A genomic island in *Brucella* involved in the adhesion to host cells:

identification of a new adhesin and a translocation factor

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Abstract

Adhesion to host cells is the first step in the virulence cycle of any pathogen. In Gram-negative bacteria, adhesion is mediated, amongst other virulence factors such as the lipopolysaccharides, by specific outer-membrane proteins generally termed adhesins that belong to a wide variety of families and have different evolutionary origins. In *Brucella*, a widespread zoonotic pathogen of animal and human health concern, adhesion is central as it may determine the intracellular fate of the bacterium, an essential stage in its pathogenesis. In the present paper we further characterized a genomic locus that we have previously reported encodes an adhesin (BigA) with a bacterial immunoglobulin-like domain (BIg-like). We found that this region encodes a second adhesin, which we have named BigB; and PalA, a periplasmic protein necessary for the proper display in the outer membrane of BigA and BigB. Deletion of *bigB* or *palA* diminishes the adhesion of the bacterium and overexpression of BigB dramatically increases it. Incubation of cells with the recombinant BIg-like domain of BigB induced important cytoskeletal rearrangements and affected the focal adhesion sites indicating that the adhesin targets cell-cell or cell-matrix proteins. We additionally show that PalA has a periplasmic localization and is completely necessary for the proper display of BigA and BigB, probably avoiding their aggregation and facilitating their transport to the outer membrane. Our results indicate that this genomic island is entirely devoted to the adhesion of *Brucella* to host cells.

Introduction

Adhesion to either specific tissues or target host cells is the first step in the virulence cycle of almost all pathogenic bacteria. This process is central, as it may determine specific replicative niches, tissue tropism as well as the outcome of the immune response, impacting on the efficiency of the infection. Bacterial adhesion is mediated by dedicated outer membrane molecules that target specific proteins or glycolipids on the surface of the host cells, promoting attachment and, eventually, invasion if the pathogen has an intracellular life cycle. Bacterial adhesins exhibit high selectivity for target molecules, recognizing molecular motifs with a high degree of specificity comparable to enzymes and immunoglobulins (Klemm & Schembri, 2000). There are many different families of adhesins that vary depending on the Gram nature of the bacteria, the host cell as well as the virulence cycle of the pathogen. They play pleiotropic roles, enhance competition with other bacteria and find their niche within the host, facilitating the establishment of pathogenesis and modulating the alterations of the cellular functions (Patel, Mathivanan, & Goyal, 2017).

Adhesins are responsible for pathogen persistence causing, amongst other symptoms, tissue inflammation in the host. Gut mucosa, lungs and bladder are some examples of inflammation mediated by adhesin immune activation (Conover et al., 2016; Mil-Homens, Pinto, Matos, Arraiano, & Fialho, 2017; Moore, Boren, & Solnick, 2011; Palmela et al., 2018). The cytoskeleton is intrinsically involved in inflammation and its plasticity is an important component

of host cells. Cytoskeleton is exploited by pathogens by either triggering actin rearrangements that can cause cell invasion that lead to their own phagocytosis or by zippering, where invasion occurs first by a direct contact between the adhesin and its ligand which activates cytoskeletal components that culminate in the closure of the phagocytic cup and bacterial internalization (Cossart & Sansonetti, 2004; Patel et al., 2017; Pizarro-Cerda & Cossart, 2006).

Many secretion mechanisms for different adhesins in a wide variety of bacteria have been described depending on their Gram classification. In Gram negative bacteria, one of the most studied mechanisms is the chaperone-usher assisted surface display of PapD and FimC in *E. coli*, consisting of an assembly platform or usher and a periplasmic chaperone, which complexes with the future adhesin avoiding proteolytic degradation and premature polymerization (Jones et al., 1993).

Brucellaceae are Gram-negative zoonotic intracellular pathogens of veterinary and human health importance that cause important economical losses and sanitary problems, particularly in endemic regions (Byndloss & Tsolis, 2016; Corbel, 1997). Although their virulence is completely dependent on the capacity of the bacteria to replicate intracellularly to reach their niche (Byndloss & Tsolis, 2016; O'Callaghan et al., 1999; Sieira, Comerci, Sanchez, & Ugalde, 2000; R. A. Ugalde, 1999), adhesion to specific tissues is a central step in the pathogenesis of the bacterium that allow *Brucella* to invade professional and non-professional phagocytes (Detilleux, Deyoe, & Cheville, 1990; Pizarro-

Cerda et al., 1998). Several adhesins that target different host cell proteins have been identified in *Brucella* (Castaneda-Roldan et al., 2006; Posadas, Ruiz-Ranwez, Bonomi, Martin, & Zorreguieta, 2012; Ruiz-Ranwez, Posadas, Estein, et al., 2013; Ruiz-Ranwez, Posadas, Van der Henst, et al., 2013).

Our laboratory has identified a genomic region, named Bab1_2009-Bab1_2012 gene cluster, unique to *Brucella* that is involved in the adhesion and invasion to host cells and important in the oral route of infection (Czibener & Ugalde, 2012). This region, with horizontally transmitted features, encodes four open reading frames present only in the genus *Brucella* and with no detectable homology with known proteins. Further analysis of this region determined that it encodes a powerful adhesin (BigA) with a bacterial immunoglobulin-like (Big-like) domain that targets the bacteria to the cell-cell junction membrane in polarized cells promoting the adhesion and invasion of epithelial cells through cytoskeleton modifications (Czibener et al., 2016).

In the present study we provide new insight into the importance of the Bab1_2009-Bab1_2012 genomic cluster in adhesion, characterizing two new proteins that belong to this island. One of them is a novel adhesin, which has a BIg-like domain, similar to the one present in BigA. Moreover, our results demonstrate that another gene of this region, with no apparent homology or conserved domains has a major role in the correct localization of these adhesins in the outer membrane of *Brucella*. Our results indicate that the

Bab1_2009-Bab1_2012 gene cluster plays a central role in the adhesion of *Brucella*.

Experimental Procedures

Media and culture conditions

Brucella strains were grown at 37°C in Tryptic Soy Broth (TSB). *E. coli* strains were grown at 37°C in Luria–Bertani broth. If necessary, media was supplemented with the appropriate antibiotics at the indicated final concentrations: Ampicillin, 100 μg/ml; Kanamycin, 50 μg/ml and Nalidixic Acid, 5 μg/ml.

Recombinant DNA techniques, mutant and plasmid constructions

Construction of B. abortus

2308 Δbab1_2012 mutant strain

Regions flanking the *bab1_2012* gene were amplified and ligated using the recombinant PCR technique (Czibener & Ugalde, 2012). The resulting fragment was digested with EcoRI and XbaI and ligated to the pK18mobSacB plasmid digested with the same enzymes. The primers used for PCR amplification were: CC46 (5′- CCGGAATTCCACTCGGCAGTGGGTCTGTT -3′) and CC47 (5′- TTTACCCTCTAATATAATTA -3′) to amplify a 500 bp upstream region and CC48 (5′- ATATTAGAGGGTAAAGTTTAAAAATGAAAAAGTTAC -3′) and CC49 (5′- GCTCTAGATATGCGCTTCGCACTTTTCG -3′) to amplify a 500 bp downstream region; CC46 and CC49 were used for the overlapping PCR.

Construction of B. abortus 2308 Δbab1_2011 mutant strain

Regions flanking the *bab1_2011* gene were amplified and ligated using the recombinant PCR technique (Czibener & Ugalde, 2012). The resulting fragment was digested with EcoRI and BamHI and ligated to the pK18mobSacB plasmid digested with the same enzymes. The primers used for PCR amplification were: PL4 (5′- CCGGAATTCATCATTCAGGAGCAAAAATG-3′) and PL5 (5′- GATATTACGGCTTTGGGATC-3′) to amplify a 500 bp upstream region and PL6 (5′- CAAAGCCGTAATATCTGATTTGGAGATACTTACTTT-3′) and PL7 (5′- CGCGGATCCTAATGGCGGTGTTATC -3′) to amplify a 500 bp downstream region; PL4 and PL7 were used for the overlapping PCR.

In both cases, the resulting plasmids were introduced into *B. abortus* 2308 by biparental mating using the *E. coli* S17-λpir strain. Double recombination events (Km^s Sac^r) were selected, and the gene knockout was confirmed by genomic PCR.

Construction of plasmid pBBR4-MCS4-BigB-3xFlag and pBBR4-MCS4-PalA-3xFlag:

For the construction of the vector expressing a C-terminal 3xFLAG-tagged version of gene Bab1_2012 (BigB) and Bab1_2011 (PalA) the plasmid pBBR1-MCS4-3xFLAG (Dohmer et al., 2014) was used. DNA fragments containing these genes were separately amplified by PCR from *B. abortus* 2308 genomic DNA using primers BAB1_2012F (5′- CGGAATTCACTATCAATACGTATTCTAT-3′) and BAB1_2012R (5′- CGCCATGGCTATTACCCTTGTAGAAAGAA -3′) for the Bab1_2012 gene, and

PL8 (5′- GGAATTCTTCGGGAAATAGTCCAG-3′) and PL9 (5′-CATGCCATGGCAAAACAATTATCACGTGC-3′) for Bab1_2011. Both PCR products were digested with EcoRI and NcoI restriction enzymes and cloned in plasmid pBBR1-MCS4-3xFlag in the same sites generating an in-frame fusion to the 3xFLAG epitope. The resulting plasmids were named pBBR-Bab1_2012-3xFlag and pBBR-Bab1_2011-3xFlag respectively and introduced in the *B. abortus* strains (wild type and mutant) by bi-parental mating for overexpressed and complemented strains for these genes. The expression of BigA-3xFLAG and PalA-3xFLAG was confirmed by Western blotting.

Construction of pQE30 IgBigB-3xFLAG-HIS

In order to generate the poly-histidine-tagged IgBigB/3xFlag recombinant protein a DNA fragment was first amplified from *B. abortus* genomic DNA using primers PL1 (5′ - GGGGTACCCCAAGCGTAAGCTTTCC -3′) and PL2 (5′-CGATCATGGCTATTACCCTTGTAGA -3′) the PCR product was digested with KpnI and NcoI and cloned in pBBR1-MCS4-3xFLAG (Dohmer, Valguarnera, Czibener, & Ugalde, 2014) in the same sites. The fragment was subsequently amplified using primers PL1 and PL3 (5′-CGATCATGGCTATTACCCTTGTAGA -3′) and the PCR fragment was digested with KpnI and PstI and subcloned in the pQE30 vector in the same sites.

Construction of pBBR1-MSC4-BigAsfGFP and pBBR1-MSC4-BigB sfGFP:

With the purpose of generating superfolder GFP-tagged proteins the plasmids pBBR1-MCS4-sfGFP and pBBR2-MCS4-sfGFP (Dinh & Bernhardt, 2011; Valguarnera et al., 2018) were used. DNA fragments spanning genes Bab1_2009 (*bigA*) and Bab1_2012 (*bigB*) were separately amplified by PCR from *B. abortus* 2308 genomic DNA using primers CC64- (5'-CCCAAGCTTACTTTAGTAAAGACATACT-3') and CC65 (5'-CGCGGATCCCCGGAGGAGATGGTGGC -3') for BigA-sfGFP gene, and PL10- (5'-GGAATTCCTTCTAATAAATATAAGTATCTATAATCTAT-3') and PL11 (5'-GACTAGTTATTACCCTTGTAGAAAGAACA-3') for BigB-sfGFP gene. Both PCR products were digested with HindIII and BamHI for BigA-sfGFP and EcoRI and SpeI for BigB-sfGFP and cloned in pBBR1-MCS4-sfGFP and pBBR2-MCS4-sfGFP in the same sites generating an in-frame fusion with the sfGFP. The expression was confimed by Western blotting. The resulting plasmids were separately introduced in the *B. abortus* strains 2308 (wild type), 2308/Δ*palA* and 2308/PalA-3xFLAG by bi-parental mating.

All constructs were confirmed by sequence analysis.

Protein expression and purification

Recombinant poly-histidine-tagged BIg-BigB and BIg-BigA were expressed in *E. coli* and purified using nickel-affinity chromatography under denaturing conditions in the presence of urea 6M. Briefly, *E. coli* was grown at 37°C at 250 r.p.m., and the expression was induced with IPTG at $OD_{600}=0.6$. 3 hrs post-

induction cells were harvested and broken by sonication. The resulting lysate was centrifuged at 16000 g and the inclusion bodies (insoluble) washed two times and resuspended in Imac A buffer (500mM NaCl, 50mM Tris-HCl pH 7.6 and 20mM imidazole) with 6M urea and applied to a HisTrapTM HP column (GE, Healthcare). The protein was eluted with the same buffer with 300mM of imidazole. The eluted fractions were pooled and dialyzed against re-folding buffer (50mM Tris- HCl pH 7.6, 1mM NaCl, 0.2mM DTT, 0.1mM EDTA, 10% glycerol, 500mM L-Arginine and 1mM PMSF) followed by a second dialysis against PBS. The product of this was concentrated by ultrafiltration and used as the source of recombinant protein.

Antibody production and purification

Anti-Bab1_2012 antiserum was performed inoculating mice (a first inoculation and two boosters at 17 and 32 days after the primo injection) with 100 µg in the first and 50 µg in the boosters of the recombinant protein in Freund's adjuvant. Two weeks after the last booster, mice were evaluated for their response by Western blot and the positive animals were euthanized and the serum extracted.

Intracellular replication assays

Antibiotic protection assays were performed in the human cell lines HeLa (ATCC), the murine macrophage-like J774 A.1 (ATCC) and the dog cell line

MDCK (ATCC) as described in (Czibener et al., 2016; J. E. Ugalde, Czibener, Feldman, & Ugalde, 2000). Cells were seeded in 24-well plates in suitable culture medium at 10^5 cells ml^{-1} and incubated overnight at 37°C for HeLa and J774 A.1 cells, and the appropriate time until confluence for MDCK (once they have reached confluence they were left for an extra 48 hrs to differentiate apical and basolateral membranes). *Brucella* strains were grown in TSB with the appropriate antibiotics for 24 hrs and diluted in culture medium prior to infection. The suspension was added at the different multiplicity of infections and centrifuged at 1000 g for 10 min. After 1 hr of incubation at 37°C, cells were washed and fresh medium containing 100 µg ml^{-1} of streptomycin and 50 µg ml^{-1} of gentamicin was added. At 4, 24 and 48 hrs post-infection, cells were washed and lysed with 0.1% Triton-100X. The intracellular CFU were determined by direct plating on TSB agar plates.

Immunofluorescence microscopy

Cells were seeded on glass coverslips and infected or not depending on the experiment performed. For infections, at different times post-infection, cells were washed three times with PBS and fixed for 15 min in 4% paraformaldehyde and then processed for immunofluorescence labelling. For cells treated with recombinant BigB and/or recombinant BigA, different concentrations (depending on the experiment) of the purified protein in PBS were added to the monolayer, and at different times post-treatment cells were

processed for imaging. After either the infection or treatment with recombinant BigB coverslips were washed three times with PBS, incubated for 15 min with PBS added with 50mM NH₄Cl in order to quench free aldehyde groups. Coverslips were then blocked, incubated with the primary antibodies in a PBS, 5% bovine serum albumin, 10% horse serum and 0.1% saponin solution for 1 h at room temperature, washed in PBS and then stained with phalloidin rhodamin and incubated with the secondary antibodies in PBS, 10% horse serum, 5% bovine serum albumin and 0.1% saponin solution under the same conditions. The coverslips were mounted onto glass slides using FluorSave Reagent (Calbiochem). Cells were observed in an EPI fluorescence microscope (Nikon- Eclipse T2000 or an Olympus IX81 attached with a confocal module) using a 60X oil immersion objective, with 1.40 NA and 1.42NA respectively. Projections were saved in OIFF format (Olympus original format) and imported to Image J (NIH, Bethesda, MD), where images were edited and merged using RGB format.

The secondary antibodies used were goat anti-mouse or goat anti-rabbit Alexa Fluor 568 or 488 (Molecular Probes, Invitrogen Co.) at a 1:4000 dilution. For DNA staining, 40,6-diamidino-2- henylindole DAPI dye at 0.5 mg ml^{-1} (final concentration) was used.

Adhesion and internalization assays

To determine adhesion and invasion, infected cells were fixed for 15 min in 4% paraformaldehyde (pH 7.4) at room temperature at 4 h post-infection. Coverslips were washed three times with PBS, incubated for 10 min with PBS added with 50 mM NH$_4$Cl and, incubated with the primary antibody rabbit anti-*Brucella* polyclonal antibody (dilution 1:1500) in a PBS, 5% BSA solution. Afterwards permeabilization with saponin was performed and then cells were washed and incubated with the other primary antibody mouse anti-M84 (anti-O-antigen) monoclonal antibody (dilution 1:1000) (Nielsen, Kelly, Gall, Nicoletti, & Kelly, 1995) in a PBS, 5% BSA, 0.1% saponin solution followed by incubation with the secondary antibodies (Alexa Fluor 568 or 488, Molecular Probes, Invitrogen Co) in a PBS, 5% BSA, 0.1% saponin solution. The coverslips were mounted as described before. Invasion was determined as the number of bacteria positive for both labels versus the ones positive for the anti-mouse labelling. Adhesion was determined counting the number of bacteria associated per 100 cells.

Bacterial staining

For staining of Bab1_2012 on total bacteria, a saturated culture was diluted in order to obtain 10^6 UFC ml^{-1} and the cells fixed in 4% paraformaldehyde for 20 min, washed with PBS and incubated in suspension with a 1:25 dilution of the anti-Bab1_2012 policlonal antibody (refer to the preceding texts) for 8 h. After two washes with PBS the cells were incubated with a 1:2000 dilution of a

secondary anti-mouse conjugated to Alexa 568 for 1 h. After two washes with PBS bacteria were deposited on polylysine treated glasses and mounted for confocal observation.

Cell transfection

Transfection of HeLa cells with plasmids expressing DsRed-zyxin and mCherry-paxillin (Arregui, Balsamo, & Lilien, 1998; Hernandez, Sala, Balsamo, Lilien, & Arregui, 2006) was performed in 24-well tissue-culture plates using lipofectamine 2000 (Invitrogen Corp, Carlsbad, CA, USA) as recommended by the manufacturer.

Analysis of protein expression and subcellular localization

Whole bacteria

Brucella whole cell extracts were resuspended in Laemmli sample buffer and heated to 100°C for 5 min. Samples were submitted to SDS-PAGE (10% or 15% depending on the assay) and transferred to nitrocellulose membranes. The presence of 3xFLAG or sfGFP-tagged proteins was carried out by immunoblot analysis using mouse anti-Flag M2 monoclonal antibody (Sigma-Aldrich dilution, 1:5000) or anti-GFP monoclonal antibody (1:2000), and IRDye secondary anti-mouse antibody (LI-COR, Inc.).

Periplasmic and Cytoplasmic Localization Assay

Fractionation assays were performed as described previously described (Dohmer et al., 2014). *B. abortus* strains were grown in TSB for 16–24 h at 37 °C until an A_{600} of 1 was reached, and 2.5 x 10^{10} bacterial cells were centrifuged for 10 min at 3300 g. The pellets were washed with physiological solution, centrifuged for 10 min at 3300 x g, and resuspended in 1 ml of 0.2 M Tris-HCl (pH 7.6). One milliliter of 0.2M Tris-HCl (pH 7.6), 1M sucrose, and 0.25% Zwitterion 3-16 solution was added to the cell suspension and incubated for 10 min at room temperature. The samples were centrifuged for 30 min at 8000 x g, and the pellets were separated from the supernatants and stored at -20 °C until used for western blot analysis.

Preparation of total membrane fractions

Total membrane fractions were prepared as previously mentioned (Del Giuduce et al, 2013). Briefly, cells were harvested at 8000 × g, resuspended in buffer A (15 mM Tris-HCl [pH 8], 0.45 mM sucrose, 8 mM EDTA [pH 8], 0.4 mg/ml, lysozyme 5mg/ml) and incubated for 15 min at 4°C. Then, cells were centrifuged (8000 × g, 4°C, 15 min) and sonicated in buffer B (50 mM Tris-HCl [pH 7.6], 5 mM MgCl2, 2 mM phenylmethylsulfonyl fluoride [PMSF], deoxyribonuclease I [DNAse]; 4 watts 60 seconds pulse and 60 seconds rest for 30 min at 4°C). The sonicated cells were centrifuged, and supernatant was recovered. Supernatant was diluted 1:4 in buffer C (1 M Tris-HCl [pH 8.0], 1mM PMSF) and insoluble membrane fractions were recovered by ultracentrifugation (10 PSIG, 4°C, 90 min, (Airfuge-Beckman Coulter) and pellet was homogenized

in 50 mM Tris-HCl, pH 8.0. Pellets and supernatants were stored until western blot analysis.

Western blot analysis of sub localization of Brucella proteins

The pellets and supernatants from total membrane fractions, periplasmic and cytoplasmic were processed for Western blotting using an anti-FLAG M2 monoclonal antibody (1:10000), anti-GroEL (1:2000), anti-OMP-19 (1:2000), and anti-OMP2b (1:2000), provided by Dr. Axel Cloeckaert as primary antibodies (Cloeckaert, de Wergifosse, Dubray, & Limet, 1990; Cloeckaert et al., 1991), and IRDye secondary anti-mouse antibody (LI-COR, Inc.). All antibodies were diluted in TBS, 1% nonfat milk, 0.1% Tween 20 solution. Detection was performed using the Odyssey imaging system (LI-COR, Inc.).

Microscopy analysis of Brucella

Exponential-phase cultures of *B. abortus* BigB-sfGFP strains expressing the BigB gene tagged with super folder GFP fluorescent fusion protein was placed on a microscope slide that was layered with a pad of 1% agarose in phosphate buffered saline (PBS) as previously described (Hallez et al., 2007; Ruiz-Ranwez, Posadas, Estein, et al., 2013). For image acquisition, samples were examined on an IX81 microscope with an Olympus FV1000 confocal module (60X PLAPO objective, numerical aperture [NA] of 1.42). For each observation under the microscope, at least three fields were randomly selected for analysis. Images were processed with the Image J program (NIH, Bethesda, MD).

Co-Immunoprecipitation of PalA-3xFLAG and BigA-sfGFP

B. abortus strains expressing 2308 PalA-3xFLAG /BigA-sfGFP and the *B. abortus* control strain 2308 BigA-sfGFP were cultivated in TSB for 16–24 h at 37 °C. Cells were harvested at 8000xg, resuspended in 50 ml of buffer A (20 mM Tris-HCl pH 7.6; 0.5 M Sucrose; 10 mM EDTA; 400 µg/ml lysozyme) and incubated for 15 min at 4 °C. Then, cells were centrifuged (8000 x g, 15 min) and sonicated in 2 ml of buffer B (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM MgCl2, 1 mM PMSF, DNase). The resulting lysate was centrifuged twice, and supernatants were recovered and mixed with IP buffer (150 mM NaCl, 1 mM EDTA, 0.25% Nonidet and 0.25% Triton X-100). For immunoprecipitations, 40 ul of anti-FLAG M2 affinity gel (Sigma-Aldrich) pre-equilibrated with IP buffer was mixed with supernatants and incubated at 4 °C overnight on a rotating platform. The gel suspension was washed at 4°C, 5 times with TBS on a rotating platform, and bound proteins were eluted with 2x Laemmli sample buffer without reducing agent. The presence of 3xFLAG and sfGFP-tagged proteins was evaluated by western blot analysis using anti-FLAG rabbit polyclonal antibody (1:5000), anti GFP rabbit polyclonal antibody (1:5000) and IRDye secondary anti-rabbit antibody (LI-COR, Inc.). All antibodies were diluted in TBS, 1% nonfat milk, 0.1% Tween solution. Detection was performed using the Odyssey imaging system (LI-COR, Inc.).

Results

Bab1_2012 encodes a second adhesin of the *bab1_2009-bab1_2012* gene cluster

As indicated above, *bigA* (gene *bab1_2009* in *B. abortus* 2308 (Chain et al., 2005)) encodes an adhesin that targets the bacterium to the cell-cell interaction membrane and promotes adhesion and invasion of the host cell (Czibener et al., 2016). In order to determine if any other of the three genes encoded in this region are also adhesins we analyzed the putative proteins in search of motifs that could suggest this type of function. Interestingly, analysis of Bab1_2012 showed a weak homology (using the https://toolkit.tuebingen.mpg.de/tools/hhpred) with bacterial immunoglobulin-like domains present in several adhesins of different pathogens and in BigA (Czibener et al., 2016). Also, in silico analysis predicted three transmembrane segments (SMART- EMBL). To evaluate if Bab1_2012 is an adhesin a deletion mutant was constructed and intracellular replication assays in non-phagocytic cells were performed. As can be observed in Figure 1A, the Δ*bab1_2012* mutant showed a significant reduction in the intracellular number of bacteria at the early stages of the infection curve (4 hrs post-infection) in HeLa cells and this reduction was also observed in a polarized cell line as Madin-Darby Canine Kidney (MDCK) cells (Figure 1B). This phenotype was also observed in the macrophagic cell line J774 A.1 (Supplementary Figure 1). To determine if this phenotype was the result of a reduction in the adhesion, invasion or capacity to

survive intracellularly, adhesion and invasion assays in HeLa cells were performed comparing the wild type, mutant and complemented strains. Figure 1, panels C and D shows that the Δ*bab1_2012* mutant had a reduced capacity to adhere to host cells and, consequently, a reduced invasion in comparison to the wild type parental strain. Interestingly, and as we have observed with BigA (Czibener et al., 2016), the complemented strain overexpressing Bab1_2012 from a multicopy plasmid, resulted in a strain with a dramatic increase in its adhesion capacity (Figure 1D).

To determine the localization of Bab1_2012 in the cells we expressed a 3xFlag-tagged version of the protein in *B. abortus* and determined its subcellular localization by fractionation experiments (see Experimental procedures). As can be observed in Figure 2A, Bab1_2012 fractioned with total membranes, indicating that it is either an inner or an outer membrane associated protein. Further periplasmic extraction experiments (Figure 2B) showed that the protein product of *bab1_2012* is located in the periplasm or outer membrane, which, in combination with the result of its association with total membranes, confirms that Bab1_2012 is located in the outer membrane.

To further confirm that Bab1_2012 is exposed in the outer membrane we developed a polyclonal antibody against the Ig-like domain of the protein and performed an immunofluorescence with non-permeabilized cells of the *B. abortus* expressing Bab1_2012 from a medium copy plasmid and the deletion mutant strains. As can be observed in Figure 2C, the protein was found on the

bacterial surface confirming that Bab1_2012 is an exposed outer membrane protein.

Altogether these results indicate that Bab1_2012 codes for an adhesin that mediates attachment to host cells. Consequently we renamed this gene *bigB* for Bacterial Immunoglobulin Gene B.

BigB induces cytoskeletal rearrangements of host cells

We have previously reported that treatment of cells with the recombinant bacterial immunoglobulin-like domain of BigA induces profound cytoskeletal rearrangements that results, upon longer treatments, in the detachment of the cells from the plate (Czibener et al., 2016). As BigB has a BIg-like domain as well, we wanted to determine if this domain of BigB induces similar effects. For this purpose we cloned the region of the gene coding the Ig-like domain (amino acids 120 to 399 of the coding region), produced it in *E. coli* as a double poly-histidine-3xFLAG tagged protein and purified it by affinity chromatography as indicated in Experimental procedures. HeLa cells were treated either with the recombinant BigB, with the recombinant BigA or a combination of both. As can be observed in Figure 3A, BigB produced rounding of the cells that detached from the plate, as the BigA-treated cells, but at higher concentrations and longer times of incubation. Additionally, when observed under the confocal microscope we noticed that the treated cells seemed to have a higher height than the control group. To determine this we measured the height of the cells performing a Z stack of cells incubated with BigA, BigB or both together, which

gave us an indirect quantitative measurement of the cytoskeleton modifications. Figure 3B shows that incubation with either BigA or BigB induced a doubling in the cell height indicating that the proteins induce a profound rearrangement of the cytoskeleton. Interestingly incubation with a combination of both proteins did not have an additive effect.

To further confirm that the incubation of cells with BigB affects their adhesion we transfected HeLa cells with the focal adhesion markers zyxin-DSRed or paxillin-mRFP, treated them with the recombinant BigB, fixed the cells and stained with Phalloidin as indicated in Materials and Methods. As can be observed in Figure 3C, treatment of the transfected cells with BigB for 4 hrs induced significant changes in the focal adhesion sites as evidenced by the disappearing of both zyxin and paxillin markers at the borders of the cells treated with BigB. Consistent with this, the constrained form observed in the treated HeLa cells proved that the cytoskeleton structure was also affected by this adhesin. Taken together, these results provide proof that BigB alters focal adhesion sites triggering cytoskeletal rearrangements in non-phagocytic cells.

Bab1_2011 is periplasmic protein involved in the adhesion of *Brucella*

The Bab1_2009-Bab1_2012 genomic region encodes four open reading frames present only in the *Brucella* genus and with no detectable homology to other protein in databases (Czibener & Ugalde, 2012). Since we have shown that at least two of these proteins are adhesins (BigA and BigB) this prompted

us to evaluate the function of the other genes present in the region (*bab1_2010* and *bab1_2011*). For this we generated deletion mutants in both open reading frames and performed intracellular replication curves in HeLa cells with the resulting mutants. Deletion of *bab1_2010* resulted in a strain with a reduced intracellular survival at early time points but we were not able to complement it (data not shown). Deletion of *bab1_2011* resulted in a strain with a moderate but statistically significant reduction in the intracellular CFUs at 4 hrs post-infection (Figure 4A) that was complemented with the gene in *trans*. To determine if this reduction was the consequence of a reduced adhesion or invasion we performed adhesion/invasion assays by immunofluorescence as indicated above. As can be observed in Figure 4B the *B. abortus* Δ*bab1_2011* strain showed a statistically significant reduction in the adhesion capacity compared to the wild type parental strain and, interestingly, complementation of the mutant resulted in a strain with an increased adhesion, as we have also observed for BigA and BigB. As expected, due to the reduced adhesion of the strain, invasion was also affected in the mutant strain (Figure 4C). These results indicate that Bab1_2011 is either an adhesin *per se* or affects the activity of other adhesins (such as BigA and BigB).

Bab1_2011 encodes a protein of 129 amino acids conserved within the *Brucella* genus but with no detectable homology to other proteins and no canonical signal peptide. To gain further insight into the function of Bab1_2011 we constructed a *B. abortus* strain expressing a 3xFlag tagged version of the

protein from a replicative plasmid (see Experimental procedures) and performed subcellular fractionation assays to determine the subcellular localization of the protein. As can be observed in Figure 4 panels D and E, Bab1_2011 was not found associated with total membranes but partitioned in both the periplasmic plus outer membrane and the cytoplasmic fractions indicating that it is a periplasmic protein, therefore discarding Bab1_2011 to be an adhesin.

Altogether these results strongly suggest that Bab1_2011 is a protein that probably affects the adhesion of *Brucella* indirectly by regulating the function or the display of the other molecules of the Bab1_2009-Bab1_2012 genomic region.

Bab1_2011 is necessary for the insertion of BigA and BigB in the outer membrane

Our results indicate that Bab1_2011 is a periplasmic protein involved in the adhesion process and, since it is present in a locus that encodes two adhesins (BigA and BigB) we hypothesized that it could be involved in the translocation and/or insertion of these proteins in the outer membrane. To determine if this is the case we expressed 3xFlag-tagged versions of BigA and BigB in the wild type and *Δbab1_2011* strains and analyzed their subcellular localization by performing total membrane purifications. A first unexpected observation was that BigA, in the *Δbab1_2011* strain, migrated in an SDS-PAGE of total extracts

as a 70 kDa protein instead of 40 kDa as it was observed in the wild type strain (Czibener et al., 2016). (Figure 5A). This migration pattern was not altered even when higher concentrations of DTT, urea or protein alkylating agents were used strongly suggesting that BigA is probably covalently linked to itself or another protein but not through a disulfide bond. Moreover, as can be observed in Figure 5B, BigA was not found associated with total membranes as we have previously described in the wild type strain (Czibener et al., 2016). To determine if this phenomenon was also observed with BigB we performed the same extraction protocol with the *Δbab1_2011* (BigB-3xFlag) strain and determined its membrane association. As shown in Figure 5C, in the Δ*bab1_2011* mutant BigB lost its membrane localization too although in this case the protein had a migration as expected according to its predicted molecular weight. Both extractions were performed in parallel with the corresponding wild type strain. Overall, these results indicate that Bab1_2011 has a critical role in regulating the localization in the outer membrane of the two adhesins present in the same locus. For this reason, we renamed Bab1_2011 as *palA* for periplasmic adhesin locator A.

One possibility for the phenotype observed with the Δ*palA* mutant is that neither BigA nor BigB could be translocated to the periplasm. To determine if this is the case we performed periplasmic extractions with the *B. abortus* Δ*palA* strain expressing the Flag-tagged versions of the proteins and determined their localization by western-blot. Figure 5 panels D and E shows that both BigA and

BigB retain their periplasmic localization in the mutant indicating that their translocation to this compartment is not affected. To corroborate that neither BigA nor BigB have their translocation to the periplasm affected in the $\Delta palA$ mutant we constructed translational fusions to the superfolder-GFP (sGFP) of both proteins and compared their localization in the wild-type and mutant strains by confocal microscopy. As can be observed in Figure 6A-B the fusion BigA-sfGFP showed a bipolar localization in both strains, demonstrating that although BigA in the $\Delta palA$ mutant is located in the periplasm, the bipolar localization is not affected in the mutant Also, as it was previously described in Czibener et al., 2016 BigA in the wild type strain is exposed in the membrane surface and its localization is polar. In the case of the fusion BigB-sfGFP the construct was unstable in the $\Delta palA$ strain and we were not able to obtain reproducible results. Altogether these results indicate that the absence of PalA impairs the insertion of both BigA and BigB in the outer membrane remaining in the periplasmic space and, in the case of BigA, covalently linked to another protein or aggregated. This impairment could be the consequence of an indirect cause or because PalA interacts, either directly or in a complex, with BigA and BigB, stabilizing them in the periplasm, avoiding aggregation, and facilitating their transport to the outer membrane. To determine if BigA directly interacts with PalA we performed co-immunoprecipitation assays with total extracts of *B. abortus* strains co-expressing PalA-3xFlag and BigA-sfGFP. As can be observed in Figure 6C, BigA-sfGFP co-immunoprecipitated with PalA

demonstrating that they interact either directly or indirectly in the bacterium. In the case of BigB-sfGFP, we were not able to observe co-immunoprecipitation with PalA even though multiple attempts with different conditions of buffers and detergents were performed.

Discussion

Adhesion to tissues and host cells is the initial, and in many cases a bottleneck step, in the virulence process of nearly all pathogens. The capacity to adhere and eventually invade target cells can determine tissue tropism, virulence cycle as well as immune response and, as a consequence, resolution of the infection. In bacteria, adhesion is mediated, amongst other proteins, by adhesins, a heterogeneous group of outer membrane molecules that target a wide spectrum of proteins, carbohydrates and lipids in the plasma membrane of host cells (Patel et al., 2017). Successful interaction of bacteria via these adhesins paves the way for host colonization and pathogenesis.

While adhesion has been extensively studied in many bacteria, in *Brucella*, a zoonotic pathogen of animal and human health concern with an intracellular life cycle, not much has been conducted to understand how the bacterium adheres and invades. To date few adhesins in the *Brucella* genus have been identified: the SP41 and the autotransporters (BmaC, BtaE and BtaF) and almost none of their cellular targets have been identified (Castaneda-Roldan et al., 2006; Posadas et al., 2012; Ruiz-Ranwez, Posadas, Estein, et al., 2013; Ruiz-Ranwez, Posadas, Van der Henst, et al., 2013).

Our group has identified a genomic region (genes Bab1_2009 to Bab1_2012 in *B. abortus* 2308) with horizontally transmitted features and demonstrated that is involved in the adhesion to host cells (Czibener & Ugalde, 2012). This region encodes four open reading frames conserved in all *Brucella* species but none

of them have homology to known proteins. We have recently characterized one of these genes (*bigA*) and shown that it encodes an adhesin that targets the bacteria to the cell-cell junction membrane in confluent epithelia cell cultures (Czibener et al., 2016). BigA has an exposed bacterial immunoglobulin-like (BIg-like) domain present in many bacterial proteins whose function ranges from enzymes to pilli, fimbria and adhesins. It is proposed that these domains mainly mediate protein-protein interactions and that its wide distribution in nature is due to its energetically favorable folding (Bodelon, Palomino, & Fernandez, 2013). Several pilli and fimbria, that are central for the adhesion of the bacterium to different substrates, as well as the Intimin of enterohemorrhagic *Escherichia coli* or the Invasin A of *Yersinia enterocolitica* that mediate the invasion process of the pathogen have these BIg-like domains (Bodelon et al., 2013; Mikula, Kolodziejczyk, & Goldman, 2013).

Analysis of the other three genes present in the *Bab1_2009-Bab1_2012* genomic island showed that Bab1_2012 (BigB) also has a putative BIg-like domain although the homology is weaker than the domain present in BigA. Adhesion and invasion assays with deletion or overexpressing strains as well subcellular fractionation experiments indicated that BigB is also an adhesin located in the outer membrane of *B. abortus*.

Moreover, as with BigA (Czibener et al., 2016), treatment of HeLa cells with the recombinant purified BIg-like domain of BigB also resulted in significant changes in the cytoskeletal and focal adhesion structures strongly suggesting

that, as what we determined with BigA, BigB probably targets proteins in either cell-cell of cell-substrates interactions. These ligands are probably involved in cytoskeleton modifications that will subsequently trigger the entry of *Brucella* to host cells as it has been reported in other bacterial adhesins like CagL, an important adhesin of *Helicobacter pylori* involved in these types of rearrangements as well as inflammation (Backert, Tegtmeyer, & Fischer, 2015). The other two remaining open reading frames in the genomic island are *bab1_2010* and *bab1_2011* that code for putative proteins with no detectable homology or known domains. Deletion of *bab1_2011* (*palA*) resulted in a strain with a reduced adhesion capacity even though the protein showed a periplasmic localization, suggesting that it is not an adhesin *per se*. Cellular fractionation assays of BigA and BigB in the Δ*palA* mutant, as well as co-immunoprecipitation experiments, showed that PalA stabilizes both adhesins in the periplasm allowing translocation and the correct insertion in the outer membrane, probably avoiding their aggregation. It remains unclear how PalA mechanistically mediates this translocation/insertion. The simplest mechanism is that PalA could act as a chaperone, directly interacting with BigA and BigB, preventing the exposure of hydrophobic patches present in the transmembrane domains, thus avoiding aggregation during its translocation to the outer membrane. Previous studies in other Gram-negative bacteria demonstrated the presence of periplasmic chaperones like PapD and FimH, which are necessary for pili assembly (Soto & Hultgren, 1999). Alternatively, PalA could be part of a

periplasmic secretion system that mediates the translocation of both adhesins to its final localization in the outer membrane. In this hypothesis, absence of PalA will probably impair secretion and the adhesins would remain periplasmic, as we have observed.

Despite the function of PalA, BigA and BigB have to be translocated to the outer membrane to be functional and to achieve this, a specific mechanism has to be necessarily involved. One of the most studied mechanisms for the export of adhesins are the autotransporters, like the *E. coli* intimin and the invasin (Inv) and YadA from *Yersinia enterocolitica* (Leibiger, Schweers, & Schutz, 2019). In these autotransporters translocation occurs by a two-step mechanism mediated by different domains present in the adhesins. First they are targeted to the periplasm by a canonical signal peptide and, once in this compartment, they use a passenger domain that interacts with the peptidoglycan to be finally inserted in the outer membrane through a beta barrel domain (Leibiger et al., 2019; Leo, Oberhettinger, Schutz, & Linke, 2015). In these cases there are additional proteins that act as chaperones and/or insertion complexes that help to translocate these adhesins to the outer membrane (Lazar & Kolter, 1996; Leibiger et al., 2019; Sklar, Wu, Gronenberg, et al., 2007; Sklar, Wu, Kahne, & Silhavy, 2007). BigA and BigB do not seem to belong to these families of autotransporters as they don´t have the domain structure typical of these proteins. Even though we cannot discard that BigA and BigB use a similar

mechanism to be transported to the outer membrane it is highly probable they are translocated in a yet unknown way.

The outcome of the interaction between *Brucella* and its host cells relies on the first interaction of the bacterium with the plasmatic membrane of the target cell. In this report, we further characterized the Bab1_2009-Bab1_2012 genomic island, and found that, not only encodes two adhesins, but it also regulates, through PalA, the correct display of them in the outer membrane. Further work will be required to establish how PalA achieves this, how BigB interacts with the host cell as well as the identification of the receptors for both adhesins.

Figure legends

Figure 1. Bab1_2012 is involved in the adhesion to non-phagocytic cells.

A. Intracellular replication curve of *B. abortus* 2308 (wild type), *B. abortus* Δ*bab1_2012* and complemented (Δ*bab1_2012*(Bab1_2012)) strains in HeLa cells (MOI 1:500). **** *P<0.0001*, two-way ANOVA, Tukey's multiple comparisons test. **B.** Intracellular survival of *B. abortus* 2308 (wild type), *B. abortus* Δ*bab1_2012* and complemented (Δ*bab1_2012*(Bab1_2012)) strains in MDCK cells at 4 hrs post-infection (MOI 1:2000). **** *P<0.0001*, one-way ANOVA, Bartlets test. **C.** Invasion assay with *B. abortus* 2308 (wild type), *B. abortus* Δ*bab1_2012* and complemented (Δ*bab1_2012*(Bab1_2012)) strains in HeLa cells at 4 hrs post-infection (MOI 1:1000). * *P<0.01,* two-way ANOVA, Tukey's multiple comparisons test. **D.** Adhesion assay with *B. abortus* 2308 (wild type), *B. abortus* Δ*bab1_2012* and complemented (Δ*bab1_2012*(Bab1_2012)) strains in HeLa cells at 4 hrs post-infection (MOI 1:1000). * *P<0.01,* unpaired T-test.

All assays were performed at least three times.

Figure 2. Bab1_2012 localizes in the outer membrane. A. Western blot analysis of total membranes or soluble fraction (supernatant) prepared with the *B. abortus* 2308 (pBBR-Bab1_2012-3xFlag) strain. **B.** Western-blot analysis of the subcellular localization of Bab1_2012-3xFlag fusion performed with the *B. abortus* 2308 (pBBR-Bab1_2012-3xFlag) strain. Perip. + OM, periplasm plus

outer membrane. Bab1_2012, FLAG staining; Omp19, outer membrane protein 19; GroEL, cytoplasmic chaperonin. **C.** Immunofluorescence microscopy of non-permeabilized cells of *B. abortus* 2308 (pBBR-BigB-sfGFP) and *B. abortus* 2308 *Δbab1_2012* with a polyclonal mouse anti Ig-Bab1_2012.

Figure 3. BigB induces cytoskeletal rearrangements in treated HeLa cells.
A. Images of HeLa cells treated with 100 μg of purified recombinant BigB bacterial-immunoglobulin like domain for 4 hrs fixed and stained with phalloidin (red). Non-treated; incubated with BSA. **B.** Cell height of HeLa cells treated with the recombinant bacterial-immunoglobulin like domains of BigA and BigB, individually and in combination. **** *P<0.0001*, two-way ANOVA Tukey's Test. Non-treated; incubated with BSA. Right image is to show how the Z-stack was measured. **C.** Confocal images (60x) of HeLa cells transfected with plasmids that express mRFP Paxillin or DS-red Zyxin (both in red) and treated over-night with 0.1 mg/ml of recombinant BigB bacterial-immunoglobulin like domain. Cells were stained with phalloidin (white). Mock: 0.1 mg/ml of heat-denaturalized recombinant BigB. Scale bar: 10 μm.

Figure 4. Bab1_2011 is a periplasmic protein involved in the adhesion of *Brucella* to non-professional phagocytes. A. Intracellular replication curve of *B. abortus* 2308 (wild type), *B. abortus Δbab1_2011* and complemented (*Δbab1_2011*(Bab1_2011)) strains in HeLa cells (MOI 1:500). *** *P<0.001*, two-

way ANOVA, Tukey's multiple comparison test**. B.** Adhesion assay with *B. abortus* 2308 (wild type), *B. abortus* Δ*bab1_2011* and complemented (Δ*bab1_2011*(Bab1_2011)) strains in HeLa cells at 4 hrs post-infection (MOI 1:1000). ** *P<0.01* **** *P<0.0001*. **C.** Invasion assay with *B. abortus* 2308 (wild type), *B. abortus* Δ*bab1_2011* and complemented (Δ*bab1_2011*(Bab1_2011)) strains in HeLa cells at 4 hrs post-infection (MOI 1:1000). *** *P<0.001*. **D.** Western blot analysis of total membranes or soluble fraction (supernatant) prepared with the *B. abortus* 2308 (pBBR-Bab1_2011-3xFlag) strain. **E.** Western-blot analysis of the subcellular localization of Bab1_2011-3xFlag fusion performed with the *B. abortus* 2308 (pBBR-Bab1_2011-3xFlag) strain. Perip. + OM, periplasm plus outer membrane. Bab1_2011, FLAG staining; Omp19, outer membrane protein 19; GroEL, cytoplasmic chaperonin.

Figure 5. Deletion of Bab1_2011 affects the localization of BigA and BigB in the outer membrane but does not affect their translocation to the periplasm. A. Western blot analysis with an anti-Flag antibody of total extracts of strains 2308 (BigA-3xFlag) and Δ*bab1_2011* (BigA-3xFlag). **B.** Western blot analysis with an anti-Flag antibody of total membranes or soluble fraction (supernatant) prepared with the Δ*bab1_2011* (BigA-3xFlag) strain. **C.** Western blot analysis with an anti-Flag antibody of total membranes or soluble fraction (supernatant) prepared with the Δ*bab1_2011* (BigB-3xFlag) strain. **D.** Western-blot analysis of the subcellular localization of BigA-3xFlag fusion performed with

the Δ*bab1_2011* (BigA-3xFlag) strain (Δ*palA* (BigA-3xFlag). **E.** Western-blot analysis of the subcellular localization of BigB-3xFlag fusion performed with the Δ*bab1_2011* (BigB-3xFlag) strain (Δ*palA* (BigB-3xFlag). Omp19, outer membrane protein 19; Omp2b, outer membrane protein 2b; GroEL, cytoplasmic chaperonin. Perip. + OM, periplasm plus outer membrane.

Figure 6. PalA and BigA interact directly or indirectly. A and B. Immunofluorescence and DIC images of the wild type and Δ*palA* mutant strains expressing a BigA-super-folder GFP fusion protein (BigA-sfGFP) showing how the fusion shows a bi-polar localization. **C.** Co-immunoprecipitation (Co-IP) analysis of protein extracts from the *B. abortus* 2308 strain co-expressing PalA-3xFlag and BigA-sfGFP. As a negative control a *B. abortus* 2308 strain expressing only the BigA-sfGFP was used. Immunoprecipitation was performed with an anti-Flag monoclonal antibody coupled to sepharose and the immunoprecipitates analyzed by western blot with an anti-GFP antibody. Input were the crude extracts prior to the immunoprecipitation.

Supplementary Figure 1. Intracellular replication curve of *B. abortus* 2308 (wild type), *B. abortus* Δ*bab1_2012* and complemented (Δ*bab1_2012*(Bab1_2012)) strains in J774 A.1 cells (MOI 1:500). **** *P<0.0001,* ** *P<0.01,* two-way ANOVA, Tukey's multiple comparison test**.**

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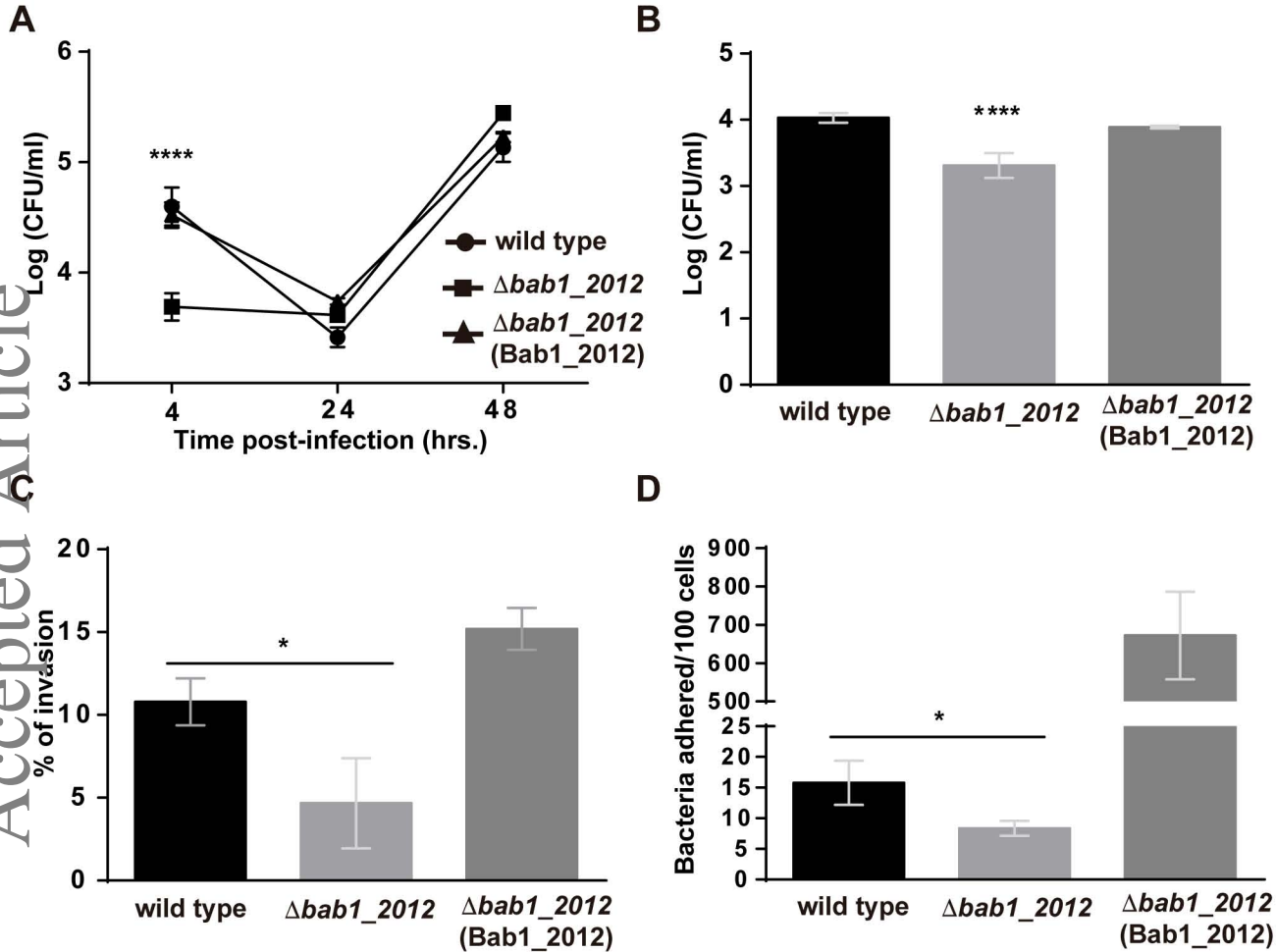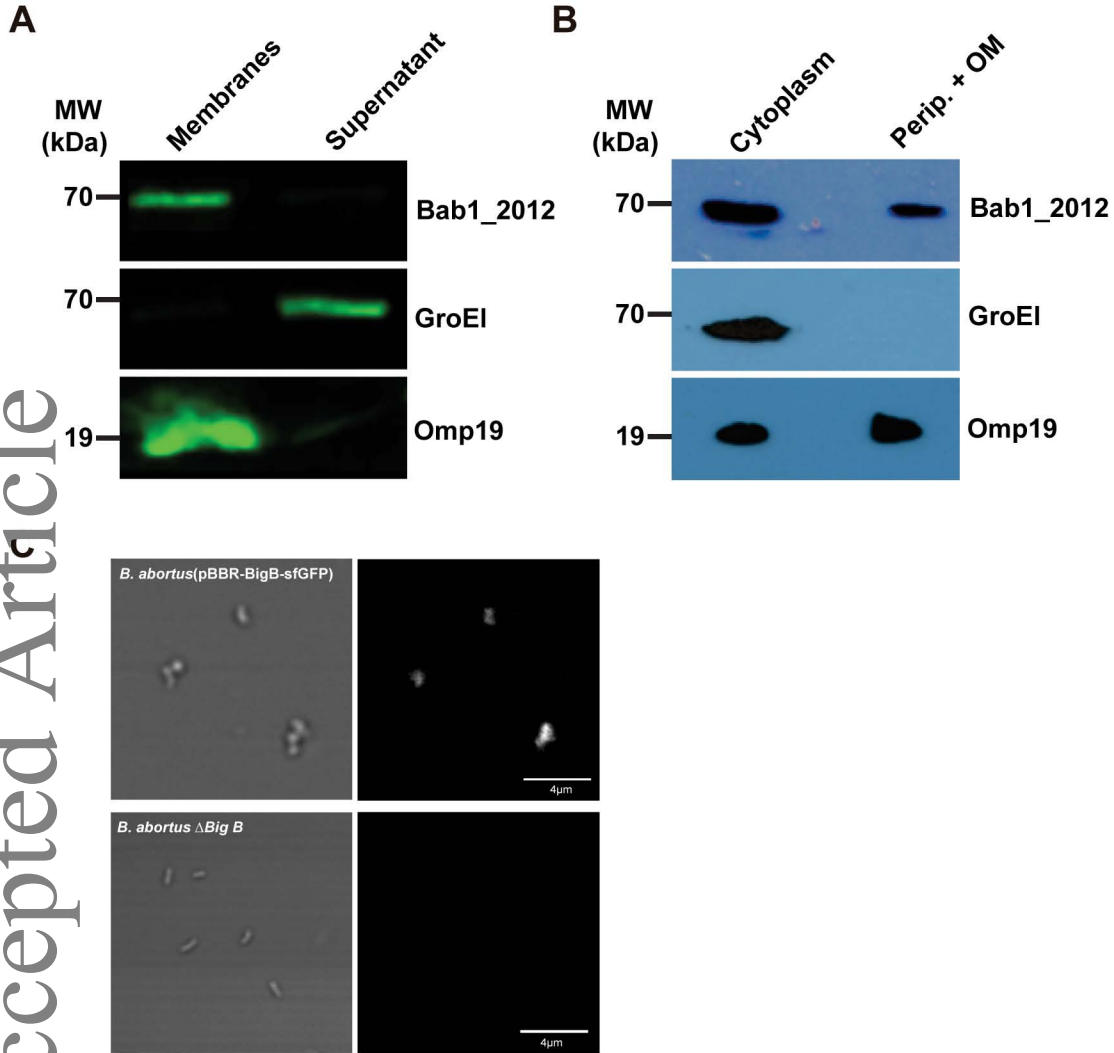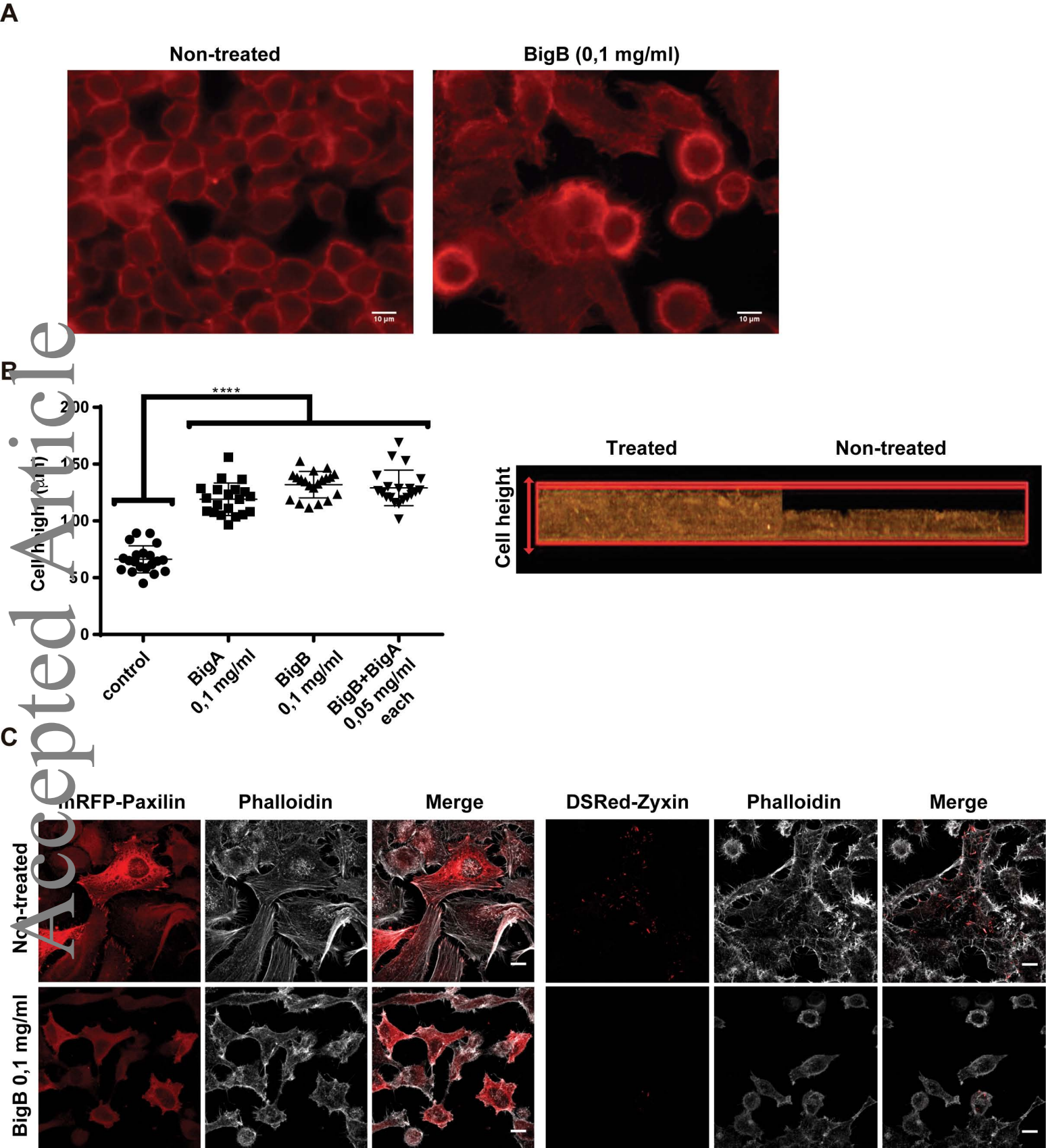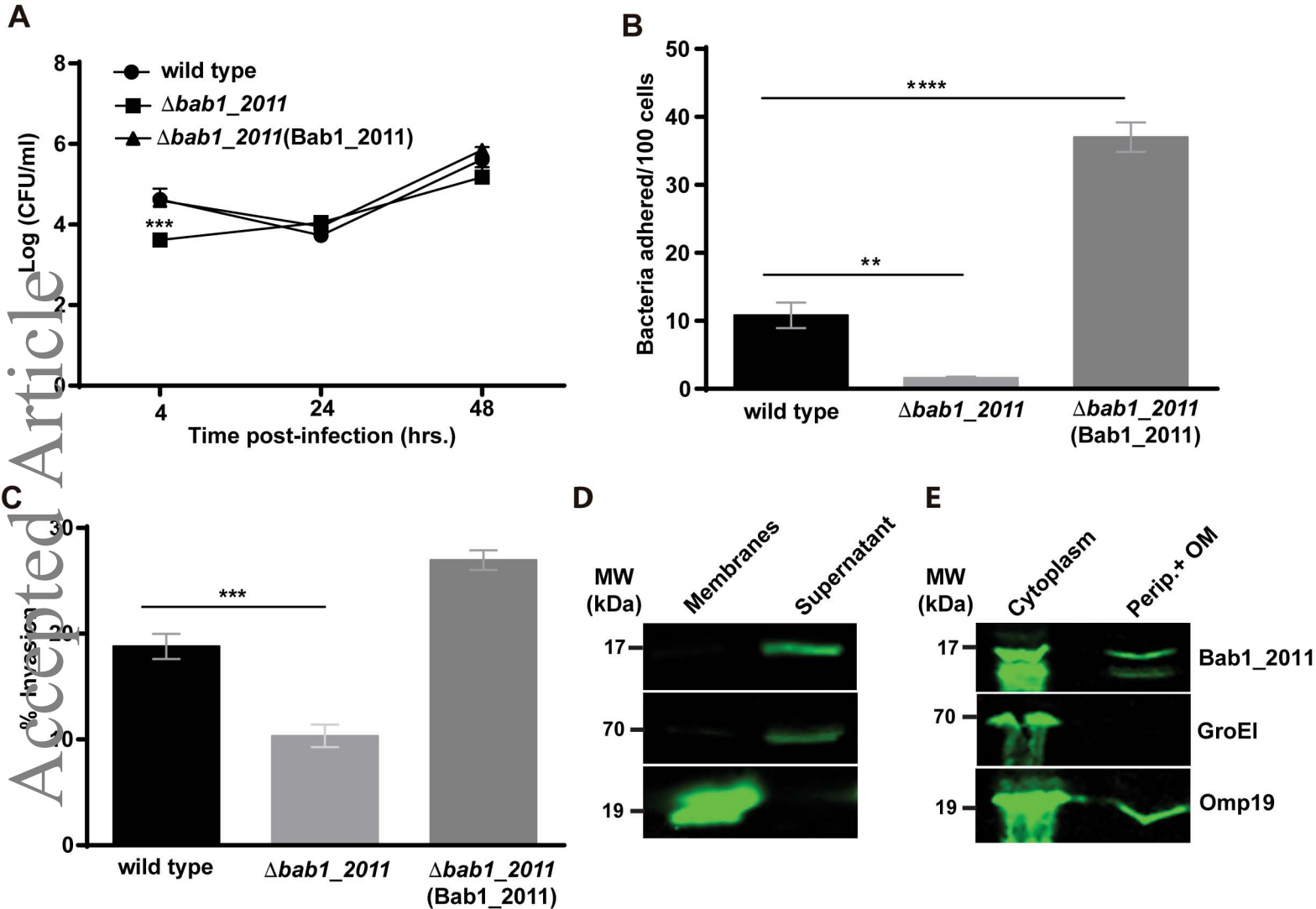
Figure 1

A

MW (kDa) | Membranes | Supernatant
70 — Bab1_2012
70 — GroEl
19 — Omp19

B

MW (kDa) | Cytoplasm | Perip. + OM
70 — Bab1_2012
70 — GroEl
19 — Omp19

B. abortus(pBBR-BigB-sfGFP)

4μm

B. abortus Δ*Big B*

4μm

Figure 2

Figure 3

Figure 4

A

MW (kDa)

wild type (BigA-3xFlag)   Δ*bab1_2011* (BigA-3xFlag)

70 —

40 —

α-Flag

B

MW (kDa)

Membranes   Supernatant

70 —   Δ*bab1_2011* (BigA-3xFlag)

70 —   GroEL

19 —   Omp19

C

MW (kDa)

Membranes   Supernatant

70 —   Δ*bab1_2011* (BigB-3xFlag)

70 —   GroEL

40 —   Omp2B

D

MW (kDa)

Cytoplasm   Perip.+OM

70 —   Δ*palA* (BigA-3xFlag)

70 —   GroEL

40 —   Omp2B

E

MW (kDa)

Cytoplasm   Perip.+OM

70 —   Δ*palA* (BigB-3xFlag)

70 —   GroEL

40 —   Omp2B

Figure 5

A

wild type (BigA-sfGFP)

1 µm

B

Δ*palA* (BigA-sfGFP)

1 µm

C

MW (kDa)	wild type BigA-sfGFP	wild type BigA-sfGFP PalA-3xFlag	
IP α-Flag WB: αGFP 70			◄ BigA sfGFP
WB: α-Flag 17			◄ PalA-3xFlag
Input WB: αGFP 70			◄ BigA sfGFP

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Figure 6