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BigA is a novel adhesin of *Brucella* that mediates adhesion to epithelial cells

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Summary

Adhesion to cells is the initial step in the infectious cycle of basically all pathogenic bacteria, and to do so, microorganisms have evolved surface molecules that target different cellular receptors. *Brucella* is an intracellular pathogen that infects a wide range of mammals whose virulence is completely dependent on the capacity to replicate in phagocytes. Although much has been done to elucidate how *Brucella* multiplies in macrophages, we still do not understand how bacteria invade epithelial cells to perform a replicative cycle or what adhesion molecules are involved in the process. We report the identification in *Brucella abortus* of a novel adhesin that harbours a bacterial immunoglobulin-like domain and demonstrate that this protein is involved in the adhesion to polarized epithelial cells such as the Caco-2 and Madin–Darby canine kidney models targeting the bacteria to the cell–cell interaction membrane. While deletion of the gene significantly reduced adhesion, over-expression dramatically increased it. Addition of the recombinant protein to cells induced cytoskeleton rearrangements and showed that this adhesin targets proteins of the cell–cell interaction membrane in confluent cultures.

Introduction

Pathogenic bacteria have evolved a wide range of strategies to escape host defence mechanisms and create a safe, in many cases intracellular, replicative niche. For many pathogens, these strategies involve escaping the innate and adaptive components of the immune system. Epithelial tissues impose a physical barrier and are one of the first defence strategies pathogens encounter during infection which must be successfully trespassed in order to progress in the infectious process (Kim *et al*., 2010). In order to establish a systemic infection, pathogens must be able to invade these tissues inflicting the minimal damage possible during the process. To do this microorganisms have evolved many mechanisms devoted to modulate different cellular activities that lead to either a transient colonization of these cells as an intermediate step or to 'relax' the tight junctions that maintain the integrity of the epithelium facilitating a paracellular translocation (Kim *et al*., 2010; Barreau and Hugot, 2014). For colonization of certain epithelium barriers (for example the intestinal one) bacterial pathogens have evolved two major strategies. Internalization by a 'zippering' process involves a surface bacterial protein binding with high affinity to adherence molecules of the mammalian cell surface that ultimately ends in endocytosis. The 'trigger' process is exploited by pathogens that induce massive cytoskeletal changes at the site of contact causing membrane ruffling and promoting the internalization of the bacteria into a micropinocytic vacuole (Sansonetti, 2002). *Yersinia pseudotuberculosis* is a paradigm of zippering entry requiring Inv, an outer membrane protein that interacts with β1 integrins (α3β1, α4β1, α5β1 and αVβ1), which are involved in adherence of epithelial cells to the extracellular matrix (Isberg and Leong, 1990; Sansonetti, 2002; Mikula *et al*., 2013). Inv is able to oligomerize and bind β1 integrins with an affinity constant much higher than fibronectin. This high affinity binding may account for the transition between adherence and internalization, as the cytoplasmic domain of β1 integrins transmits signals to the cytoskeleton that mediate rearrangements and internalization (Sansonetti, 2002). A canonical example of a bacteria that uses the trigger process is *Salmonella* spp., which promotes its own entry in epithelial cells by means of a type III secretion system, inducing a robust and transient membrane ruffling that ends in the internalization of the bacteria into vesicles that ultimately evade fusion with lysosomes promoting intracellular replication (Patel and Galan, 2005).

Brucella spp. are Gram-negative facultative intracellular bacteria that cause brucellosis, a worldwide-distributed zoonosis that affects a broad range of mammals, ranging

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from domestic and wild animals to humans. Brucellosis remains endemic in many developing countries, where it causes important economic losses (Corbel, 1997). In humans, brucellosis is a serious debilitating disease and infection depends on contact with infected animals or their products by direct ingestion of the bacteria or exposure to aerosols through which the pathogen accesses an epithelium. After the initial phase of the infection bacteria survive within mononuclear phagocytes, and the infected monocytes (or macrophages) play a crucial role in the dissemination of the bacteria to specific locations of the body (spleen, brain, heart and bones) leading in many cases to a chronic infection (von Bargen *et al*., 2012). Most of the work aiming at understanding the pathogenesis of *Brucella* has focused on elucidating the molecular mechanisms that enable intracellular replication in professional phagocytes, but few has been done to characterize the initial interaction of the bacteria with *bonafide* epithelial cells, most probably the first line of defence it encounters during the infectious process. To date, few reports have described the interaction of *Brucella* with polarized epithelial cells, and the only manuscript was mainly focused on the inflammatory response but not on the molecular determinants that mediate attachment and invasion (Ferrero *et al*., 2012).

Adhesion to host cells is the first step in the virulence process of many pathogens. Adhesins are widely distributed in microbial pathogens, and they bind a wide range of surface molecules depending on the life style and cells they target during the infectious cycle. In *Brucella* several adhesins have been described to date but few have been tested in a *bonafide* epithelial cell model. SP41, the first adhesin identified in *Brucella*, was demonstrated to be involved in adhesion and invasion of epithelial-like cells (HeLa) by antibody inhibition and infection assays using null mutants (Castaneda-Roldan *et al*., 2006). The gene coding for SP41 is the *ugpB*, and codes for a periplasmic glycerol-3-phosphate binding ATP-binding cassette transporter present in several pathogenic and non-pathogenic bacteria and localizes to the outer membrane. Recently, several proteins have been identified as novel adhesins (Posadas *et al*., 2012; Ruiz-Ranwez *et al*., 2013a; Ruiz-Ranwez *et al*., 2013b). In a series of papers the authors identified three autotransporters (BmaC, BtaE and BtaF) and demonstrated that they are important for binding to HeLa and A549 cells as well to several extracellular matrix proteins like fibronectin and hyaluronic acid.

We have recently identified a region in *Brucella abortus* that codes for four open reading frames with no known homologues and conserved in all *Brucella* species (Czibener and Ugalde, 2012). Deletion of this 5 kb pair region significantly affects adhesion and invasion of host cells, indicating that it encodes an adhesin. Interestingly, one of the proteins encoded by a gene in this region has a domain with significant homology to bacterial immunoglobulin-like (BIg-like) domains present in some adhesins (Czibener and Ugalde, 2012; Bodelon *et al*., 2013). In the present work we have advanced in the characterization on this gene and demonstrate that it codes for an adhesin that mediates attachment and invasion of host cells. Moreover, we demonstrate that this protein targets the bacteria to the cell–cell junction membrane in Madin–Darby canine kidney (MDCK) and Caco-2 cells and that *Brucella* performs a complete replicative cycle in MDCK cells modulating the actin cytoskeleton during the different phases of the intracellular replication process.

Results

Deletion of Bab1_2009 *affects adhesion to and invasion of HeLa cells*

We have recently identified a region in chromosome 1 of *B. abortus*, conserved in all *B.* species, that is involved in the adhesion and invasion of the bacteria to host cells (Czibener and Ugalde, 2012). This 5 kb pair region encodes four open reading frames with no homologues described in the literature and present only in the genus *Brucella* (Fig. S1). One of the genes present in this region (*Bab1_2009*) codes for a protein with a domain homologous to BIg-like domains present in several adhesins such as the Intimin of enterohemorrhagic *Escherichia coli* strains (Czibener and Ugalde, 2012) (Fig. S1). In order to explore the role *Bab1_2009* might play in the pathogenesis of *Brucella* we generated a deletion mutant strain in the gene and evaluated its intracellular replication capacity in HeLa cells. As can be observed in Fig. 1A, deletion of the gene significantly affected the early stages of the intracellular replication cycle although the mutant replicated with the same kinetics during the latter times points (24 and 48 h). Moreover, expression of the gene from a multicopy plasmid significantly increased the number of intracellular bacteria recovered at all times post-infection tested (Fig. 1B). The reduction in the intracellular bacteria observed in the mutant, as well as the increased number recovered in the over-expressing strain, particularly at 4 h post-infection, prompted us to evaluate if these strains had an altered adhesion and/or invasion capacity. To determine these parameters, HeLa cells were infected with the wild type, mutant and over-expressing strains, and at different times post-infection, the cells were extensively washed, fixed and the bacteria stained with an anti O-antigen monoclonal antibody. To quantify adhesion we determined, by immunofluorescence, the number of bacteria associated per 100 cells. On the other hand, invasion was determined performing an in/out staining (refer to Experimental procedures section) and quantifying the number of intracellular/extracellular *Brucella* per 100

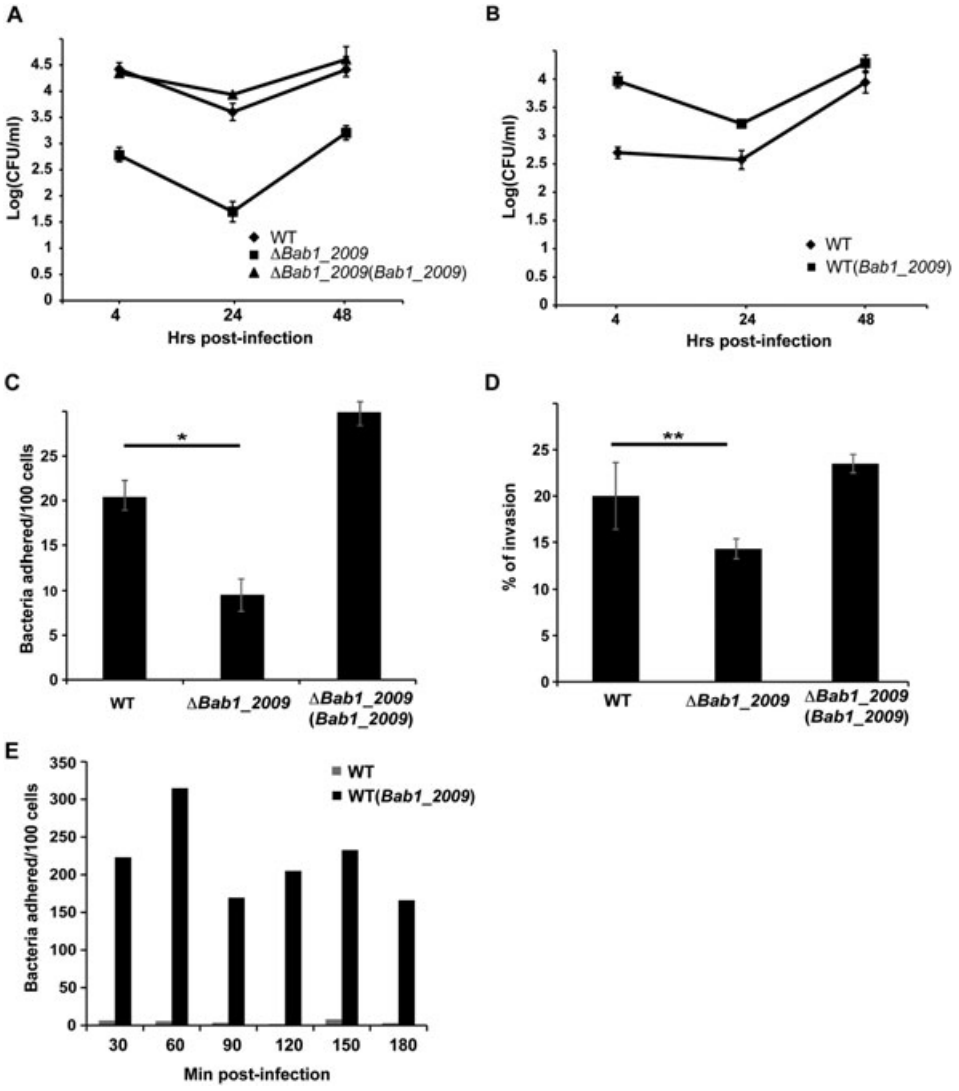

Fig. 1. *Bab1_2009* is involved in the adhesion to host cells.
A. Intracellular replication curve of *B. abortus* 2308, *B. abortus* Δ*Bab1_2009* and complemented strains in HeLa cells (MOI 1:500).
B. Intracellular replication curve of *B. abortus* 2308 and *B. abortus* 2308 (pBBR-*Bab1_2009*-3xFlag) strains in HeLa cells (MOI 1:500).
C, D. Adhesion (C) and invasion (D) assays of *B. abortus* 2308, *B. abortus* Δ*Bab1_2009* and complemented strains in HeLa cells at 4 hrs post-infection. $^{*}P < 0.0005$, $^{**}P < 0.001$ (MOI 1:1000). E. Adhesion of the *B. abortus* 2308 (pBBR-*Bab1_2009*-3xFlag) strain in HeLa cells (MOI 1:50). All assays were performed at least three times.

bacteria. As shown in Fig. 1 C and D, the mutant strain displayed a significant defect in both the adhesion and the invasion of HeLa cells, phenotype that was complemented by the expression *in trans* of the gene. Interestingly, the wild-type strain over-expressing the product of *Bab1_2009* showed a dramatic increase in its adhesion capacity (Fig. 1E).

Bab1_2009 *localizes in the bacterial outer membrane*

Results of the adhesion assays with the deletion mutant suggested that *Bab1_2009* could code for an adhesin. In order to determine the sub-cellular localization of *Bab1_2009* we first tested if the protein is present in the bacterial membrane. For this we performed a total membrane preparation (refer to Experimental procedures section) with the *B. abortus* strain expressing a 3xFLAG tagged version of the gene and determined presence of the protein in the membrane or supernatant by Western blot. Fig. 2A shows that the protein was found exclusively in the membrane fraction indicating that it is membrane associated. Moreover, sub-cellular fractionation experiments with the same strain demonstrated that the protein has periplasmic or outer membrane localization (Fig. 2B). This result, together with the fact that it is a membrane protein, indicated that *Bab1_2009* localizes in the outer membrane. To confirm this we developed a polyclonal antibody against the Ig-like domain of the protein (refer to Experimental procedures section and in the succeeding texts for additional information) and performed an immunofluorescence with the *B. abortus Bab1_2009* over-expressing and the deletion mutant strains. As can be observed in Fig. 2C, the protein was found in the bacterial surface in discrete spots confirming that *Bab1_2009* is an exposed outer membrane protein. To determine if the surface exposition is necessary for the function of *Bab1_2009* we performed a gentamicin protection assay to determine invasion, at 4 h post-infection, prior incubation of the bacteria with the anti-Ig-like domain antibody.

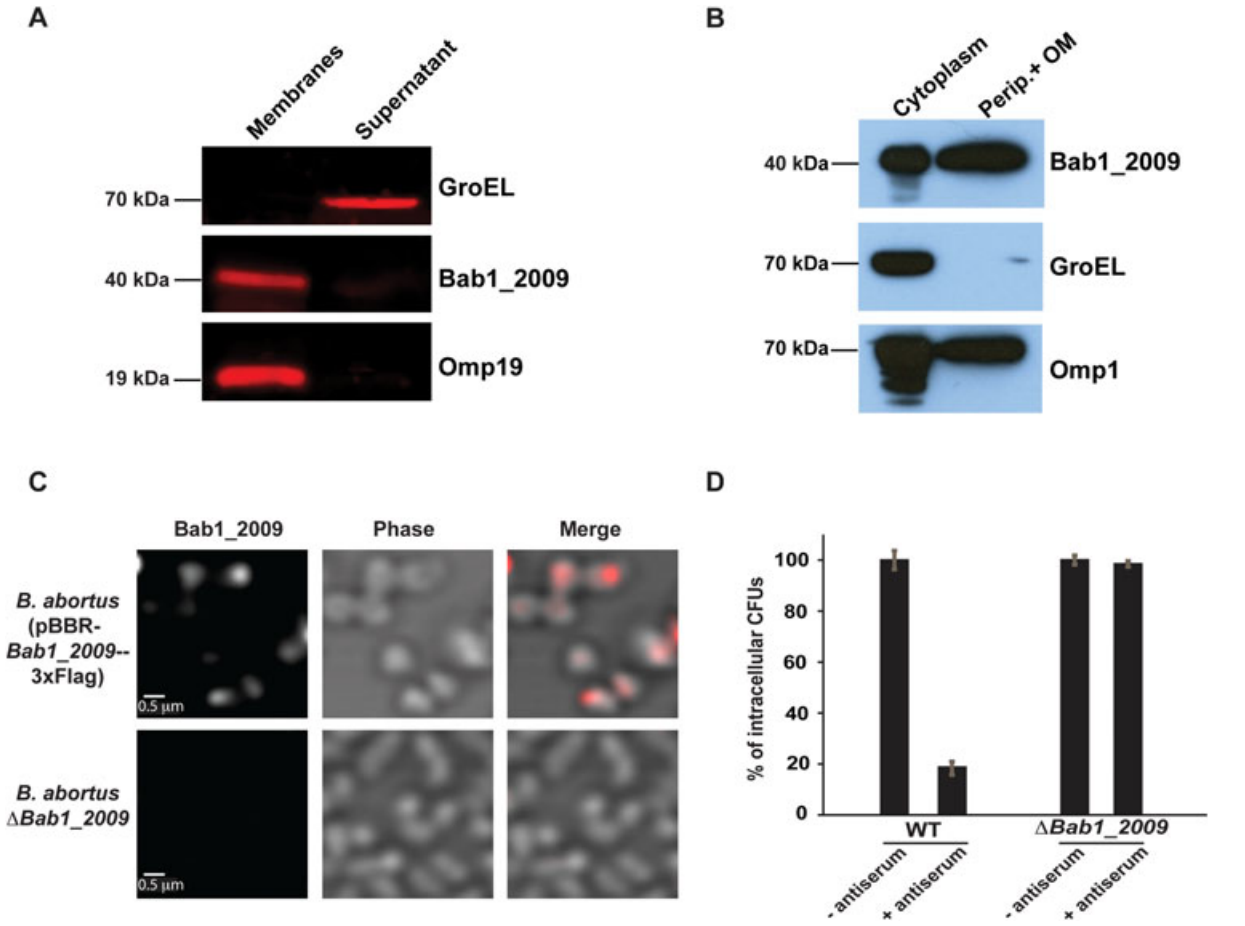

Fig. 2. *Bab1_2009* localizes in the outer membrane.
A. Western blot analysis of total membranes or supernatants prepared with the *B. abortus* 2308 (pBBR-Bab1_2009-3xFlag) strain. Bab1_2009, FLAG staining; Omp19, outer membrane protein 19; GroEL, cytoplasmic chaperonin.
B. Western blot analysis of the sub-cellular localization of Bab1_2009-3xFlag fusion performed with the *B. abortus* 2308 (pBBR-Bab1_2009-3xFlag) strain. Perip. + OM, periplasm plus outer membrane. Bab1_2009, FLAG staining; Omp1, outer membrane protein 1; GroEL, cytoplasmic chaperonin.
C. Confocal images of *B. abortus* 2308 (pBBR-Bab1_2009-3xFlag) and *B. abortus* Δ*Bab1_2009* stained with an antiserum anti-BIg-like domain of *Bab1_2009*. Red, anti-BIg-like staining.
D. Gentamicin protection assay with HeLa cells at 4 h post-infection performed with the wild type and the Δ*Bab1_2009* deletion strains prior incubation of the bacteria with the anti-BIg-like antiserum.

As can be observed in Fig. 2D, pre-incubation of the bacteria with the antiserum significantly decreased the number of intracellular bacteria in the wild-type strain but had no effect on the *Bab1_2009* mutant strain, indicating that the protein function is dependent on the outer membrane localization.

Altogether, these results indicate that *Bab1_2009* codes for an adhesin that mediates attachment/adhesion to host cells and that it is localized in the outer membrane of the bacteria. Consequently, we renamed this gene *bigA* for BIg gene A.

Brucella *in Caco-2 and MDCK cells*

Most of the works aiming at understanding the interaction between *Brucella* and the host cells have been performed on macrophages or non-professional phagocytes such as the epithelioid cell line HeLa, but very little has been done with *bonafide* epithelial cells. For these reasons, and the fact that the first cell type *Brucella* encounters during the infectious process are probably in the respiratory or the oral/gastric mucosa (Tsolis *et al*., 2008; Gorvel *et al*., 2009), we decided to explore in more detail the interaction of *Brucella* with two epithelial cell models: the human epithelial colorectal adenocarcinoma (Caco-2) and the MDCK cell lines (Gaush *et al*., 1966; Chantret *et al*., 1988). Because these cells have the ability, when reaching confluence, of forming epithelium-like structures, we focused on the initial interaction of *Brucella* with these cells, particularly attempting to identify the sites of adhesion and invasion of the bacteria in a confluent monolayer. Because the *B. abortus* Δ*bigA* mutant showed a significant difference in the intracellular CFUs compared

with the parental wild-type strain during the initial phases of the intracellular cycle in HeLa cells, we initially measured the number of viable bacteria at 4 h post-infection in Caco-2 and MDCK cells by an antibiotic protection assay. As can be observed in Fig. 3 A and B, *B. abortus* was able to invade both cell lines and the mutant showed a significant decrease in its intracellular survival (between tenfold and 50-fold depending on the cell type). Moreover, adhesion assays in MDCK cells showed that this difference was because of a decrease in its adhesion capacity (Fig. 3C), while the over-expressing strain (*B. abortus bigA*) showed a dramatic increase in its adhesion (Fig. 3 D and E), demonstrating that *bigA* also plays a role in the adhesion to these cells. It is important to point out here that in the images shown the number of bacteria in the wild-type strain, even though lower with respect to the over-expressing strain, does not seem to be so dramatically less as in the quantification panel. The reason of this apparent difference is that while to detect a cell with wild-type bacteria attached we had to search several fields, in the case of the over-expressing strain every field analysed had a large number of bacteria. Similar results were observed with Caco-2 cells (not shown).

When performing the adhesion analysis in Caco-2 and MDCK cells with the *B. abortus bigA* over-expressing strain, we observed that a significant number of bacteria were found associated with the cell–cell interaction membrane as opposed to the rest of the cell body plasmatic membrane. To quantify if this was the case we set up the following experimental approach. We infected confluent Caco-2 or MDCK cells with the *bigA* over-expressing *B. abortus* strain, and at different times post-infection, the cells were fixed and stained to detect *Brucella* with an anti-O-antigen monoclonal antibody and the cortical actin of the cells with phalloidin. Each preparation was analysed by confocal microscopy quantifying the number of bacteria associated with the cell–cell interaction membrane or with the rest of the cell body. To achieve this, once the images were taken they were processed and a mask was generated around the membranes involved in the interaction between cells. With this mask set up we quantified the number of bacteria in contact with it, or in contact with the rest of the cell body. For a reference we calculated the percentage of the area occupied by the mask and the one occupied by the rest of the cell body in each image. A bacterium was considered in contact with the cell–cell interface if it was in contact with this mask. If a random distribution of the bacteria occurs during the initial interaction with the cells, the ratio

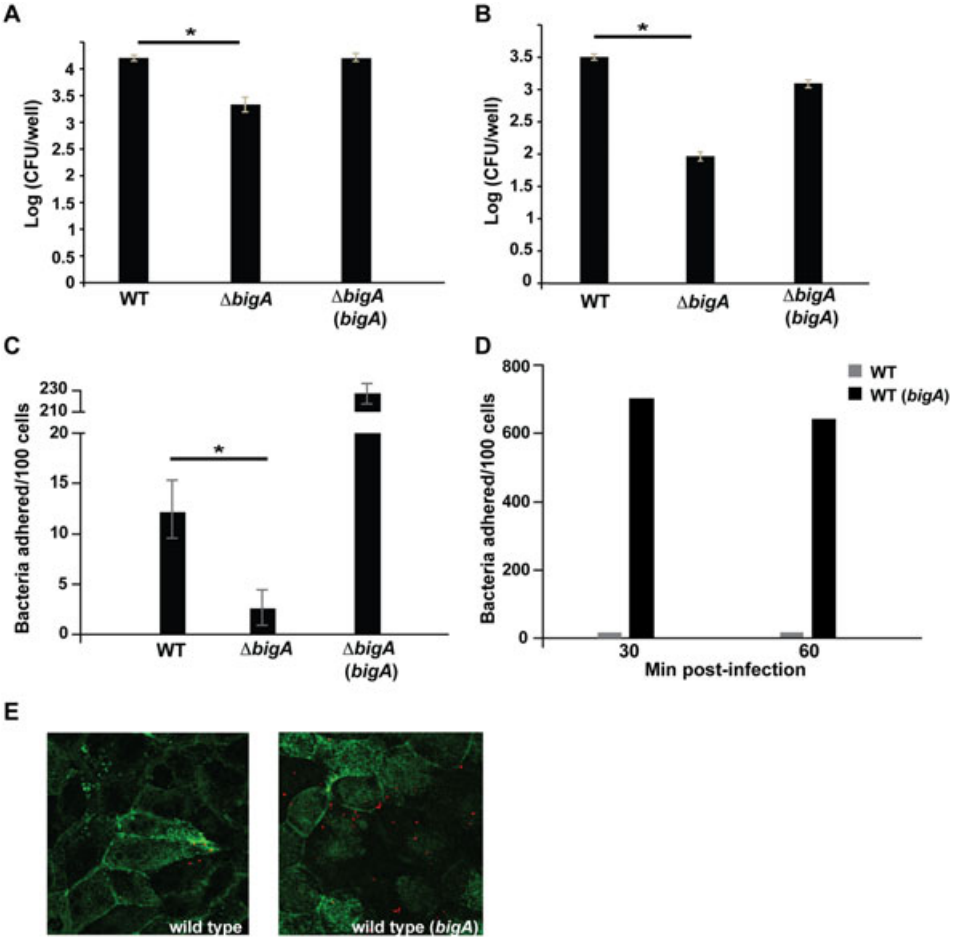

Fig. 3. BigA is involved in the adhesion to and invasion of MDCK cells.
A. Intracellular survival of *B. abortus* 2308, *B. abortus* Δ*bigA* and complemented strains in Caco-2 cells at 4 h post-infection. $^*P < 0.05$ (MOI 1:500).
B. Intracellular survival of *B. abortus* 2308, *B. abortus* Δ*bigA* and complemented strains in MDCK cells at 4 h post-infection. $^*P < 0.005$ (MOI 1:500). Assays were performed at least three times.
C. Adhesion assay of the *B. abortus* Δ*bigA* strain to MDCK cells at 2 h post-infection. $^*P < 0.0005$ (MOI 1:1000).
D. Adhesion assay of the *B. abortus* 2308 (pBBR-BigA3xFlag) strain to MDCK cells at 2 h post-infection (MOI 1:50).
E. Representative images of the quantification of panel D. Red, *Brucella*; green, phalloidin.

between the percent of bacteria versus the percent of the area of either the cell–cell membrane or the rest of the cells body should be close to 1, any deviation from this ratio implies a non-random distribution. Fig. 4 shows that in both cells types and at all times tested, the ratio was higher than 1 in the cell–cell junction membrane and lower in the rest of the plasmatic membrane (cell body), indicating that the bacteria preferentially binds to the membranes involved in the cell–cell interaction. To confirm that this phenomenon was also observed in the wild-type 2308 *B. abortus* strain we infected confluent MDCK cells, and at 2 h post-infection, fixed the cells and stained the bacteria and actin as indicated in the preceding texts. As can be observed in Fig. S2, *B. abortus* also showed a cell–cell junction membrane association. It is important to note that while in the over-expressing strain almost every filed analysed in the microscope had bacteria associated with the cells, in the case of the wild type strain this was not the case and we had to search several fields to detect attached bacteria.

The observation that *Brucella* is able to invade MDCK cells prompted us to evaluate the complete intracellular cycle by immunofluorescence microscopy. For this we used a GFP-live-actin MDCK cell line that stably expresses a GFP-tag that binds to F-actin (microfilaments) (Riedl *et al*., 2008), infected it with the over-expressing *bigA B. abortus* strain (*B. abortus bigA*) because of its higher infectivity and fixed the samples at 4, 24 and 48 h post-infection for confocal analysis. Fig. 5 shows representative images of each time point post-infection using these cells. As shown in the preceding texts, at 4 h post-infection, many bacteria were found in close proximity to the cell–cell junction membrane and were found associated with F-actin either as a phagocytic cup or completely surrounded by it (Fig. 5 A and B). To further confirm this membrane association we infected a MDCK cell line stably transfected with a plasmatic membrane marker fused to GFP (Ac-GFP1-Mem) (Dragoi and Agaisse, 2014) (see Experimental procedures section) and analysed localization of the bacteria at 4 h post-infection. For this assay we additionally stained the cells with an anti-P58 antibody that stains the basolateral membranes of the polarized MDCK cells (Kierbel *et al*., 2007) that indicated that the cells were polarized (Fig. S3). The images show that the p58 marker is clearly located at the basolateral plasmatic membrane, indicating that the conditions used in these assays induce polarization of the cells. As shown in the preceding texts, and confirmed in Fig. 5C, we found many bacteria associated with the plasmatic membrane during the initial stages of the intracellular cycle (4 h post-infection), and we were able to find many bacteria showing the plasmatic membrane surrounding them (arrow in Fig. 5D). Additionally, a p58

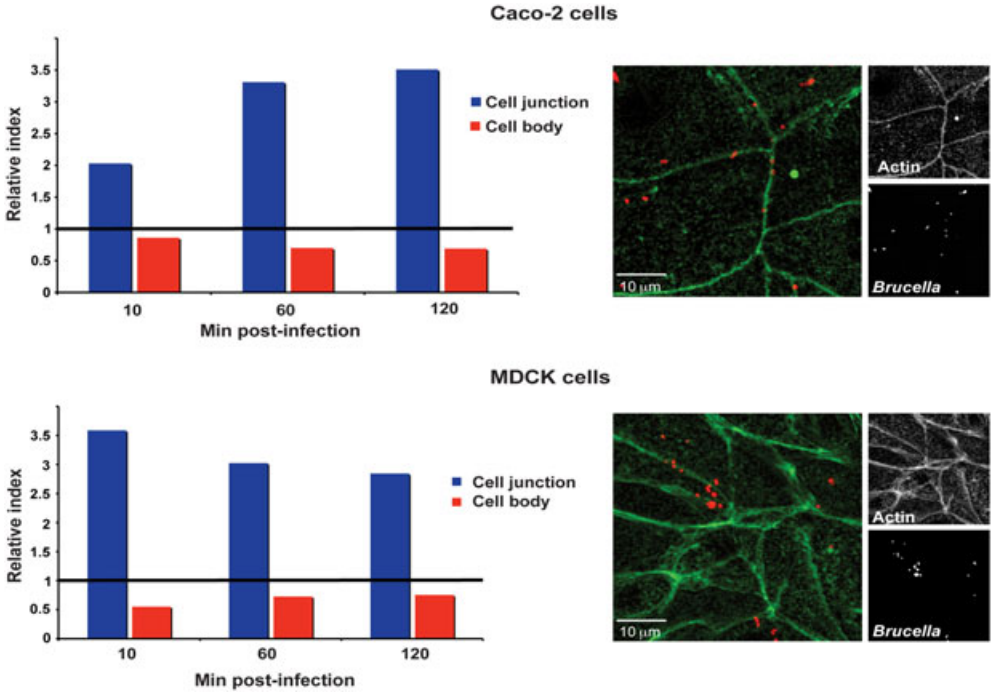

Fig. 4. *Brucella* is targeted to the cell-cell interaction membrane during invasion in confluent epithelial cells. Relative index calculated as the ratio between the percent of bacteria positioned either over the cell–cell junction membrane or the rest of the cells body divided by the percent of the area of the cell–cell junction membrane or the percent of the area of the cells body in each field. A total of 300 bacteria were used for the index. The index was calculated for three time points post-infection in Caco-2 and MDCK cells. A random distribution of the bacteria would imply a relative index close to one; a deviation from this ratio indicates a non-random distribution. On the right representative images of each assay are shown. Red, *Brucella*; green, phalloidin.

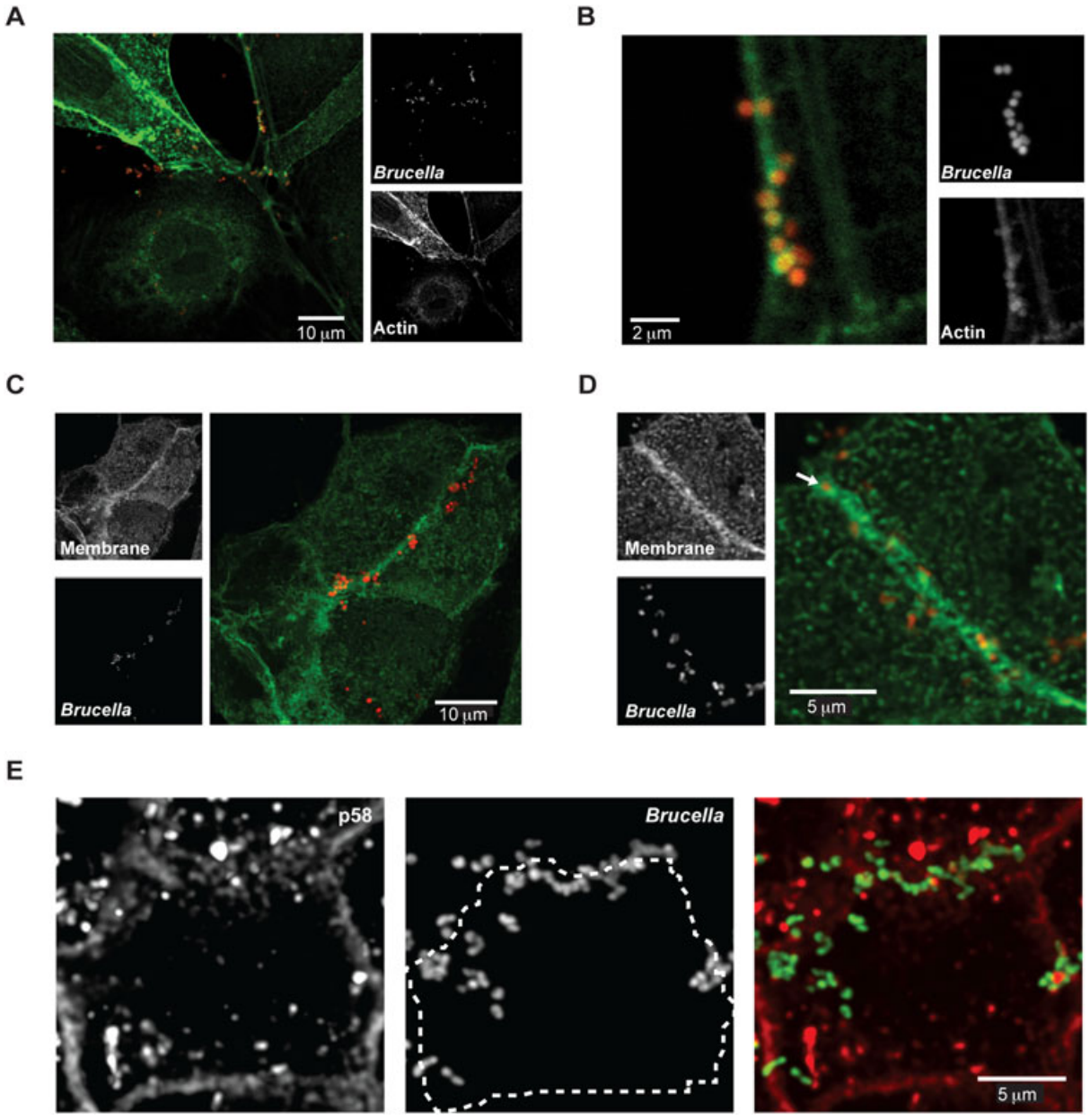

Fig. 5. Early interaction of *Brucella* with MDCK cells.
A. Confocal images of MDCK GFP-live-actin cells infected with *B. abortus* at 4 h post-infection.
B. Inset of the 4 h post-infection image.
C, D. Confocal images of MDCK stably transfected with the AC-GFP1-Mem marker (green) and infected with *B. abortus* (red) at 4 h post-infection.
E. Confocal images of MDCK cells infected with *B. abortus* (green) for 4 h and stained with an anti-P58 antibody (red). Dotted line, p58 staining.

staining of these infected cells showed that many bacteria were found associated with the basolateral membrane (Fig. 5E).

At 24 h post-infection almost all the *Brucella* were found in small proto-niches with no signs of actin association (Fig. 6A). At 48 h post-infection, we found many perinuclear intracellular niches each with a large number of bacteria. Surprisingly, F-actin appeared in the niches as a mesh-like structure and the bacteria were arranged in groups of 3 to 10 cells each (Fig. 6 B and C). These structures were completely disassembled when the infected cells were treated with cytochalasin D for 40 min (it was not possible to detect replicative niches), indicating that they are tethered by this actin mesh structure (Fig. 7; cytochalasin D). Moreover, removal of cytochalasin D and recovery of the cells for an extra 3 h resulted in complete

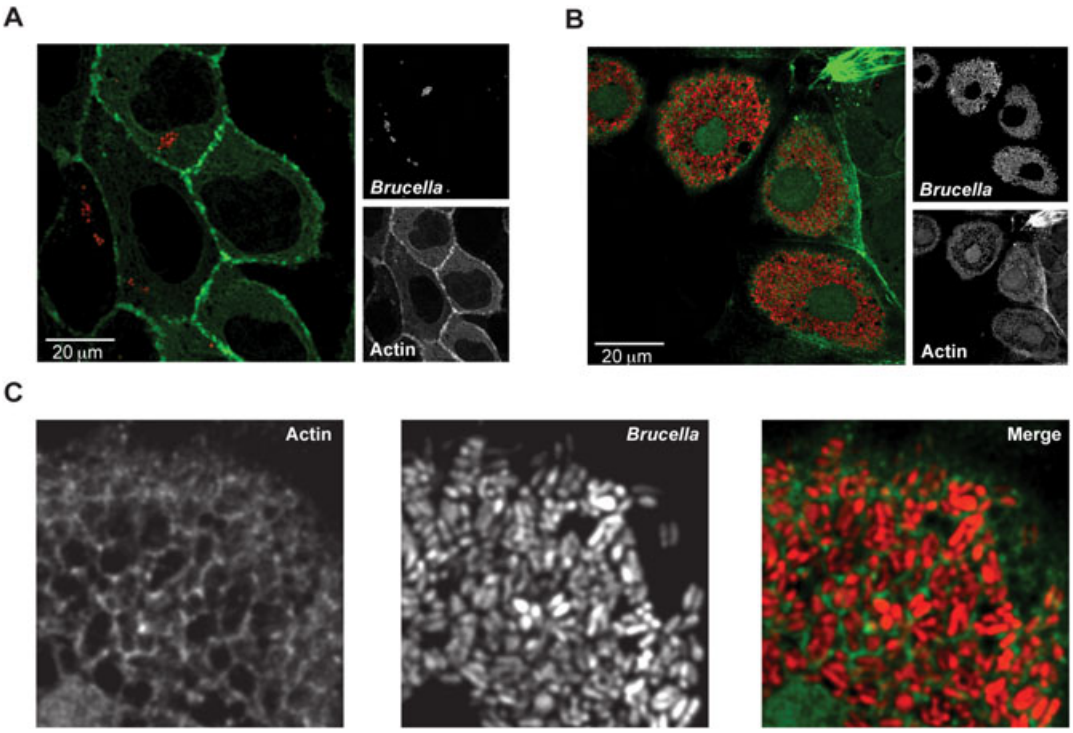

Fig. 6. *Brucella* replicates in MDCK cells and the replicative niche is surrounded by actin.
A. Confocal images of MDCK GFP-live-actin cells infected with *B. abortus* at 24 h post-infection.
B. Confocal images of MDCK GFP-live-actin cells infected with *B. abortus* at 48 h post-infection C. Inset of the 48 h post-infection image. Red, *Brucella*; green, GFP-live-actin (F-actin).

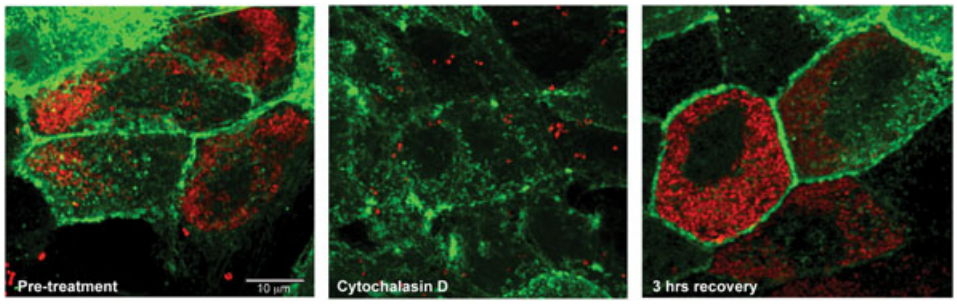

Fig. 7. The replicative niche of *Brucella* in MDCK cells is tethered by actin. Confocal images of MDCK GFP-live-actin cells infected with *B. abortus* for 48 h (pre-treatment), treated for 40 min with cytochalasin D (cytochalasin D) and treated with cytochalasin D for 40 min and left to recover for an extra 3 h (3 h recovery). Red, *Brucella*; green, GFP-live-actin (F-actin).

re-establishment of the niches (Fig. 7; recovery), indicating that the biochemical information required for maintenance of these structures is conserved even if actin is de-polymerized. It is worth noticing that while in the pre-treated and recovered cells the replicating bacteria seemed to be arranged in a certain number of stacks, the ones treated with cytochalasin D were dispersed in a higher number of stacks in the *z*-axis, which resulted in an apparent less number of bacteria per image.

BigA targets the focal and cell–cell adhesion sites

To further advance in understanding the cellular target of BigA, we cloned the region of the gene coding the BIg-like domain, produced it in *E. coli* as a double poly-histidine-3xFLAG tagged protein and purified it by affinity chromatography (Fig. S4). The purified protein was initially added to HeLa cells to determine if it was possible to observe any specific localization by immunofluorescence using a monoclonal anti-FLAG antibody. Surprisingly, HeLa cells treated with this protein showed a rearrangement of its cytoskeleton and focal adhesion sites as determined by transfection of the cells with constructions expressing fusions of Zyxin and Paxillin to DS-Red and mCherry respectively and Phalloidin staining (Fig. 8 A–C). Zyxin and Paxillin are focal adhesion markers and Phalloidin stains cortical actin and is a good marker of cytoskeleton integrity. In the case of Zyxin (Fig. 8A), while the control cells showed the focal adhesion sites at the border of the cells,

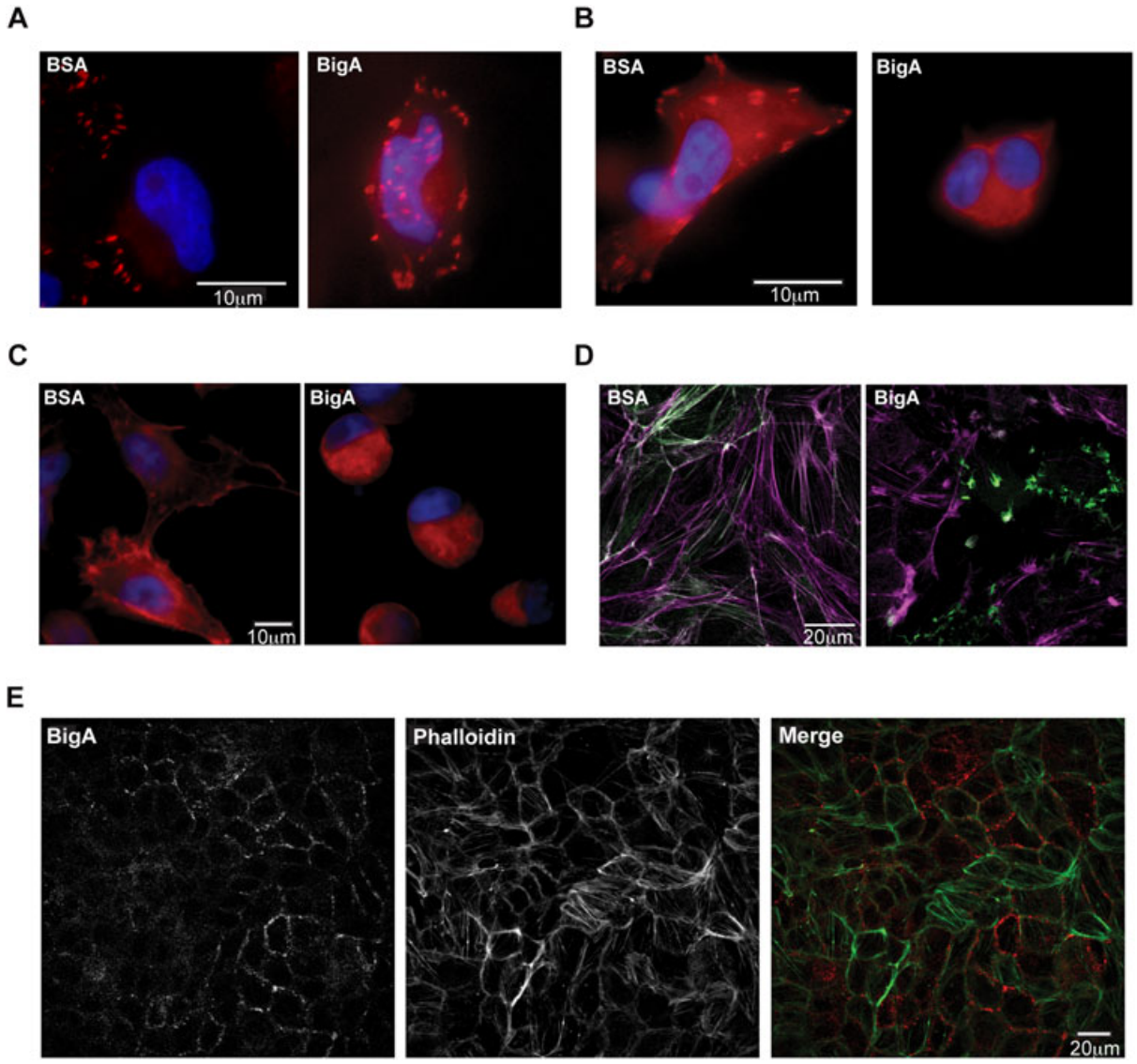

Fig. 8. BigA alters focal adhesion sites in HeLa cells and is targeted to the cell-cell junction membrane in MDCK cells affecting the cytoskeleton structure.
A, B. Epifluorescence microscopy images of HeLa cells transfected with plasmids that express DS-red and mCherry fusions to Zyxin (A) and Paxillin (B) respectively and treated with 50 μg of recombinant BigA for 4 h.
C. Epifluorescence microscopy images of HeLa cells treated with 50 μg of recombinant BigA for 4 h and stained with phalloidin (red).
D. Confocal images of MDCK GFP-live-actin (green) cells treated or not with 50 μg of recombinant BigA for 24 hrs and stained with phalloidin (magenta).
E. Confocal image of MDCK cells treated with recombinant BigA for 4 h. Green, phalloidin; red, BigA.

treatment with BigA induced a contraction of the cell and the focal adhesion sites were found in the body of the cell. Focal adhesion sites harbouring Paxillin were completely disassembled upon treatment with BigA (Fig. 8B). This was not observed when the cells were treated with the same concentration of bovine seroalbumin (BSA) as a control demonstrating that BigA alters adhesion molecules triggering cytoskeletal rearrangements.

In an effort to characterize in more detail the role BigA plays in the interaction with epithelial cells, and in view of the results obtained with treated HeLa cells, we added the recombinant BIg-like domain to confluent MDCK cells and determined the localization by confocal immunofluorescence microscopy at 4 h post-treatment. Fig. 8E shows that the protein localized preferentially to the cell–cell interaction membrane, strongly suggesting that the receptor/receptors of BigA are localized in these regions. To determine if BigA generated a similar effect on these cells as the one observed with HeLa, we treated MDCK cells for 24 h with 50 μg of the recombinant protein and monitored the morphology of the cells over time. As can be observed in Fig. 8D, treatment of MDCK GFP-live-actin cells resulted in the loss of the cytoskeletal structure as was evidenced by phalloidin staining.

Discussion

Epithelial barriers are the first lines of defence microorganisms normally encounter when initiating an infectious cycle and, for this reason, pathogens display a wide variety of virulence strategies devoted to circumvent them. As a general rule, pathogenic bacteria have two mayor invasion strategies: either they actively induce their own internalization in epithelial cells via a triggering process or invade through a zippering process that involves adhesins that target surface receptors (Sansonetti, 2002). A third possibility is the paracellular route; this is breaching the epithelial barriers (relaxing the cell–cell junction) and penetrating through the cells to reach systemic sites (Barreau and Hugot, 2014). While much has been done in several important pathogens in order to understand the way they circumvent different epitheliums, in *Brucella* most of the works performed have been focused on the interaction of the bacteria with phagocytes, particularly in the mechanisms that allow subversion of the phagocytic pathway and intracellular bacterial replication. For this reason we have, comparably, less knowledge on how *Brucella* interacts with epithelial cells, and more specifically, there are few adhesion molecules identified or what they target in the host cells (Castaneda-Roldan *et al*., 2006; Posadas *et al*., 2012; Ruiz-Ranwez *et al*., 2013a; Ruiz-Ranwez *et al*., 2013b).

We have recently identified a genomic region in *B. abortus* with horizontally transmitted features that is involved in the adhesion to host cells (Czibener and Ugalde, 2012). This region encodes four open reading frames conserved in all *Brucella* sequenced to date but none of them have homology to known proteins. The results presented here demonstrate that the protein encoded in the open reading frame *Bab1_2009*, which we have named *bigA*, is a potent adhesin that targets the bacteria to the cell–cell junction membrane in confluent epithelia cell cultures. This protein has a BIg-like domain that we have shown is exposed in the surface of the bacterium. This Ig-like superfamily domain is present in many bacterial proteins whose function ranges from enzymes to pilli, fimbria and adhesins (Bodelon *et al*., 2013; Mikula *et al*., 2013). In all cases it is proposed that these domains mainly mediate protein–protein interactions and that its wide distribution in nature is because of its energetically favourable folding (Bodelon *et al*., 2013). Several pilli and fimbria, which are central for the adhesion of the bacterium to different substrates, as well as the Intimin of enterohemorrhagic *E. coli* or the Invasin A of *Y. enterocolitica* that mediate the invasion process of the pathogen, have these Ig-like domains (Bodelon *et al*., 2013; Mikula *et al*., 2013). In all cases these domains have the function of recognizing either receptors of the cells or different substrates. Moreover, this domain belongs to the Ig superfamily present also in many eukaryotic adhesion molecules like cadherins, integrins and selectins that mediate the cell–cell or cell–matrix interaction in a wide range of tissues. Our results strongly suggest that BigA binds to one of these surface molecules targeting the bacteria to the cell–cell junction membrane in the process. Addition of the recombinant BIg-like domain to HeLa and MDCK induced profound focal adhesion sites and cytoskeletal rearrangements, indicating that this domain targets surface receptors involved in adhesion. Moreover, a localization analysis performed on confluent MDCK cells showed that the protein localized to the cell–cell interaction plasmatic membrane. At this stage we do not know if these alterations are the consequence of a signal transduction triggered by the protein upon binding or if it is the result of titrating one or several adhesion proteins of the cell. It could be hypothesized that the BIg-like domain binds to domains of the Ig-like superfamily present in several adhesion molecules displacing their normal interaction with other proteins of either an adjacent cell or with the solid matrix and, thus, interrupting their normal homo and heterotypical interactions. More works will be needed in order to understand the molecular mechanisms of action of BigA and to be able to identify its receptor/s.

With the goal of gaining a better insight on the intracellular cycle of *B. abortus* in epithelial cells, we infected a GFP-live-actin expressing MDCK cell line and followed the infection over a 48 h time frame. Our analysis revealed that *Brucella* modulates the actin recruitment and loss to the *Brucella* containing vacuole: in the early phases of the invasion process it recruits actin to the endocytic cup, which is afterwards lost during its intracellular trafficking until it reaches the replicative niche where it is again acquired and structured in a mesh-like structure that appears necessary to tether and maintain the niches together. These observations suggest that *Brucella* actively modulates actin dynamics during its intracellular trafficking in these cells, and that most probably, it does it by secreting specific virulence factors devoted to this. One interesting observation was the fact that treatment with cytochalasin D of 48 h infected cells harbouring replicative niches resulted in complete disassembly of them indicating that the polymerized actin is necessary to tether the niches during the replication phase. Moreover, after the treatment with the de-polymerization agent and a 3 h recovery, we observed that the niches and the actin mesh re-formed indicating that the bacteria probably drive the signals necessary to contain these structures in a certain stage of the intracellular cycle.

In sum, the results presented here show that *Brucella* has evolved specific strategies to adhere, invade and replicate in epithelial cells, probably the first cell type the pathogen encounters during the infectious cycle. This may have important consequences for their interaction with phagocytes since, when they reach these immune cells,

the bacteria probably performed one or more replication cycles in an epithelium. How this initial cycle impacts the way *Brucella* confronts phagocytes is a still completely unknown field of research.

Experimental procedures

Media and culture condition

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^{-1}; Kanamycin, 50 μg ml^{-1} and Nalidixic Acid, 5 μg ml^{-1}.

Recombinant DNA techniques, mutant and plasmid constructions

Construction of the ΔbigA mutant strain. To construct the *B. abortus* 2308 Δ*bigA* mutant strain, the regions flanking the *bigA* gene were amplified and ligated using the recombinant PCR technique (Czibener and 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 as follows: CC23 (5′-CCGGAATTCTGATTAGAATTGATAAACTG-3′) and CC24 (5′-GATAATTCTTTAAAA AGTAT-3′) to amplify a 500 bp upstream region and CC25 (5′-TTTTAAAGAATTATCGGCCGTC TTATACTATTCAGA-3′) and CC22 (5′-CGCGGATCCAGACCGG AATCTGACTGTAC-3′) to amplify a 500 bp downstream region; CC23 and CC22 were used for the overlapping PCR.

The resulting plasmid was introduced into *B. abortus* 2308 by bi-parental mating using the *E. coli* S17-λ*pir* strain.

Plasmid constructions. For the construction of the vector expressing a C-terminal 3xFLAG-tagged version of gene *Bab1_2009* (*big*A), the plasmid pBBR1-MCS4-3xFLAG (Dohmer *et al*., 2014) was used. A DNA fragment spanning the gene was amplified by PCR from *B. abortus* 2308 genomic DNA using primers CC15 (5′-CTAGCTAGCACTTTAGTAAAGACATACT-3′) and CC16 (5′-CATGCCATGGCCCGGAGGAGATGGTGGC-3′), and the PCR product was digested with NheI 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 plasmid was named pBBR-BigA3xFlag and introduced in the *B. abortus* strains (wild type and mutant) by bi-parental mating.

In order to generate the poly-histidine-tagged BigA/3xFlag (pBigA-FLAG-HIS) a DNA fragment of 225 bp was amplified from *B. abortus* genomic DNA using primers FM9 (5′-CGCGGATCCAGGTATGATATGTCAGAT-3′) and FM11 (5′-CGGAATTCACATTGATCTGAAACCTT-3′) the PCR product was digested with BamHI and EcoRI, cloned in pBAD/3xFlag (Spano *et al*., 2008) in the same sites and subsequently subcloned in the pQE30 vector in sites BamHI and HindIII.

All constructs were confirmed by sequence analysis.

Protein expression and purification

Recombinant poly-histidine-tagged BigA was expressed in *E. coli* and purified using nickel-affinity chromatography under denaturing conditions in the presence of urea 6 M. Briefly, *E. coli* was grown at 37°C at 250 r.p.m., and the expression was induced with IPTG at $OD_{600nm} = 0.6$. 3 h post-induction; cells were harvested and broken by sonication. The resulting lysate was centrifuged at 16 000× and the inclusion bodies (insoluble) washed two times and resuspended in Imac A buffer (500 mM NaCl, 50 mM Tris-HCl pH 7.6 and 20 mM imidazol) with 6 M urea and applied to a HisTrap^{TM} HP column (GE, Healthcare). The protein was eluted with the same buffer with 100 mM of imidazol. The eluted fractions were pooled and dialysed against re-folding buffer (50 mM Tris-HCl pH 7.6, 1 mM NaCl, 0.2 mM DTT, 0.1 mM EDTA, 10% glycerol, 2 mM Zwittergent and 1 mM 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_2009* 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.

Purification of the serum was performed as follows: Recombinant BigA (200 μm) was coupled to an Affi-gel 10 resin (BioRad) according to the manufacturers instructions and loaded into a column. After three washes with PBS the serum from the immunized mice was loaded into the column, washed three times with PBS, an the retained antibodies eluted from the column with 100 mM glycine pH 2,5. The eluted Igs were initially observed by SDS-PAGE and silver staining before using them.

Intracellular replication assays

Antibiotic protection assays were performed in the human cell lines HeLa (ATCC), Caco-2 (ATCC) and the dog cell line MDCK (ATCC) as described in Ugalde *et al*. (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 cells and the appropriate time until confluence for Caco-2 and MDCK (once they have reached confluence they were left for and extra 48 hrs). *Brucella* strains were grown in TSB with the appropriate antibiotics for 24 h and diluted in culture medium prior to infection. The suspension was added at the different multiplicity of infections (1:500 for HeLa and 1:1000 for Caco-2 and MDCK) and centrifuged at 300 g for 10 min. After 1 h 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 h post-infection, cells were washed and lysed with 0.1% Triton-100X. The intracellular CFU were determined by direct plating on TSB agar plates.

Cell transfection

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

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 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 BigA coverslips were washed three times with PBS, incubated for 15 min with PBS added with 50 mM NH_4Cl in order to quench free aldehyde groups. Coverslips were then blocked, incubated with the primary antibodies in a PBS, 5% bovine serum albumin and 0.1% saponin solution for 1 h at room temperature, washed in PBS and then 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 immunofluorescence microscope (Nikon-Eclipse T2000 or an Olympus IX81 confocal microscope) using a 60X oil immersion objective. Projections were saved in TIFF format and imported to ADOBE PHOTOSHOP CS where images were 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 NH4Cl and, before permeabilization, incubated with the primary antibody rabbit anti-*Brucella* polyclonal antibody (dilution 1:1500) in a PBS, 5% BSA solution. Then cells were washed and incubated with the other primary antibody mouse anti-M84 (anti-O-antigen) monoclonal antibody (dilution 1:1000) 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_2009* 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 purified anti-*Bab1_2009* antibody (refer to the preceding texts) for 1 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.

Membrane isolation, periplasmic extractions and Western blot analysis

Total membrane preparation was performed as described in Guidolin *et al*. (2015). Periplasmic extractions were prepared as previously described (Dohmer *et al*., 2014). Briefly, the strains were grown in TSB for 16–24 h until they reached an OD of 1 and 2.5×10^{10} cells were centrifuged at 3300 g for 10 min. The pellets were washed twice with PBS buffer and resuspended in 1 ml of 0.2 M Tris-HCl pH 7.6. One millilitre of 0.2 M Tris-HCl pH 7.6, 1 M Sucrose and 0.25% Zwittergent 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 g, the pellets separated from the supernatants and stored at −20 C until they were used. The pellets and supernatants were processed for Western blot, using an anti-FLAG M2 monoclonal antibody (1:5000), anti-GroEL (1:2000) and anti-OMP-1 (1:2000) and anti-OMP-19 (1:2000) kindly provided by Dr Axel Cloeckaert as primary antibodies, and goat anti-mouse HRP (1:2000) (Dako) or goat anti-mouse conjugated to the infrared 680 or 800 fluorophores (Odyssey) as secondary antibodies (1:20 000 dilution). All antibodies were diluted in Tris-buffered saline (TBS), 5% non-fat milk and 0.05% Tween-20 solution.

Invasion inhibition by antiserum treatment

For the antibody-mediated inhibition assays a standard antibiotic protection assay was performed but prior to the addition of the bacteria, they were incubated for 60 min with a 1:100 dilution of the anti-serum. The total number of viable intracellular CFU at 4 h post-infection was calculated by direct plating as indicated in the preceding texts.

Generation of the AC-GFP1-Mem MDCK cell line

For the MDCK cell line stably expressing a plasmatic membrane cell marker a lentivirus carrying the AC-GFP1-Mem marker (Invitrogen) was used as described in Dragoi and Agaisse (2014). After infection, three rounds of enrichment of the cells expressing GFP were performed by FACS cell sorting and plating to finally obtain a cell line with over 80% GFP expressing positive cells.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. A. Scheme of the 5 kb base pair region with a significant deviation of the G + C content encoding four open reading frames with no homologues described in the literature and present only in the genus *Brucella*.

B. Protein alignment of a domain of the protein encoded by *Bab1_2009* with the bacterial immunoglobulin-like domain of the Intimin of *Escherichia coli*.

Fig. S2. Confocal images of MDCK cells infected with *B. abortus* 2308 and, at 2 h post-infection, washed extensively, fixed and stained with phalloidin and a *Brucella* anti-O antigen monoclonal antibody. Red, *Brucella*; green, actin.

Fig. S3. A. MDCK GFP-live-actin cells stained with the p58 antibody. Confluent MDCK cells were fixed and stained with a polyclonal anti p58 antibody that stains the basolateral membranes.

B, C. 3D reconstruction of MDCK GFP-live-actin cells stained with the p58 antibody. Green, GFP-live-actin; red, p58.

Fig. S4. A. Protein sequence of BigA and highlighted in yellow the BIg-like domain cloned and produced in *E. coli*.

B. Coomassie brilliant blue staining (left) and Western blot with a monoclonal anti-Flag antibody (right) of the purified BIg-like domain of *Brucella abortus* BigA produced in *E. coli*.