, which correlated with an increased splenomegaly (Fig. 5B, 5C and 5D). An interesting observation was that the complemented strain exhibited less bacterial load and splenomegaly than the wild type strain, which led us hypothesize that it might also have an altered LPS. Fig. S7 shows that the Δ*romA* complemented strain, expressing RomA from a plasmid, had a LPS with less assembled O-antigen (partly rough).

The increase in the spleen size was the result of an enhanced inflammatory response. Fig. 6, panel A to D, shows that the mutant induced a significantly higher inflammatory response in comparison to the wild type, measured by the production and circulation of two pro-inflammatory cytokines (TNF-α and IFN-γ). This was also confirmed by histological observation of the spleens that showed a higher cellularity of the red pulp with more macrophages and neutrophils, as well as more pronounced granulomatous lesions in the mutant (Fig. S8). Additionally, Fig. S9 shows that the Δ*romA* mutant triggered an increased inflammatory response as early as 48 hrs post-infection, indicating that the strain *per se* is significantly more pro-inflammatory and that the robust inflammation it induced was not the consequence of an increased bacterial proliferation. To further analyze the kinetics of the infection in mice; we determined spleen colonization of the mutant in comparison with the wild type parental strain at 7 and 42 days post-infection. We did not observe differences at these two time points (Fig. S10) indicating that the increased bacterial load is a phenomenon

restricted to the acute phase of the infection and it does not persist during the chronic phase. In both time points we observed an enhanced splenomegaly (not shown).

Absence of RomA alters the positioning of LptD, an LPS biosynthetic protein in the periplasmic space

The localization of RomA as well as the membrane-related phenotypes found in the Δ*romA* mutant, suggested that this protein might be involved in the organization/localization of LPS biosynthetic complexes in the periplasmic space and/or outer membrane. To evaluate if this was the case we constructed a fusion of the gene *lptD* that codifies for a protein involved in the transport of the LPS to the outer membrane [10, 21], with super-folder GFP (sfGFP) [22], and evaluated its localization in the wild type and Δ*romA* strains. As can be observed in Fig. 7A, LptD showed an altered distribution in the mutant in comparison with the parental strain. More specifically, while most of the wild type cells showed a single localization spot, the mutant cells had a more homogenous distribution. This was not observed with the inner membrane protein responsible for flipping the O-antigen-lipid intermediate to the periplasm, RfbD [9], (Fig. 7B) indicating that only some of the LPS biosynthesis proteins have an aberrant distribution in the mutant.

DISCUSSION

In the present study we have identified a novel gene (*romA*) in *Brucella*, and conserved in almost all α-proteobacteria, that codes for a small periplasmic protein with no known function. A mutant in *romA* is pleiotropic and displays several phenotypes, all related with an altered periplasm and/or outer membrane. The sensitivity of the mutant to several detergents and the fact that its membrane is more permeable to hydrophobic compounds indicated an altered outer membrane. LPS analysis showed that the mutant strain has several modifications. On one side, an altered smooth to rough ratio which results in a strain with significantly more assembled LPS compared to the wild type strain. Additionally, the LPS has an O-antigen with a higher degree of polymerization, with at least twice the amount of perosamines but substituted with formyl residues. These modifications have several implications and raises interesting questions for future studies. To our knowledge this is the first report of a mutant that has an altered smooth:rough LPS ratio, strongly suggesting that *Brucella* (and probably other members of this group) controls this equilibrium. It is tempting to speculate that a certain level of incomplete LPS is necessary to assemble or expose other outer membrane components that could be affected if the O-antigen is present in all LPS molecules. If this hypothesis is correct the amount of smooth to rough LPS could be determined by a compromise between two needs: to protect against harmful conditions encountered in the environment and to allow the assembly and positioning of a set of proteins or supramolecular structures necessary for motility, virulence, attachment and protein secretion among others. A similar hypothesis has been postulated for the length of the O-antigen and the Type III secretion system in *Shigella* [23]. The authors proposed that the length of the O-antigen is determined by two opposing necessities, its protective properties and the efficiency of the Type III injectisome. Currently we do not have a molecular explanation of why the Δ*romA* mutant has a longer O-antigen but speculate that the stoichiometry of the LPS biosynthetic machinery is probably altered and that this affects the synthesis. In this regard we have shown that LptD, involved in the transport of the LPS to

the outer membrane [9], but not the flippase located in the inner membrane, showed a mislocalization in the mutant strongly suggesting that RomA participates in the organization/assembly of this machinery in the periplasm and/or outer membrane.

It is prompting to hypothesize that the modified LPS in the *romA* mutant probably disturbs the homeostasis of the outer membrane and this impacts the virulence of the bacterium in several ways. For example, even though it was less infective in the cellular model of infection it triggered an exacerbated inflammatory response that actually increased the bacterial load in the spleens of infected mice during the acute phase of the infectious process. This inability to tune down the inflammatory response, a hallmark of the *Brucella* infection [1], is probably the result of a combination of factors, both structural and functional, although we cannot completely rule out at this stage that the modified LPS is the only component that could account for all the phenotypes. It has been described that a mutant in the *fliC* gene in *Brucella*, that codes for a component of a flagellar-like structure, induced an increased splenomegaly and showed higher bacterial loads in the spleens as well as more tissue damage, similar to the one we observed with the Δ*romA* mutant [24]. Since the flagellum is assembled in the outer membrane and because we have observed that the expression of *fliC* is not affected (not shown) it could be speculated that the altered LPS affects the assembly of this structure and this results in a similar phenotype as the *fliC* null mutant.

The fact that the mutant induces severe inflammation during the acute phase of the infectious process indicates that *Brucella* has active mechanisms to modify its cellular structure in order to tune down the immune response. This is not trivial, as it has been suggested that the default cellular structure of *Brucella* is mainly responsible for its stealthy strategy and it implies that the bacterium could actually modulate up and down the inflammatory response depending on the phase of the infectious cycle or its needs, modifying its membrane composition or structure. It could be speculated that the inflammatory balance and the necessity of the bacteria to assemble membrane structures needed for virulence must be tightly equilibrated to

establish a successful chronic infection. Under this view, *Brucella* should be able to finely counterbalance these two needs to be a successful pathogen.

ACKNOWLEDGMENTS

We thank members of the J.E.U. laboratory for useful discussions and Thomas Bernhardt from Harvard University for kindly providing the superfolder GFP coding plasmid.

This work was supported by grants PICT-PRH08-160 to C.C., PICT-PRH08-230 and PICT-1028-2014 to J.E.U., and grants from the University of San Martín to C.C. and J.E.U. C.C., S.G.A., A.S.C., K.A.P., J.M.S., J.C. and J.E.U. are members of the National Research Council of Argentina (CONICET). E.V. was supported by a fellowship of the National Research Council of Argentina (CONICET).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

E.V. and J.E.U. conceived and designed research. E.V., J.M.S, C.C., F.R.F., A.C.C., S.G.A., K.A.P. and F.G. performed the experiments. J.C. provided input in the cytokine assays determinations. A.S.C. designed the experiments and analyzed the O-antigen structure determination. E.V. and J.E.U. wrote the manuscript.

REFERENCES

1. Byndloss MX, Tsolis RM. Brucella spp. Virulence Factors and Immunity. Annu Rev Anim Biosci **2016**; 4:111-27.

2. Keestra-Gounder AM, Byndloss MX, Seyffert N, et al. NOD1 and NOD2 signalling links ER stress with inflammation. Nature **2016**; 532:394-7.

3. Salcedo SP, Marchesini MI, Degos C, et al. BtpB, a novel Brucella TIR-containing effector protein with immune modulatory functions. Front Cell Infect Microbiol **2013**; 3:28.

4. Salcedo SP, Marchesini MI, Lelouard H, et al. Brucella control of dendritic cell maturation is dependent on the TIR-containing protein Btp1. PLoS pathogens **2008**; 4:e21.

5. Spera JM, Comerci DJ, Ugalde JE. Brucella alters the immune response in a prpA-dependent manner. Microb Pathog **2014**; 67-68:8-13.

6. Spera JM, Herrmann CK, Roset MS, Comerci DJ, Ugalde JE. A Brucella virulence factor targets macrophages to trigger B-cell proliferation. The Journal of biological chemistry **2013**; 288:20208-16.

7. Spera JM, Ugalde JE, Mucci J, Comerci DJ, Ugalde RA. A B lymphocyte mitogen is a Brucella abortus virulence factor required for persistent infection. Proc Natl Acad Sci U S A **2006**; 103:16514-9.

8. Martirosyan A, Moreno E, Gorvel JP. An evolutionary strategy for a stealthy intracellular Brucella pathogen. Immunol Rev **2011**; 240:211-34.

9. Ruiz N, Kahne D, Silhavy TJ. Transport of lipopolysaccharide across the cell envelope: the long road of discovery. Nature reviews Microbiology **2009**; 7:677-83.

10. Okuda S, Sherman DJ, Silhavy TJ, Ruiz N, Kahne D. Lipopolysaccharide transport and assembly at the outer membrane: the PEZ model. Nature reviews Microbiology **2016**; 14:337-

45.

11. Conde-Alvarez R, Arce-Gorvel V, Iriarte M, et al. The lipopolysaccharide core of Brucella abortus acts as a shield against innate immunity recognition. PLoS pathogens **2012**; 8:e1002675.

12. Dohmer PH, Valguarnera E, Czibener C, Ugalde JE. Identification of a type IV secretion substrate of Brucella abortus that participates in the early stages of intracellular survival. Cellular microbiology **2014**; 16:396-410.

13. Roset MS, Garcia Fernandez L, DelVecchio VG, Briones G. Intracellularly induced cyclophilins play an important role in stress adaptation and virulence of Brucella abortus. Infection and immunity **2013**; 81:521-30.

14. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol **1959**; 37:911-7.

15. White PG, Wilson JB. Differentiation of smooth and nonsmooth colonies of Brucellae. J Bacteriol **1951**; 61:239-40.

16. Luederitz O, Risse HJ, Schulte-Holthausen H, Strominger JL, Sutherland IW, Westphal O. Biochemical Studies of the Smooth-Rough Mutation in Salmonella Minnesota. J Bacteriol **1965**; 89:343-54.

17. Ugalde JE, Czibener C, Feldman MF, Ugalde RA. Identification and characterization of the Brucella abortus phosphoglucomutase gene: role of lipopolysaccharide in virulence and intracellular multiplication. Infection and immunity **2000**; 68:5716-23.

18. Ugalde JE, Comerci DJ, Leguizamon MS, Ugalde RA. Evaluation of Brucella abortus phosphoglucomutase (pgm) mutant as a new live rough-phenotype vaccine. Infection and immunity **2003**; 71:6264-9.

19. Guidolin LS, Morrone Seijo SM, Guaimas FF, Comerci DJ, Ciocchini AE.

Interaction network and localization of Brucella abortus membrane proteins involved in the synthesis, transport, and succinylation of cyclic beta-1,2-glucans. J Bacteriol **2015**; 197:1640-8.

20. Marchesini MI, Ugalde JE, Czibener C, Comerci DJ, Ugalde RA. N-terminal-capturing screening system for the isolation of Brucella abortus genes encoding surface exposed and secreted proteins. Microb Pathog **2004**; 37:95-105.

21. Bos MP, Tefsen B, Geurtsen J, Tommassen J. Identification of an outer membrane protein required for the transport of lipopolysaccharide to the bacterial cell surface. Proc Natl Acad Sci U S A **2004**; 101:9417-22.

22. Dinh T, Bernhardt TG. Using superfolder green fluorescent protein for periplasmic protein localization studies. J Bacteriol **2011**; 193:4984-7.

23. West NP, Sansonetti P, Mounier J, et al. Optimization of virulence functions through glucosylation of Shigella LPS. Science **2005**; 307:1313-7.

24. Terwagne M, Ferooz J, Rolan HG, et al. Innate immune recognition of flagellin limits systemic persistence of Brucella. Cellular microbiology **2013**; 15:942-60.

FIGURE LEGENDS

Figure 1. **The protein product of *Bab1_1280* is a membrane-associated periplasmic protein. A**. Cell fractions from the merodiploid strain *Bab1_1280*::3xFLAG were analyzed by Western Blot with a monoclonal α-FLAG antibody. PE is periplasm and PR stands for protoplasts. Monoclonal α-Omp1 (porin) and α-GroEL (Hsp60 homologue) antibodies were used as outer membrane and cytoplasmic controls, respectively. **B.** Western blot with α-OMP1 and α-FLAG antibodies on SDS-PAGE of total membranes prepared from the *Bab1_1280*::3xFLAG strain. **C**. Total membranes from the *Bab1_1280*::3xFLAG strain were resuspended and incubated in different buffer conditions and ultracentrifuged. Pellet and supernantant fractions were analyzed by Western Blot with a monoclonal α-FLAG antibody. Sn stands for supernatant fraction.

Figure 2. **The absence of *Bab1_1280* causes pleiotropic membrane defects**. **A**. Detergent sensitivity assays. Stationary phase cultures of either the wild type 2308 or the Δ*Bab1_1280* strains were grown at 37°C, diluted to OD_{600}=1 and serial dilutions were plated in solid media containing different detergents and incubated at 37°C for CFU determination. Sarkosyl: N-lauroylsarcosine, Zwittergent: Zwittergent 3-16 and DOC: Sodium deoxycholate. The figure shows significant differences between the means of both strains for Sarkosyl (*$p<0.01$), Zwittergent (*$p=0.006$) and DOC (*$p=0.001$). **B**. N-phenyl-1-naphtylamine incorporation assay. Stationary phase cultures of either the wild type 2308 or the Δ*Bab1_1280* strains were grown at 37°C, diluted to OD_{600}=0.1 and grown at 37°C until exponential phase was reached. $7.5x10^8$ cells (resuspended in 250 µl of PBS) were used per well in 96-well black plates to measure baseline fluorescence previous to adding NPN (10 µM final concentration) and measurements were made every 18 seconds during 5 minutes. Relative Fluorescence Units (RFU) were calculated by dividing each value by the mean obtained for the baseline for each

strain (*$p<0.001$). In all cases (A-C), error bars are SD and *p*-values were calculated by the unpaired t-tests. **C**. Two-dimension thin layer chromatography (2D-TLC) of total phospholipids. Total [^{14}C]acetate-labeled lipids were extracted from cultures in the presence of choline and analyzed by 2D-TLC and autoradiography. Lipids spots corresponding to CL, PG, OL, PE, and PC are indicated. CL, cardiolipin; PG, phosphatidylglycerol; OL, ornithine lipid; PE, phosphatidylethanolamine; PC, phosphatidylcholine. **D.** Crystal violet staining. Stationary phase cultures of either the wild type 2308 or the Δ*Bab1_1280* strains were grown at 37°C, diluted to OD_{600}=1 and serial dilutions were plated in solid media and incubated at 37°C until colonies were observed. The crystal violet staining was performed as described in Experimental Procedures.

Figure 3. **RomA is required for LPS homeostasis**. **A**. Whole cell extracts of the wild type 2308 and *ΔromA* strains were analyzed by Western Blot using a rabbit polyclonal α-*Brucella* antibody. Monoclonal α-GroEL, α-Omp1, α-Omp16 and α-Omp19 antibodies were used for loading controls. **B**. Whole cells and periplasmic fractions were analyzed by Western Blot using a α-Smooth LPS (S-LPS) or a α-Rough LPS (R-LPS). **C.** Densitometry of gels in panel b. **D.** LPS was extracted from either the wild type 2308 or the Δ*romA* strains and analyzed by SDS-PAGE and Western Blot using the α-S-LPS and α-R-LPS antibodies (left panel), or SDS-PAGE and LPS silver staining (right panel).

Figure 4. **RomA is required for controlling the O-antigen length**. **A.** Aminosugar analysis by HPAEC-PAD of the oligosaccharides released after acid hydrolysis of the LPS of the wild type 2308 or the Δ*romA* strains. Comparison of the Rha4N/GlcN stoichiometry ratio in both strains. **B.** LPS was purified from the wild type 2308 Δ*romA* mutant strains and the O-antigen was released by acid hydrolysis. The released oligosaccharides were analyzed by MS in the

positive ion mode, and showed that the mutant strain has up to 25 perosamine subunits (Rha4N) per O-antigen chain (m/z=4835.5) in comparison to the 14 subunits of the wild type strain (m/z=3100.0). An inset of the wild type strain in the range of m/z=4200-5000 shows the absence of the peak corresponding to an O-antigen with 25 subunits of perosamine. Fo, formyl groups.

Figure 5. RomA is important for the intracellular cycle and its absence results in a hiperinflammatory strain. **A.** Intracellular multiplication of wild type 2308, Δ*romA* and Δ*romA* complemented strains in bone marrow derived macrophages (BMDM). *$p=0.005$ and **$p=0.002$. Error bars are SD and p-values were calculated by the unpaired t-test. **B.** Spleens extracted from the infected mice were homogenized for CFU determination by direct plating after 15 days post-infection. *$p<0.0001$. Error bars are SEM and *p*-values were calculated by the unpaired t-test. **C**. Spleens weight before homogenization. ***$p=0.0001$ and ****$p<0.0001$. Error bars are SEM. **D.** Comparative sizes of spleens of mice intraperitoneally infected with $1x10^5$ CFU per animal of either the wild type 2308, Δ*romA* and Δ*romA* complemented strains and 15 days post-infection.

Figure 6. The mutant Δ*romA* has an increased inflammatory response. A to D. Inflammatory cytokines determined in the infected animals at 15 days post-infection. Interferon gamma (IFN-γ) and Tumor necrosis factor alpha (TNF-α) levels were measured in spleens (A and C) and sera (B and D) by ELISA as described in Experimental Procedures. In all cases, significant differences were found between the means of the wild type and mutant strains and between the means of the mutant and the complemented strain. No differences were found between the wild type and complemented strain. **A**: *$p=0.03$ and ***$p=0.009$; **B**: *$p=0.02$ and **$p=0.001$; **C**: **$p=0.004$ in both groups; **D**: **$p=0.001$ in both cases. Error

bars are SEM and *p*-values were calculated using ordinary one-way ANOVA multiple comparisons between groups.

Figure 7. RomA participates in the positioning of LptD, a LPS transport protein. **A**. The outer membrane LPS transporter LptD was expressed with a C-terminal sfGFP from a pBBR4 plasmid in the *B. abortus* 2308 and Δ*romA* strains. Bacteria were grown at 37°C until exponential phase, placed on agarose pads and analyzed by fluorescence confocal microscopy. **B**. The inner membrane O-antigen flippase RfbD was expressed with a C-terminal EYFP from pTRC-EYFP in the *B. abortus* 2308 and Δ*romA* strains. Bacteria were grown at 37°C until exponential phase, placed on agarose pads and analyzed by fluorescence confocal microscopy.

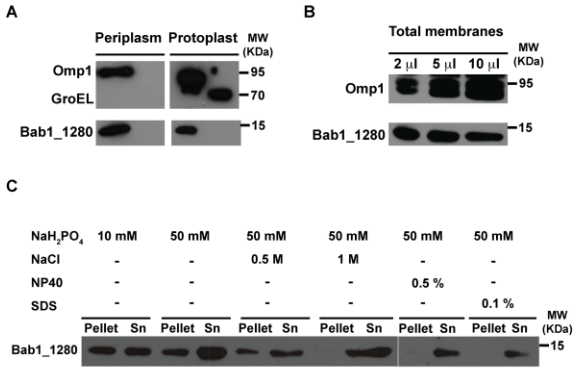

Figure 1

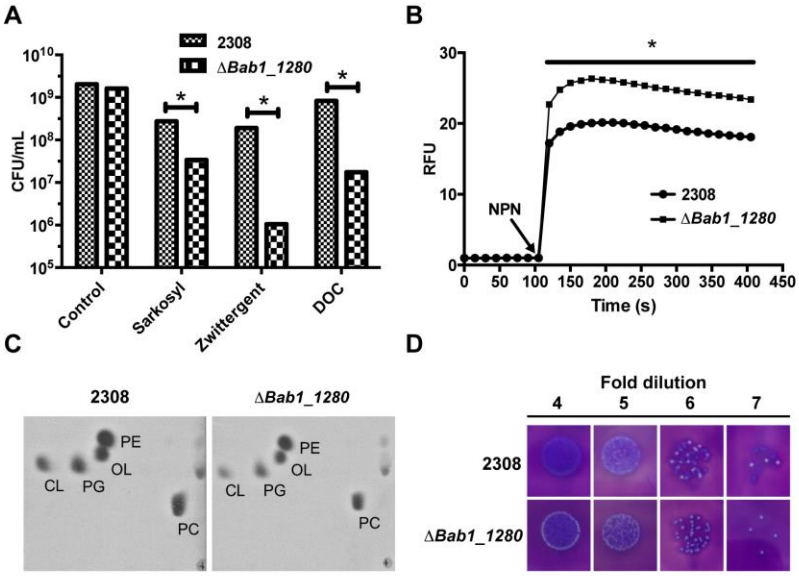

Figure 2

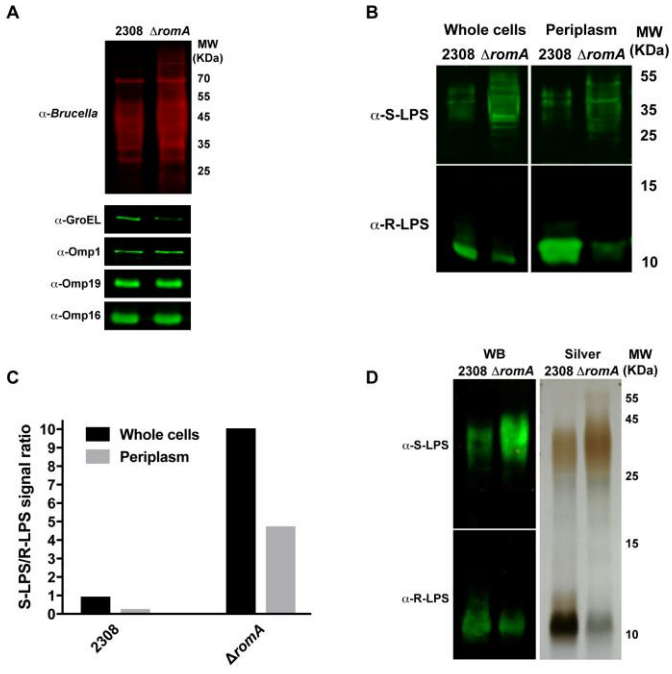

Figure 3

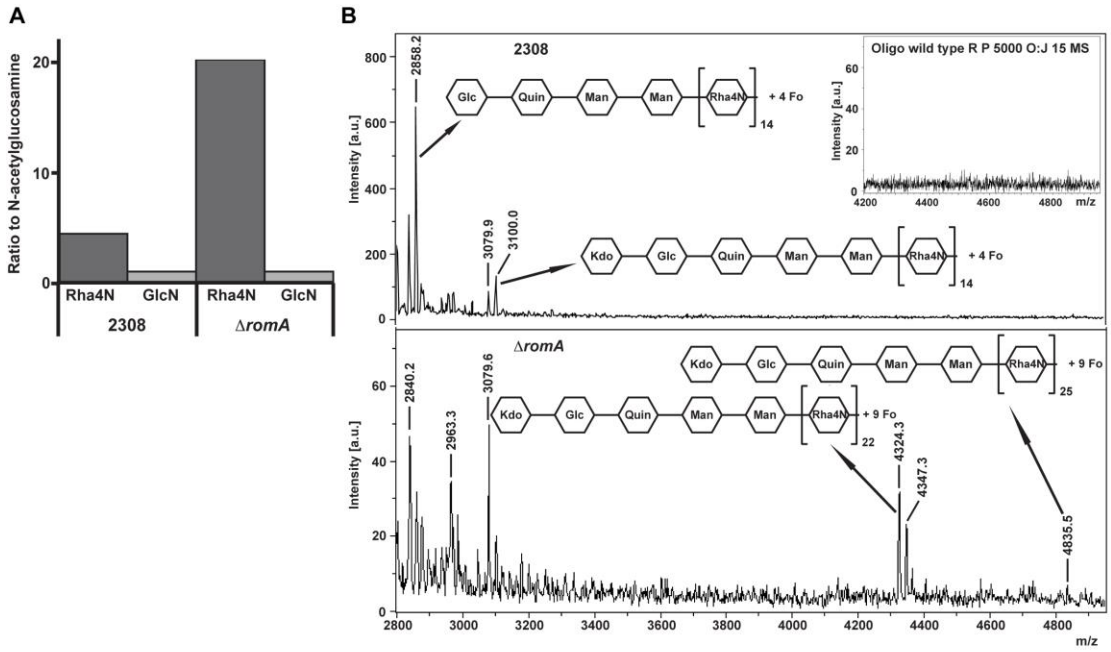

Figure 4

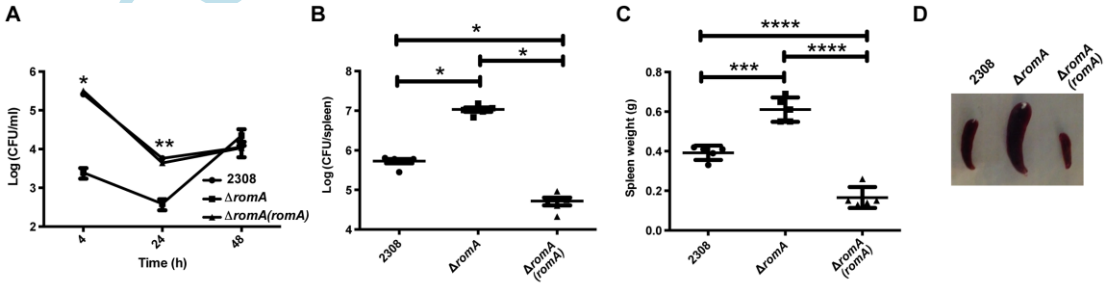

Figure 5

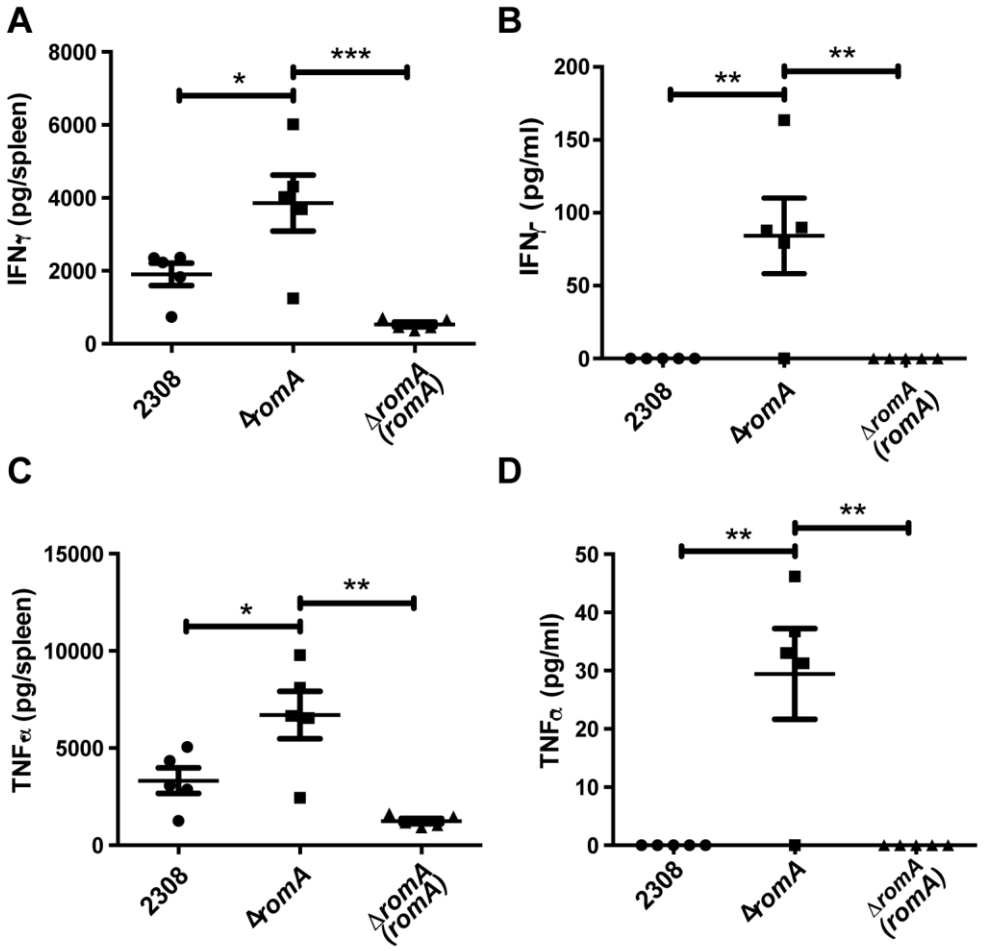

Figure 6

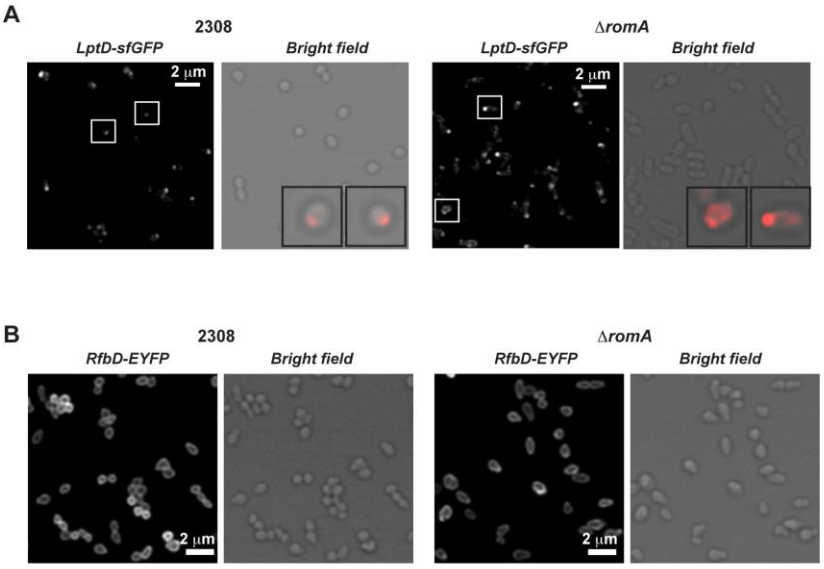

Figure 7