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BmaC, a novel autotransporter of *Brucella suis*, is involved in bacterial adhesion to host cells

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Summary

Brucella **is an intracellular pathogen responsible of a zoonotic disease called brucellosis.** ***Brucella*** **survives and proliferates within several types of phagocytic and non-phagocytic cells. Like in other pathogens, adhesion of brucellae to host surfaces was proposed to be an important step in the infection process. Indeed,** ***Brucella*** **has the capacity to bind to culture human cells and key components of the extracellular matrix, such as fibronectin. However, little is known about the molecular bases of** ***Brucella*** **adherence. In an attempt to identify bacterial genes encoding adhesins, a phage display library of** ***Brucella suis*** **was panned against fibronectin. Three fibronectin-binding proteins of** ***B. suis*** **were identified using this approach. One of the candidates, designated BmaC was a very large protein of 340 kDa that is predicted to belong to the type I (monomeric) autotransporter family. Microscopy studies showed that BmaC is located at one pole on the bacterial surface. The phage displaying the fibronectin-binding peptide of BmaC inhibited the attachment of brucellae to both, HeLa cells and immobilized fibronectin** ***in vitro*****. In addition, a** ***bmaC*** **deletion mutant was impaired in the ability of** ***B. suis*** **to attach to immobilized fibronectin and to the surface of HeLa and A549 cells and was outcompeted by the wild-type strain in co-infection experiments. Finally, anti-fibronectin or anti-BmaC antibodies significantly inhibited the binding of wild-type bacteria to HeLa cells. Our results highlight the role of a novel monomeric autotransporter protein in the adhesion of** ***B. suis*** **to the extracellular matrix and non-phagocytic cells via fibronectin binding.**

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Introduction

The extracellular milieu of the host can be an aggressive and hostile environment for pathogens. In addition to physical stresses, pathogens are exposed to other host defence mechanisms including complement deposition and opsonization. Thus, several pathogenic bacteria have evolved strategies to both attach to host cells and induce their invasion into target cells for proliferation and/or dissemination (Pizarro-Cerda and Cossart, 2006). In fact, most pathogenic bacteria express adhesins on their surfaces that mediate interaction with host cell receptors or with soluble macromolecules. This interaction offers a way to colonize and circumvent clearance from host fluids. In addition, contact of bacterial adhesins with host cell ligands mediates signalling events that may trigger or affect bacterial invasion, and even induce rapid host inflammatory responses. Bacterial adhesion to target cells is also important for some pathogens to inject effectors into host cells through specialized secretion systems. The role of filamentous adhesins, such as pili or fimbria, in the primary interaction with host cells has been widely studied in many bacterial pathogens (Kline *et al*., 2009). Over the past years, several non-polymeric adhesins with a role in early interactions of bacteria with host cells were identified. These adhesins recognize many different host molecules, including components of the extracellular matrix (ECM), such as collagen, hyaluronan and adhesive glycoproteins such as vitronectin, fibrinogen and specially fibronectin (Pizarro-Cerda and Cossart, 2006; Kline *et al*., 2009).

Brucella spp. are alpha-proteobacteria responsible for a worldwide zoonosis called brucellosis, which causes important economic losses in livestock production in several developing countries (Boschiroli *et al*., 2001; Seleem *et al*., 2010). Besides, several species of *Brucella* are also able to produce a human debilitating disease (Pappas *et al*., 2006) against which no effective vaccine is currently available (Ficht *et al*., 2009; Perkins *et al*., 2011). *Brucella* infection proceeds by ingestion or inhalation of bacteria; brucellae penetrate the mucosa,

and subsequently are transported to the lymph nodes. The spread of *Brucella* in multiple tissues is mainly mediated by macrophages. *Brucella* spp. replicates and survive for prolonged periods of time inside macrophages, non-professional phagocytes and a variety of host cells in a compartment derived from the endoplasmic reticulum (Pizarro-Cerdá *et al*., 1998; Pizarro-Cerda *et al*., 1998; Ko and Splitter, 2003; Celli and Gorvel, 2004). *Brucella* uses a stealthy strategy to escape recognition of the innate immunity and evade intracellular destruction (Martirosyan *et al*., 2011).

Little is known about the bacterial factors involved in the initial interaction between *Brucella* and host cells. Genomic analyses indicates that *pili* or other fimbria like structures are absent in several *Brucella* spp. (DelVecchio *et al*., 2002; Paulsen *et al*., 2002; Halling *et al*., 2005; Chain *et al*., 2005b; Ding *et al*., 2011). *Brucella* spp. were shown to bind to HeLa cells forming tight microcolonies on the cell surface. In addition, *Brucella* was also able to bind significantly to THP-1 macrophages (Castañeda-Roldán *et al*., 2004). It was proposed that bacterial sialic acid-binding proteins mediate the interaction of *Brucella* to sialylated host molecules (Rocha Gracia *et al*., 2002; Castañeda-Roldán *et al*., 2004). One of these proteins with affinity for sialic acid (SP41) was localized to the bacterial surface (Castañeda-Roldán *et al*., 2006). SP41 seems to be a ubiquitous protein in *Brucella* spp., suggesting that it is not a species-specific adhesin. In addition to host molecules containing sialic acid residues, *Brucella abortus* binds in a dose-dependent manner to components of the ECM, such as fibronectin and vitronectin and with less affinity, to collagen and laminin (Castañeda-Roldán *et al*., 2004). Interestingly, fibronectin has been reported inside endocytic vacuoles containing *Brucella*, suggesting that binding to fibronectin may facilitate bacterial uptake (Gay *et al*., 1986). In fact, non-opsonized *B. abortus* were proposed to penetrate via fibronectin receptors (Campbell *et al*., 1994). Taken together, all these observations indicate that brucellae might have affinity for several host ligands, probably through the interaction with multiple bacterial adhesins.

Phage display libraries have been useful to identify bacterial adhesins relevant for host colonization by different pathogens (Jacobsson and Frykberg, 1995; Heilmann *et al*., 2002; Jacobsson, 2003; Bjerketorp *et al*., 2004; Mullen *et al*., 2007). In this work, we aimed to identify *Brucella suis* genes encoding proteins that might be involved in the binding of brucellae to fibronectin (Castañeda-Roldán *et al*., 2004). To pursue this, a M13 display library (Jacobsson *et al*., 2003) of the *B. suis* genome was panned against immobilized fibronectin. Using this approach we identified a novel adhesin from the monomeric autotransporter family, which we designated BmaC. This article provides the first evidence for a role of a member from the autotransporter families in the attachment of *Brucella* to fibronectin and host cells and hallmarks the importance of the adhesion stage in the infection process of this intracellular pathogen.

Results

Identification of fibronectin-binding proteins of B. suis *by phage display*

It was previously proposed that ECM components, such as fibronectin, participate in the binding of *Brucella* to the ECM or the host cell (Castañeda-Roldán *et al*., 2004). In an attempt to identify genes coding for adhesins that mediate interaction of *Brucella* with the host, panning of a *B. suis* phage-display library against fibronectin was accomplished. To achieve this, a M13 phage-display library of fragmented genomic DNA from *B. suis* M1330 was prepared using the pG8SAET phagemid vector as previously described (Jacobsson *et al*., 2003). The resulting library consisted of 4×10^6 clones with a titre of 5.2×10^{11} colony-forming units (cfu) ml^{-1}, which together were expected to display polypeptides of varying size from all proteins encoded by the *B. suis* genome. The phage display library was screened for affinity panning against immobilized fibronectin. The clones obtained after the first panning were used to produce a phage stock for a second panning to increase the number of positive clones. This procedure was repeated until three rounds of panning were completed. Control pannings against BSA were performed simultaneously. The number of recombinant phages eluted after each round of panning against immobilized fibronectin increased sequentially as the pannings progressed (Table S1). Besides, the number of recovered phages that expressed the E-tag (Jacobsson *et al*., 2003) also increased, indicating that there was an enrichment in clones with inserts fused in the correct reading frame to the gVIII capsid phage gene (Table S1).

A total of 75 clones from pannings against fibronectin were selected for DNA sequencing. The sequences obtained were compared by BLAST searches with the complete genome of *B. suis* at the NCBI site (http://blast.ncbi.nlm.nih.gov). Given that a reliable selection is believed to be revealed by clones containing overlapping inserts (Jacobsson *et al*., 2003), recombinant clones with overlapping sequences and/or those more abundantly represented were considered to display high affinity for fibronectin. Since we aimed to identify proteins that mediate interaction with the host, those genes encoding proteins that are predicted to be extracellular or located in the outer membrane of *B. suis* were of more interest. Only BRA1148 has all these features with a high degree of confidence (Table S2). However, since several phage clones corresponding to BRA0095 and BRA0175 were

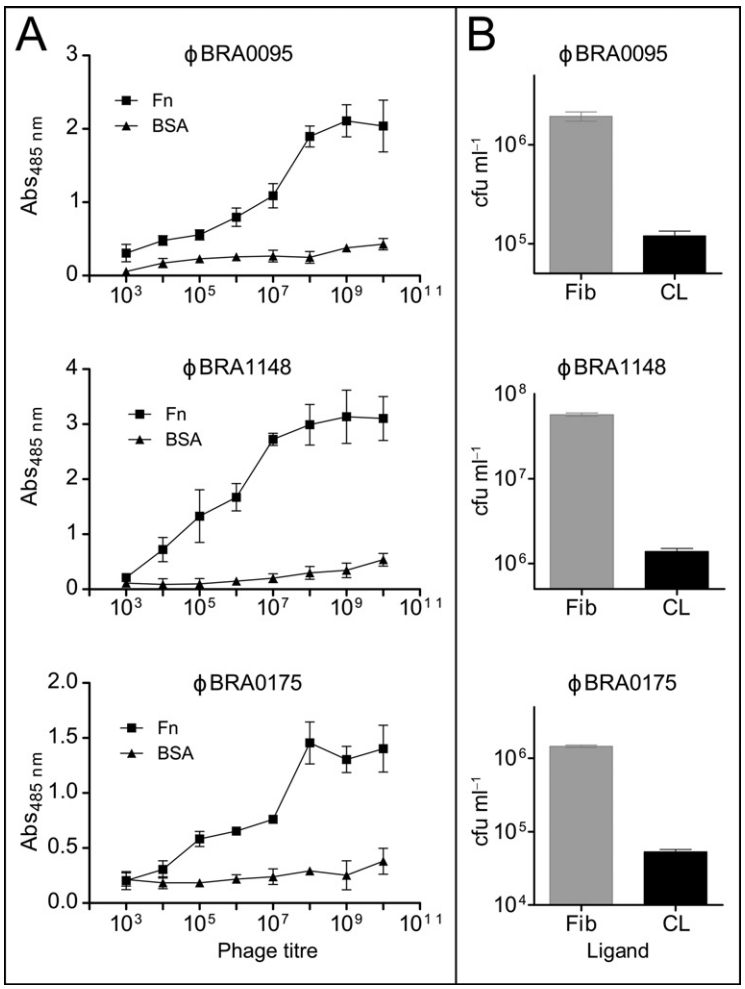

Fig. 1. Ability of the recombinant phages to bind fibronectin. Immobilized ligands are indicated, Fib: fibronectin; CL: BSA control ligand. The recombinant phage (ϕ–) is indicated above each figure.
A. ELISA assay with serial dilutions of purified recombinant phage stocks. The absorbance values obtained for each candidate are plotted against phage titre. Points represent the means ± standard deviation of values made in triplicate from a representative experiment.
B. Panning with purified phage stocks. The number of eluted phage is expressed as colony-forming units per millilitre (cfu ml^{-1}). Bar represent the means ± standard deviation of measurements made in triplicate from a representative experiment.

selected by panning selection, recombinant phages containing domains of these proteins were also submitted to further analysis.

BRA0095 encodes a small hypothetical protein of 113 amino acids length. Protein sequence analysis showed that BRA0095 does not contain an evident signal sequence or any characterized or conserved domains. However, hydrophobicity analysis indicated the presence of two putative transmembrane regions suggesting that the product of BRA0095 may be an integral membrane protein. The phage clones selected by panning with fibronectin share a region of 40 amino acids at the N-terminal end of BRA0095. *In silico* analysis of BRA0175 indicated that this gene encodes also a small hypothetical protein of 79 amino acids with no evident secretion signal peptide, transmembrane regions or any characterized domains.

BRA1148 encodes a large protein of 3420 amino acids, with a predicted molecular weight of about 340 kDa, that belongs to the type I autotransporter family (known also as monomeric autotransporters) (Loveless and Saier, 1997; Henderson *et al*., 1998; 2004; Dautin and Bernstein, 2007). The product of BRA1148 exhibits all the characteristics of proteins from this family (see below), which includes a secretion signal peptide and a β fold-C-terminal domain that is typically inserted in the bacterial outer membrane.

It was verified that the purified recombinant phages (ϕ-BRA0095, ϕ-BRA0175 and ϕ-BRA1148) expressed the E-tag (Fig. S1), confirming the expression of fusion proteins. To confirm the ability of the recombinant phages to bind fibronectin, an ELISA test was performed. The fibronectin was immobilized in the wells, confronted with increasing concentrations (from 10^3 to 10^{10}) of the phage stock and washed to eliminate unbound phages. Bound phage particles were revealed with an anti-M13 antibody conjugated to horseradish peroxidase (HRP). Immobilized BSA was used as an unrelated protein ligand. It was found that in all cases the M13 signal bound to fibronectin reaches saturation with a titre of about 10^8 cfu ml^{-1}, to levels that are several times higher than those associated to the control ligand (Fig. 1A). It is also possible to compare the fibronectin binding ability between the candidate phages and a control phage.

ϕ-BRA0175, ϕ-BRA0095 and ϕ-BRA1148 reached absorbance values 6-, 10- and 15-fold higher than the control phage (Fig. S2). The ability of the candidates to bind fibronectin was also confirmed by a panning experiment of purified phage stocks (Jacobsson *et al*., 2003). The number of eluted ϕ-BRA0095, ϕ-BRA0175 and ϕ-BRA1148 were 20-, 40- or 80-fold higher, respectively, after panning against fibronectin than against BSA (Fig. 1B). These observations confirmed that the peptides exposed on the phage surfaces exhibit higher ability to bind the original ligand (fibronectin) than unrelated ligands.

Inhibition of the binding of B. suis *to HeLa cells by recombinant phages*

Adhesins from several pathogens were shown to interact with components of the ECM in addition to cellular receptors. It was also proposed that some bacterial adhesins may interact with host cells through fibronectin molecules that are associated with the cell surface (Pizarro-Cerda and Cossart, 2006; Henderson *et al*., 2011). Based on these evidences, we hypothesized that the fibronectin-binding peptides of *B. suis* identified by phage display could have a role in adhesion to host cells. To assess this possibility, we investigated whether the phage-displayed peptides derived from BRA0095, BRA0175 and BRA1148 proteins were able to interfere with the attachment of wild-type *B. suis* to HeLa cells by blocking binding sites on these cells. HeLa cells have been extensively used as a cell culture model to study different stages of *Brucella* infection including intracellular replication (for some recent publications see: Dozot *et al*., 2006; Starr *et al*., 2008) and attachment and internalization (Castañeda-Roldán *et al*., 2004; 2006; Martin-Martin *et al*., 2009; Godefroid *et al*., 2010; Marchesini *et al*., 2011). Thus, monolayers of HeLa cells were infected with *B. suis* (moi of 1:100) in the presence or the absence of the recombinant phage and incubated for 1 h at 37°C. An unrelated stock of phages was also assayed as negative control. After washing to remove non-adherent bacteria, cell-associated brucellae were determined by cell lysis and plating bacterial dilutions on TSA. While the control phages did not affect adhesion of brucellae to epithelial cells, phages displaying portions of BRA0095, BRA0175 or BRA1148 inhibited to some extent the binding of *B. suis* to epithelial cells (Fig. 2A). These observations suggest that the peptides exposed on the phage displaced bacteria from the cell surface by their interaction with cell-associated fibronectin molecules. Since ϕ-BRA1148 blocked most efficiently the attachment of *B. suis* to HeLa cells, we decided to submit this candidate to further studies. We named BRA1148 as BmaC (*B*rucella monomeric autotransporter). Phage expressing the peptide of BmaC (ϕ-BmaC) inhibited the binding of *B. suis* to HeLa cells in a dose-dependent manner (Fig. 2B), supporting the hypothesis that *Brucella* binding sites on the cell surface were increasingly occupied by the BmaC peptide. To further support this idea, we examined the effect of adding ϕ-BmaC during HeLa infection with GFP-labelled brucellae by immunofluorescence microscopy. HeLa monolayers were also incubated with the recombinant phages in the absence of bacteria. Phages were revealed with a mouse anti-M13 comercial antibody and a Cy3-conjugated anti-mouse secondary antibody. In the absence of phages, some cell-associated GFP-labelled bacteria were observed 1 h post infection (p.i.) (not shown); at a later time (4 h), several bacterial aggregates (cell surface associated or intracellular) were detected (Fig. 3A). Interestingly, when cells were exposed only to the phages, a substantial punctate red fluorescent signal corresponding to the BmaC-phages were observed on the

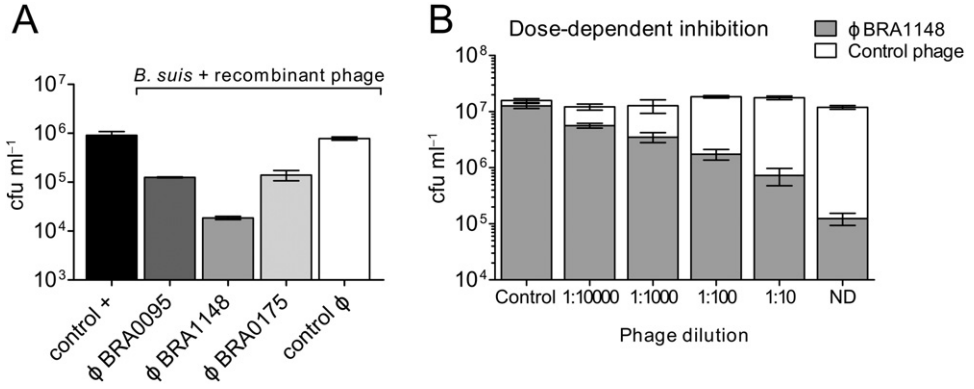

Fig. 2. A. Inhibition of *B. suis* binding to HeLa cells by the recombinant phage candidates. HeLa cell monolayers were infected with *B. suis* alone (control +), or with a mix of bacteria and recombinant phage. As a negative control an unrelated phage was included (control ϕ). Remaining adherent bacteria are expressed as colony-forming units per millilitre (cfu ml^{-1}).
B. HeLa cells were infected with mixtures of *B. suis* and suspensions of increasing titres of ϕ-BRA1148. Infection with *B. suis* alone was used as positive control, and the helper phage R408 was used as control phage. ND: non-diluted.

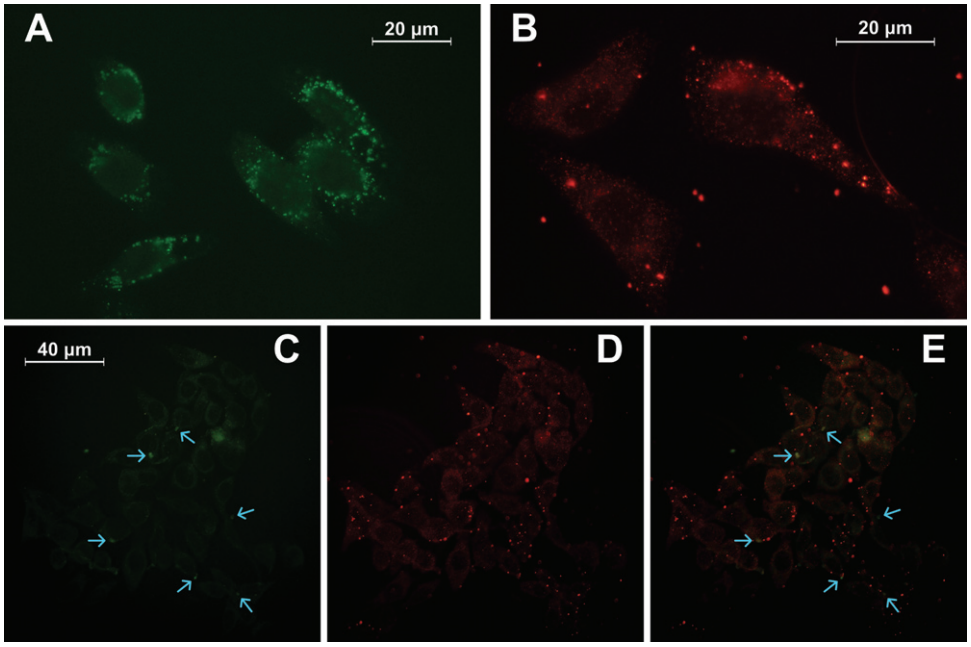

Fig. 3. Evaluation of the inhibition of *B. suis* binding to HeLa cells by ϕ-BRA1148 using immunofluorescence microscopy. HeLa monolayers were cultivated on a round coverslip in the bottom of plate wells and infected with GFP-labelled brucellae (moi of 100:1) in the absence or presence of ϕ-BRA1148 for 4 h. After elimination of non-adherent bacteria (and/or non-adherent phages), samples were fixed with paraformaldehyde and observed by CSLM.
A. HeLa cells infected with GFP-labelled brucellae (green).
B. HeLa cells incubated only with ϕ-BRA1148, immunodetected with anti-M13 and Cy3-conjugated anti-mouse antibodies (red).
C–E. HeLa cells infected with GFP-labelled brucellae in the presence of ϕ-BRA1148; (C) GFP-brucellae (green); (D) ϕ-BRA1148 (red); (E) merge. Remaining cell-associated bacteria are indicated by arrows.

cell surface after 1 (not shown) or 4 h (Fig. 3B), suggesting that the BmaC peptide interacts with the cell surface, probably through cell-associated fibronectin. As we predicted, the presence of ϕ-BmaC strongly reduced the number of cell-associated bacteria 1 h p.i. (not shown) and cell-associated or intracellular bacteria 4 h p.i. (Fig. 3C–E).

Taken together, these observations indicate that ϕ-BmaC was able to displace brucellae from the cell surface and strongly suggest that BmaC harbours adhesive activity to HeLa cells.

BmaC is a surface-associated protein and contains potential adhesin domains

Members of the type I (monomeric) autotransporter family share a common domain organization: an N-terminal secretion signal, a divergent and functional domain (passenger domain) and a conserved C-terminal region, which forms a typical β-barrel when inserted in the outer membrane (Henderson *et al*., 1998). It was shown that translocation of the passenger domain through the outer membrane of paradigmatic autotransporters is directed by the β-domain (Pohlner *et al*., 1987). The functional domain is liberated to the extracellular milieu or remains associated to the bacterial surface (Henderson and Nataro, 2001; Dautin *et al*., 2007). Autotransporters were proposed to be involved in the pathogenesis of several bacterial species, exhibiting diverse functions such as cytotoxicity, serum resistance, lipase activity, actin polymerization, adhesion to host cells and others (Stein *et al*., 1996; Nguyen *et al*., 2001; Henderson *et al*., 2004).

BmaC has all the three predicted features of autotransporters, i.e. a 72-amino-acid-long signal peptide, a 2700-amino-acid-long passenger domain and a C-terminal translocator β-domain (Fig. 4A). Analysis of the BmaC protein sequence using the domain-search tools of TIGR, Pfam and NCBI indicated that the passenger region contains several putative domains related to adhesion functions, including a pertactin domain (Leininger *et al*., 1991; Benz and Schmidt, 1992), a filamentose haemagglutinin domain (FHA) (Arico *et al*., 1993; Hannah *et al*., 1994) and several repetitive conserved motifs of the ShdA autotransporter that were proposed to be involved in the binding and persistence of *Salmonella enterica* serotype Typhimurium to the intestine (Kingsley *et al*., 2002; 2004a) (Fig. 4A). Interestingly, the portion of the BmaC passenger domain selected by phage display against fibronectin (Fig. 4A) showed 44% similarity with a conserved domain of the ShdA adhesin, which was shown to

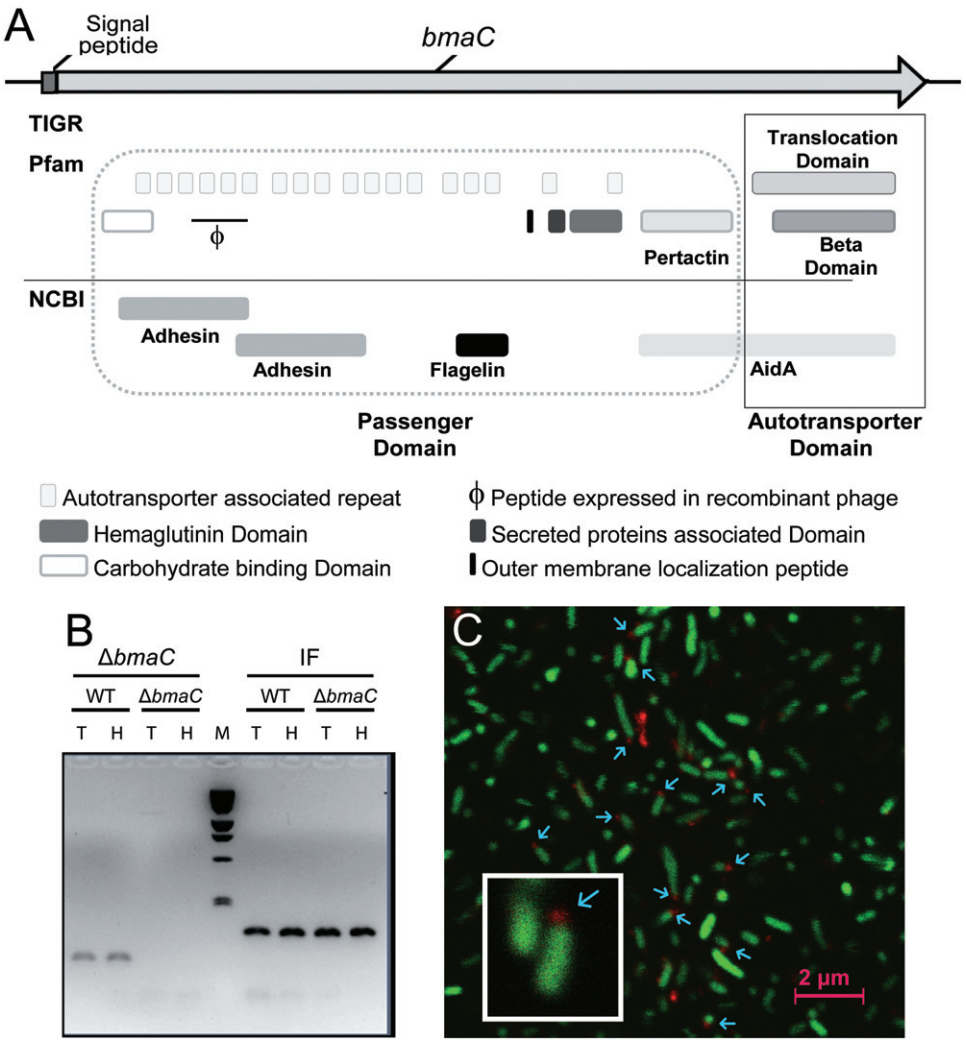

Fig. 4. A. BmaC protein domain organization; BmaC bears all the characteristic autotransporter domains. NCBI and PFAM prediction tools indicate that the passenger domain harbours several potential adhesion and repetitive motifs.
B. Expression of *bmaC* was evaluated by RT-PCR. The *bmaC* gene is expressed in the wild-type (wt) strain (but not in the Δ*bmaC* mutant) in TS broth (T) and in HeLa conditioned medium (H) (see *Experimental procedures*); the housekeeping IF gene was used as an internal control.
C. BmaC is a surface-exposed polar protein. Fixed wild-type GFP-brucellae were incubated with mouse anti-BmaC antisera and revealed with anti-mouse Cy-3-conjugated secondary antibody. Arrows in the image (and the inset) indicate the fluorescent signal (red) corresponding to BmaC.

bind fibronectin (Kingsley *et al*., 2002; 2004b). To note, ShdA, was also able to bind collagen (Kingsley *et al*., 2002). To investigate whether the portion of BmaC expressed on the phage exhibits affinity to other components of the ECM, the ability of the BmaC-phage to bind type I collagen, hyaluronic acid, and vitronectin was evaluated by panning (Fig. S4). Since some sialic acid-rich proteins were proposed to mediate adherence of brucellae to epithelial cells and macrophages (Rocha Gracia *et al*., 2002; Castañeda-Roldán *et al*., 2004), fetuin (a protein rich in sialic acid residues) and colominic acid (a homopolymer of *N*-acetylneuraminic acid) were also included in the assay. Interestingly, after panning, the numbers of BmaC-phages eluted from the wells coated with fibronectin and type I collagen were about 200- and 10-fold higher, respectively, than those eluted from wells coated with other ligands. This suggests that the BmaC peptide has more ability to bind fibronectin and type I collagen than the control ligand and other ligands included in the assay (Fig. S4). It was proposed that ShdA mimics the binding of fibronectin to heparin (Kingsley *et al*., 2004b). However, 100 µg ml^{-1} or 1 mg ml^{-1} heparin did not inhibit the binding of ϕ-BmaC to fibronectin (data not shown), suggesting that the interaction between the BmaC peptide and fibronectin does not involved a similar molecular mechanism.

To confirm the surface presentation of BmaC, immunofluorescence microscopy of GFP-labelled bacteria was

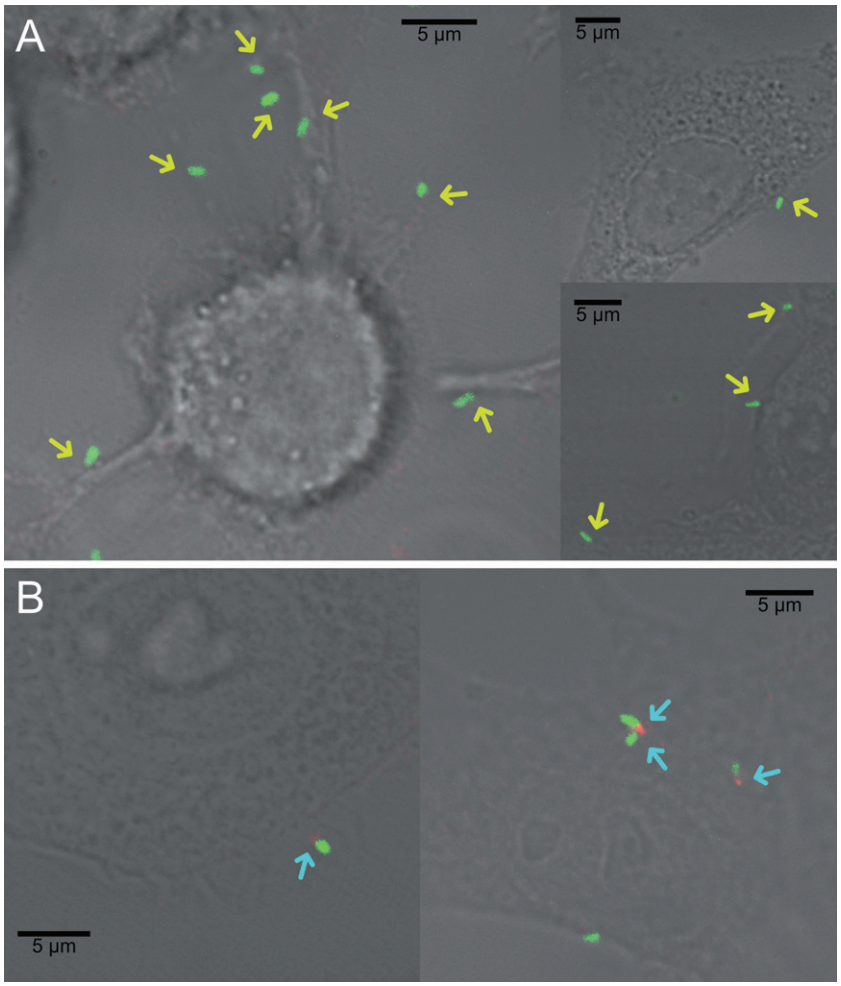

Fig. 5. Polar adhesion of *B. suis* to HeLa cells.
A. HeLa monolayers were cultivated on a round coverslip in the bottom of plate wells and infected with wild-type GFP-labelled brucellae. Samples were fixed with paraformaldehyde and observed by CSLM. Polar adhered bacteria are indicated by arrows.
B. BmaC protein exposed on the bacterial pole that interacts with the cell. Wild-type GFP-brucellae were seeded on HeLa cells. BmaC protein was detected by immunofluorescence. Arrows indicate the fluorescent signal (red) corresponding to BmaC.

performed. We first looked for conditions in which *bmaC* was expressed. RT-PCR showed that *bmaC* is expressed in both TSB and HeLa-conditional media (Fig. 4B). We took advantage of the phages expressing the BmaC peptide and used them to generate a polyclonal antiserum against BmaC in mouse. The presence of BmaC in whole cells and in the extracellular medium was analysed by Western blot using the anti-BmaC antibodies. A single band corresponding to the BmaC protein was observed by Western blot of whole-cell extracts (Fig. S5A). We did not detect any protein band in the extracellular protein fraction (not shown). These observations suggest that BmaC either is not cleaved after secretion or remains associated to the cell surface. An exponential culture of fixed wild-type GFP-*Brucella* was incubated with the mouse anti-BmaC-phage antisera and revealed with anti-mouse Cy-3-conjugated secondary antibody. A polar fluorescent signal (red) was observed in several but not all bacteria (Fig. 4C). Essentially all the cells with a detectable signal displayed BmaC at only one pole, indicating that BmaC (probably the passenger domain) is polarly exposed on the cell surface. This is not surprising since it was previously shown that several monomeric autotransporters are exposed on the bacterial surface at one pole (Charles *et al*., 2001; Jain *et al*., 2006). The observation that a considerable number of bacteria did not show a polar fluorescent signal may be due to a low level of *in vitro bmaC* expression and/or to some loss of the surface-associated passenger domain during the immunofluorescence procedure.

To investigate the relevance of the polar localization of BmaC, HeLa cells were inoculated with GFP-labelled *B. suis* and bacterial association with the cell surface was observed by confocal microscopy after 20 min of incubation. We observed different bacterial distributions on the cell surface; some small bacterial aggregates were observed in contact with the cell, especially in the boundary between cells. Single bacteria were found interacting through one of their poles with the cell surface on both the cell body and cell protrusions (Fig. 5A). Then we asked whether single bacteria are associated with the cells through the BmaC pole. By immunofluorescence, using antibodies against the BmaC fibronectin-binding peptide, we occasionally observed the red polar BmaC signal in

bacteria associated with the cell; in all cases the signal was located at the pole interacting with the cell (Fig. 5B). This low level of BmaC signal may be due to the difficulty of the anti-BmaC antibodies to access the fibronectin-binding peptide of BmaC, which we propose that is bound to cell-associated fibronectin (see below). In fact, we often observed a polar BmaC signal in bacteria that are not associated to the cell. Taken together, these observations support the idea that the polar localization of BmaC could be relevant in the interaction with host cells *in vivo*.

A deletion bmaC *mutant is impaired in the adhesion of* B. suis *to immobilized fibronectin and host cells*

In order to investigate the contribution of BmaC in the adhesion of *B. suis* to host cells, a clean deletion mutant of the *bmaC* gene was constructed. The absence of the *bmaC* gene transcript was confirmed by RT-PCR (Fig. 4B) and Western blot (Fig. S5A). Dot-blot analysis of purified whole membrane fractions showed a clear BmaC signal in the wild-type strain that was absent in the Δ*bmaC* mutant (Fig. S5B). The absence of BmaC on the surface of the Δ*bmaC* mutant was also confirmed by immunofluorescence (Fig. S3).

The ability of BmaC to mediate the binding of *B. suis* to fibronectin was tested in an *in vitro* binding assay. Wild-type, Δ*bmaC* or the KI *bmaC* (complemented) strains were added to the wells coated with immobilized fibronectin. The Δ*bmaC* mutant showed a 40-fold reduction in the number of bacteria bound to fibronectin compared with wild-type bacteria. A copy of the *bmaC* gene in the complemented strain restored the binding to wild-type levels (Fig. 6). We also evaluated whether the fibronectin-binding peptide expressed on the phage surface (ϕ-BmaC) competes for the binding of the wild-type *B. suis* to fibronectin. While a control phage did not have any effect on the binding of *B. suis* to fibronectin, ϕ-BmaC reduced this binding by about 15-fold (Fig. 6). These results provide further evidence that BmaC mediates the attachment of *B. suis* to immobilized fibronectin.

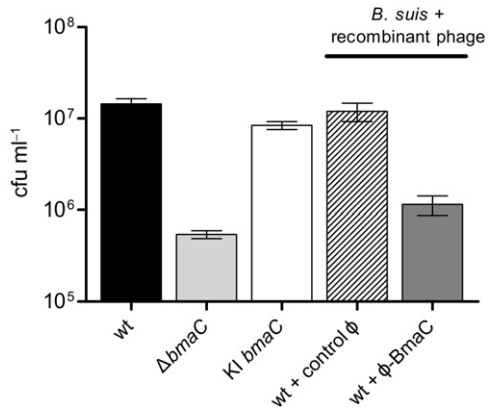

Fig. 6. *In vitro* binding of *B. suis* to immobilized fibronectin. TSB cultures of the wild type, Δ*bmaC* mutant or KI *bmaC*-complemented strain were added to wells coated with Fn. Adherent bacteria were harvested and cfu ml^{-1} were determined. Inhibition of binding with recombinant phage: a mix of wild-type culture and recombinant phages (control ϕ: helper phage; ϕ-BmaC: phage displaying a portion of BmaC protein) was added to Fn-coated wells and adherent bacteria were determined.

The ability of the Δ*bmaC* mutant of *B. suis* to attach, invade and replicate intracellularly was evaluated in a HeLa cell infection assay. At 1 h p.i., the Δ*bmaC* mutant showed a marked reduction (45-fold) in the binding to HeLa cells compared with the wild-type strain (Fig. 7A). Intracellular bacteria also decreased in cells infected with the Δ*bmaC* mutant compared with the wild-type strain (Fig. 7A). However, the relative number of intracellular bacteria (intracellular/adherent) was almost identical in both strains (Fig. 7A), thus indicating that the process impaired in the Δ*bmaC* mutant was the attachment to the epithelial cells and not internalization. To confirm that Δ*bmaC* is compromised in the binding to HeLa cells, a competition experiment with the wild-type strain was performed. A monolayer of HeLa cells was infected with equal bacterial numbers of both isogenic strains, and adherent and intracellular bacteria were determined as described in *Experimental procedures*. We observed that the wild-type strain outcompeted the Δ*bmaC* mutant in the binding to HeLa cells (Fig. 7B). Furthermore, after cell lysis only wild-type intracellular bacteria were recovered (Fig. 7B). These observations show that the BmaC adhesin confers a competitive advantage to adhere and, as a consequence, invade HeLa cells. Analyses of intracellular replication at longer incubation times (4, 24 and 48 h p.i.) revealed that, although the initial number of intracellular bacteria (1 and 4 h p.i.) decreased in the Δ*bmaC* mutant, no differences between the intracellular replication rates of the wild-type and the mutant strain were found (Fig. S6B). This observation indicates that once the Δ*bmaC* mutant reaches the intracellular compartment, replication proceeds as in the wild type. To provide final phenotypic confirmation, a *bmaC* knock-in complemented strain was included in the assay. Introduction of a wild-type copy of *bmaC* gene fully complemented the ability of this strain to attach and invade HeLa cells (Fig. 7A and Fig. S6B).

To further investigate the role of fibronectin in the interaction of *Brucella* with host cells we evaluated the ability of increasing dilutions of anti-fibronectin antibodies (anti-Fn) to inhibit the attachment of wild type and the *bmaC* mutant to HeLa cells. Anti-Fn inhibited the binding of the wild-type strain to HeLa cells in a dose-depending manner, by about 65-fold, indicating that cellular fibronectin participates in the binding of *B. suis* to HeLa (Fig. 7C). Surprisingly, the binding of the *bmaC* mutant to HeLa cells

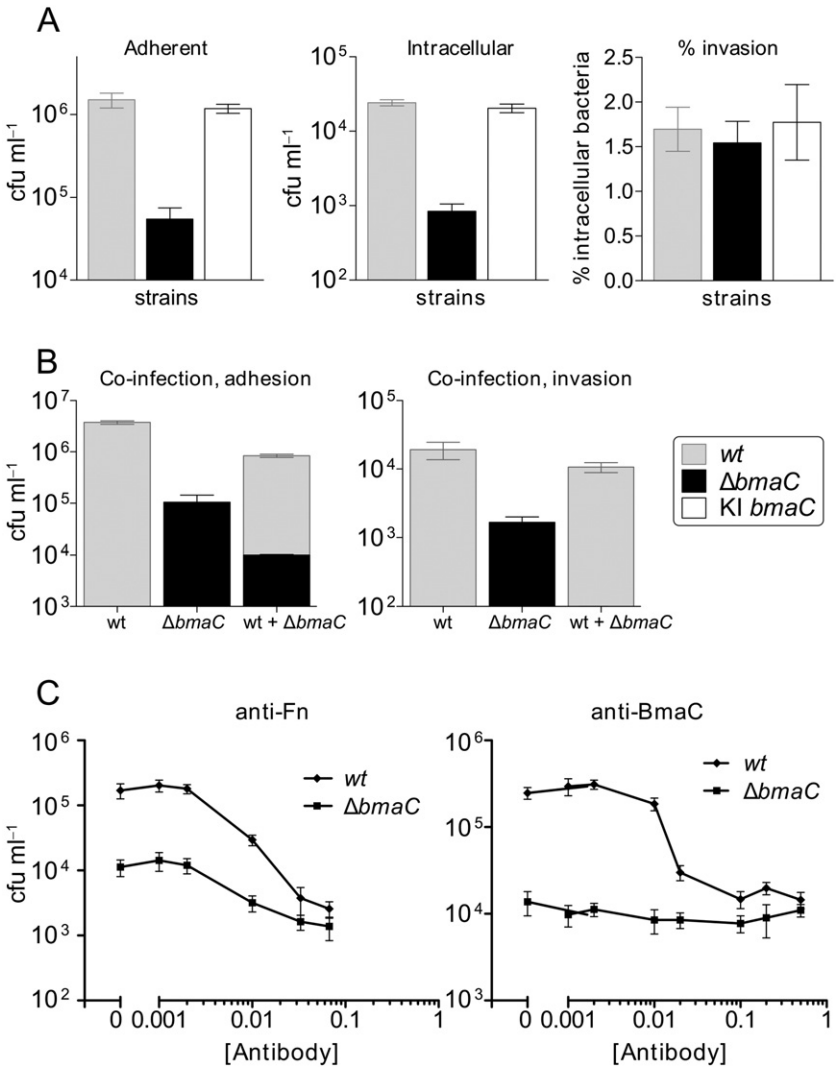

Fig. 7. Adherence of *B. suis* strains to HeLa cells.
A. HeLa cells were infected with the *B. suis* wild-type strain, the Δ*bmaC* mutant or the KI *bmaC*-complemented strain at a moi of 50. One hour p.i., the number of adherent and intracellular cells of each strain was evaluated. The invasion percentage (respect adherent) of the three strains remained unchanged.
B. In a competition experiment, HeLa cells were co-infected with equal number (cfu) of the wild type and the Δ*bmaC* mutant. Adherent and intracellular bacteria (wt and Δ*bmaC*) were determined as described in *Experimental procedures*.
C. Inhibition of *B. suis* binding to HeLa cells by antibodies. HeLa cells were infected with the wild-type strain or the Δ*bmaC* mutant as described before. Serially diluted antibodies were used to inhibit the binding. Anti-Fn: monoclonal anti-cellular fibronectin antibody (dilutions 1:10 to 1:1000). Anti-BmaC: polyclonal anti-BmaC antibody (dilutions 1:2 to 1:1000). Adherent bacteria were harvested and cfu ml^{-1} were determined.

by anti-Fn was also moderately inhibited (by eightfold) reaching levels similar to those of wild-type bacteria in the presence of anti-Fn. These observations indicate that fibronectin-dependent binding of *B. suis* to cells is mainly mediated by BmaC, but other bacterial ligands may contribute to the interaction of *B. suis* with fibronectin. We also tested the effect of anti-BmaC on the binding of the wild-type and mutant strains to HeLa cells. As expected, anti-BmaC had no effect on the binding of the Δ*bmaC* mutant but significantly reduced the binding of wild-type bacteria to HeLa cells to levels similar to the Δ*bmaC* mutant (Fig. 7C). Taken together, these results indicate that BmaC participates in the attachment of *B. suis* to host cells via fibronectin binding.

To validate the relevance of BmaC in the interaction of *B. suis* with host cells, another non-phagocytic cell culture model was tested. A monolayer of A549 epithelial cells (human type II alveolar) was infected with the wild type, the Δ*bmaC* mutant and the complemented strain and adherent and intracellular bacteria were evaluated. It was recently shown that smooth *Brucella* strains are able to attach, invade and replicate within these cells (Ferrero *et al.*, 2009). Similar to the phenotype in HeLa, the mutant was compromised in the attachment to A549 cells (Fig. 8). These observations support the concept that BmaC participates in the attachment of *B. suis* to non-phagocytic cells.

Finally, we also evaluated whether BmaC is involved in the interaction of *B. suis* with professional phagocytes. Binding assays using a monolayer of murine J774.1A macrophages showed that the Δ*bmaC* mutant adheres to the same extent than the wild type 20 min, 30 min and 60 min p.i. (not shown). As expected, similar biphasic curves of viable intracellular brucellae were observed over a 48 h course experiment of macrophages infected with the wild type, the Δ*bmaC* and the complemented strain (Fig. S6A), indicating that BmaC is not crucial for intracellular survival in the J774.1A cell line model.

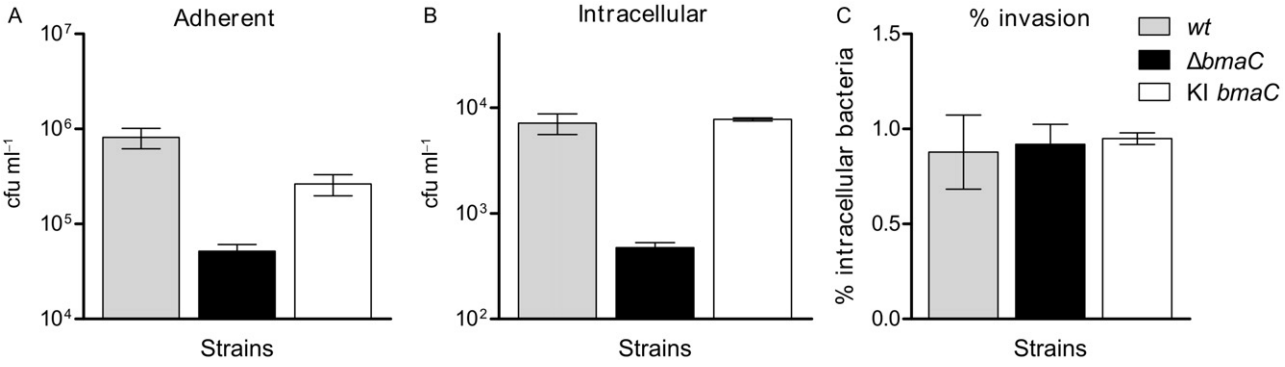

Fig. 8. The Δ*bmaC* mutant is impaired in the adherence to A549 epithelial cells.
A and B. A549 cells were infected with the *B. suis* wild-type strain, the Δ*bmaC* mutant or the KI *bmaC*-complemented strain and the number of (A) adherent and (B) intracellular cells of each strain was evaluated.
C. The invasion percentage (respect adherent) of the three strains remained unchanged.

Discussion

Host colonization by pathogenic bacteria requires binding to the host with sufficient strength either for not to be removed by the host fluids or for efficiently invade the eukaryotic cell. It was widely reported that several bacteria have evolved a broad range of molecules to promote adhesion to selected host molecules. As mentioned earlier, among proteins from the ECM, *Brucella* 'prefers' to bind fibronectin (Castañeda-Roldán *et al*., 2004). As a result of the selection process we used, which involved affinity panning against fibronectin, three recombinant phages (ϕ-BRA0095, ϕ-BRA0175 and ϕ-BmaC) were selected for further analysis. It was noted that while all three recombinant phages were capable of interacting with fibronectin, peptides exposed on the phage surface did not share a significant percentage of identity (not shown). Therefore, a common binding domain to fibronectin expressed by several adhesins of *B. suis* does not arise from these experiments, as it was observed in other known pathogens (Foster and Hook, 1998; Jacobsson, 2003; Giomarelli *et al*., 2006). Since the entire molecule of fibronectin was used as immobilized ligand, it is possible that each candidate interacts with different protein moieties (Schwarz-Linek *et al*., 2004). In addition, it was shown that fibronectin is able to bind bacterial proteins through peptide-peptide or carbohydrate-peptide interactions, involving different fibronectin domains (McKeown-Longo, 1987); thus, the possibility exists of more than one interaction mechanism between *B. suis* adhesins and the fibronectin molecule.

The use of antibodies or recombinant proteins to block the interaction of microorganisms with their host has been extensively reported; however, the use of recombinant phages to inhibit adhesion of bacterial pathogens to host cells is, to our knowledge, a novel strategy. Remarkably, the recombinant phages expressing portions of BRA0095, BRA0175 or BmaC were capable of altering the interaction between *B. suis* and HeLa cells to varying degrees, being ϕ-BmaC the phage that achieved the more efficient binding inhibition. These observations indicate that the fibronectin-binding peptides have affinity for some host ligand/receptor on the host cell. Since fibronectin can be certainly associated with the surface of different types of eukaryotic cells, including HeLa (McKeown-Longo, 1987; Wierzbicka-Patynowski and Schwarzbauer, 2003), the simplest explanation for these observations is that ϕ-BmaC (and probably ϕ-BRA0095, ϕ-BRA0175) prevents *Brucella* interaction with cell-associated fibronectin molecules. The observation that the binding of wild-type *B. suis* to immobilized fibronectin was inhibited *in vitro* by ϕ-BmaC supports this idea.

Further evidence presented in this work showed the adhesive roles of BmaC. First, adherence of *B. suis* to immobilized fibronectin or non-phagocytic cells (HeLa and A549) was significantly reduced by *bmaC* deletion; second, the Δ*bmaC* mutant was outcompeted by the wild type for adhesion to culture cells; and third, binding of *B. suis* to culture cells was inhibited by both anti-fibronectin and anti-BmaC antibodies. Taken together, our results demonstrate that BmaC mediates the binding of *B. suis* to the ECM and to non-phagocytic cells via cell-associated fibronectin.

BmaC belongs to the type I (monomeric) autotransporter protein family. *In silico* analysis clearly indicated that BmaC has several potential adhesion motifs within the functional/passenger domain that could be indeed exposed on the bacterial surface (Henderson *et al*., 2004; Dautin and Bernstein, 2007). Immunofluorescence assays confirmed that BmaC is a surface-exposed protein, supporting the hypothesis of a true adhesin. In addition, similar to other monomeric autotransporters, BmaC was located at one bacterial pole. It was suggested that orientation of the bacterial body may be relevant to

pathogen–host interactions (Jain *et al*., 2006). In fact, our observations indicate that single bacteria interact through one pole with the cell surface, probably the pole where BmaC is located. Interestingly, it was previously shown that *B. abortus* interacts with the cell surface through one pole (Pizarro-Cerdá *et al*., 1999). Therefore, it seems plausible that the polar binding might be a general feature of the *Brucella* initial interaction with host cells.

Some bacterial adhesins were also able to trigger tissue invasion (Pizarro-Cerda and Cossart, 2006). However, our results suggest that the decrease in cell internalization in the Δ*bmaC* mutant was due to impaired adhesion, indicating that the functional role of BmaC is restricted to the initial stages of infection to host cells.

It was previously reported that another protein from the type I autotransporter family (OmaA) influences survival of *B. suis* in the chronic phase of infection in the murine model (Bandara *et al*., 2005). Although the passenger domain of this protein shows more similarity to autotransporters functionally characterized as adhesins, the role of OmaA in the attachment to host cells has not been investigated yet. It would be interesting to evaluate in the future if BmaC, OmaA or other proteins from the autotransporter family of *B. suis* have complementary roles in the interaction with the host.

Interestingly, the peptide expressed on ϕ-BmaC (corresponding to 540–688 residues), showed 44% similarity to the A2-B8-A3-B9 repeat region of the ShdA autotransporter of *S. enterica* Serotype Typhimurium, which was shown to be the primary fibronectin binding site of this protein (Kingsley *et al*., 2004b). It was shown that ShdA binds to thin sections of the murine caecum with a similar pattern as fibronectin. Immunohistochemical analysis further supported that the binding of ShdA to receptors in the ECM may be a mechanism for persistent intestinal carriage of *S. enterica* Serotype Thyphimurium in mice (Kingsley *et al*., 2002). The FnIII repeat module of the Hep-2 domain of fibronectin was proposed as the binding site for ShdA adhesion, through a mechanism that may mimic binding of heparin (Kingsley *et al*., 2004b). Comparison of the BmaC and ShdA passenger domains showed that three regions of the BmaC passenger domain display similarities of around 42% with a region located between the 600 and 1200 amino acid residues of the ShdA protein, which comprises almost entirely the repeat region of the ShdA passenger domain (not shown). These observations reinforce the notion of BmaC as a fibronectin-binding protein. Similar to the role of ShdA of *Salmonella*, binding of *B. suis* to components of the ECM (independently of cell attachment) may represent a relevant mechanism to facilitate microbial colonization and dissemination in different clinical situations where damage tissue can expose the ECM. One of such scenarios is *Brucella* endocarditis, which is the main cause of brucellosis-related mortality (Reguera *et al*., 2003). Attachment of other bacterial pathogens to the ECM underlying the endothelium was proposed to contribute to the establishment of bacterial endocarditis in humans (Clarke and Foster, 2006; Sillanpaa *et al*., 2008). Interestingly, it was recently reported that *B. abortus* is able to replicate inside endothelial cells (Ferrero *et al*., 2011), which could also contribute to the destructive lesions of *Brucella* endocarditis (Jeroudi *et al*., 1987; Inan *et al*., 2010).

Although we presented several evidences supporting the idea that BmaC interacts with the surface of host cells through cell-associated fibronectin, the possibility that other subdomains of the passenger domain of BmaC recognizes other receptor/ligand on the cell surface cannot be excluded. In fact, while the fibronectin-binding domain isolated by phage display is only 113-amino-acid-long, the whole passenger domain is 2700-amino-acid-long (297 kDa) and exhibits several other putative adhesion motifs (Fig. 4A). One possibility is that interaction of the BmaC fibronectin-binding domain to cell-associated fibronectin acts to stabilize the interaction of other adhesion domain of BmaC with an additional host receptor. Other possibility is that the fibronectin molecule may act as a bridge between a host receptor and BmaC. Alternatively, BmaC may be a multifunctional adhesin, able to contribute to the adherence of *B. suis* to the ECM and different host cell ligands during the course of infection.

Several analyses revealed a remarkable similarity between the genomes of different species of *Brucella* (Rajashekara *et al*., 2004; Ficht, 2010). It was proposed that differences in pathogenicity, tissue tropism and host preferences may be the consequence of dissimilar gene inactivation of cell surface components, as a result of the process of adaptation to a specific animal host (Chain *et al*., 2005a). These types of adaptive species-specific mechanisms were proposed for other pathogens, such as *Yersinia* (Parkhill *et al*., 2001) and *Bordetella* (Parkhill *et al*., 2003). Comparison between *B. suis* and *B. melitensis* genomes indicated that some of the few regions with significant differences correspond to genes encoding predicted surface proteins (Paulsen *et al*., 2002). Based on sequence analysis, we found that genome areas of all three candidates isolated by phage display exhibit variability among species greater than the overall average variability (Rajashekara *et al*., 2004). The BmaC autotransporter appears to have functional orthologues in *Brucella microti* (BMI_II1154), *Brucella ovis* (BOV_A1053), *B. abortus* (BAB2_1107) and in all the marine species, while it is clear that the homologue gene is likely inactive in *Brucella canis*, due to inactivating nonsense mutations. In *B. melitensis,* the homologue gene (BMEII0148/9) also differs from its *B. suis* counterpart,

since it carries several mutations, which probably make the BMEII0148/9 inactive. In addition, the presumed 'functional' BmaC orthologues show a range in the overall similarity to BmaC, from 99% for BOV_A1053 from *B. ovis*, to 48% for some portions of BMEII0148 from *B. melitensis* (not shown). Thus, BmaC (and its orthologues) could be part of a set of surface factors that might contribute to host preference.

Viadas *et al*. used DNA microarrays to determine global profiles of transcription in *Brucella* spp. The *bmaC* orthologue of *B. abortus* showed a very low level of expression *in vitro* (Viadas *et al*., 2009) but was upregulated in the *bvrR/bvrS* mutant background (Viadas *et al*., 2010). These authors suggested that some groups of genes with low level of *in vitro* expression could be required for the intracellular life, or even encoding virulence factors whose expression is activated upon contact with host factors. A subset of these genes would be regulated by the BvrR/BvrS two-component system to adjust the *Brucella* physiology to the shift expected to occur during the transit from the extracellular to the intracellular niche (Viadas *et al*., 2010). In *B. suis*, we found that *bmaC* is expressed in both TSB and HeLa-conditioned media. It will be interesting to investigate in the future whether *bmaC* is regulated by conditions that mimic the extracellular or intracellular host environment and if *bmaC* expression increases upon contact of the bacteria with the cell surface.

Some adhesins (especially those from the autotransporter families) participate in bacterial self-association and attachment to abiotic surfaces in addition to bacterial adherence to different cell types. AidA and Ag43 from *Escherichia coli* are archetypal examples of this double function (Henderson *et al*., 1997; Sherlock *et al*., 2004). In the case of the BmaC autotransporter, we did not found any evidence of this dual function; in fact, the Δ*bmaC* mutant was able to autoaggregate as well as the wild type, and the attachment to abiotic surfaces was not impaired (not shown).

Evidences presented in this work and other previously reported (Rocha Gracia *et al*., 2002; Castañeda-Roldán *et al*., 2004; 2006) indicate that special attention should be given to the mechanisms underlying adhesion of *Brucella* to components of the ECM and host cells. Besides, it will be necessary to investigate the impact of BmaC (and the other candidates identified in this work) in the virulence of *B. suis* using an animal model, such as mice (Silva *et al*., 2011). Future work in our lab will be also directed to determine whether the presence of a functional BmaC protein orthologue in the different species of *Brucella* and/or the differences within the passenger domain of the different BmaC homologues contribute to host or tissue specificity and the clinical manifestations of brucellosis. It will be also interesting to dissect the BmaC protein and explore the role of subdomains of the passenger domain in the interaction with the host.

Experimental procedures

Bacterial strains and growth conditions

Brucella strains used in this study (*B. suis* M1330 [ATCC 23444] and derived strains) were all grown in tryptic soy broth (TSB, Bacto) medium with the appropriate antibiotics (nalidixic acid, 10 μg ml^{-1}; chloramphenicol, 6 μg ml^{-1} and kanamycin 25 μg ml^{-1}). *E. coli* strains DH5α, Top10 and TG1 were used as the recipient strains for cloning and were routinely grown in Luria–Bertani (LB) medium. Antibiotics (ampicillin, 50 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1} and kanamycin, 50 μg ml^{-1}) were added when needed. The standard growth temperature for all bacterial strains was 37°C.

Cell infection assays

Murine macrophagic J774A.1 cells (ATCC TIB-67), HeLa or lung epithelial A549 (ATCC CCL 185) cells were seeded in 24-well plates (5×10^4 cells per well) and inoculated (at moi 20:1 and moi 50:1 or 100:1 respectively) with the wild-type *B. suis* M1330, the Δ*bmaC* mutant or the complemented strain (KI *bmaC*). Multiwell plates were placed in a 5% CO_2 atmosphere at 37°C. After 1 h for HeLa and A549 cells or 20 min, 30 min and 60 min for J774A.1, wells were washed three times with phosphate-buffered saline (PBS) (pH 7.4) and the number of total cell-associated *B. suis* bacteria was determined: cells were treated for 10 min at 37°C with 0.1 ml of 0.1% Triton X-100 in deionized sterile water, and lysates were serially diluted in PBS and plated on TS agar with the appropriate antibiotic to determine cfu.

To determine the number of intracellular viable *B. suis* bacteria, a standard gentamicin protection assay was performed. Briefly, cells were infected as mentioned above; after 1 h incubation wells were washed three times with PBS and further incubated with cell culture medium containing 50 μg ml^{-1} gentamicin and 50 μg ml^{-1} streptomycin to eliminate remaining extracellular brucellae. After 1 h, cells were washed three times with PBS and treated for 10 min with 0.1 ml of 0.1% Triton X-100 as described above. Lysates were serially diluted in PBS and plated on TS agar with the appropriate antibiotic to determine intracellular cfu. The number of adherent bacteria was calculated as the difference between total cell-associated bacteria and intracellular bacteria.

For the competition experiment, HeLa cells were seeded in 24-well plates (5×10^4 cells per well) and infected with equivalent cfu of the wild-type *B. suis* M1330 and the Δ*bmaC* mutant at moi 50:1. After 1 h, wells were washed and adherent and invasive bacteria were determined as described before. Nalidixic acid was used as selective agent to distinguish between the unmarked wild-type strain and the nalidixic-resistant Δ*bmaC* mutant.

Cloning and generation of bmaC *unmarked deletion mutant from* B. suis

A deletion mutant in the *B. suis bmaC* gene was generated: PCR primers with new restriction enzymes sites in the 5′ and 3′ ends

were designed to amplify the flanking regions of the *bmaC* gene. Primers 5′-GAGCATGCGTCTAGGCGACGTTCTG-3′ and 5′-GGTACCACTCAAAATGTAGCCCGTC-3′ were used to amplify 700 bp of the upstream region of *bmaC* and primers 5′-GGTACCACGGCTCTATCGAATCGT-3′ and 5′-GTCGACTCATAGGTTCCACCCAGA-3′ were used to amplify 600 bp of the *bmaC*-downstream region. New restriction sites are underlined. Amplicons containing the flanking regions of *bmaC* were cloned into the pGEMTeasy vector, cleaved with the corresponding enzymes and ligated together into the pK18*mobsacB* mobilizable suicide vector (Km resistant) (Schafer *et al*., 1994) to make the p∆*bmaC* plasmid. To create the deletion *bmaC* mutant the p∆*bmaC* plasmid was conjugated to a nalidixic-resistant derivative of the wild-type strain *B. suis* M1330, and double recombinant clones were selected (Nal^R, Km^S, sucrose resistant). The absence of the entire open reading frame in the mutant was confirmed by PCR. In order to provide final phenotypical confirmation, a *bmaC* knock-in complemented strain was generated. The complete open reading frame and putative regulatory sequences were amplified using KOD HiFi polymerase (Novagen) and cloned into TOPO vector using TOPO XL-PCR cloning kit (Invitrogen). Reintroduction of a wild-type copy of the gene was assessed by mobilizing the *bmaC* gene, cloned in a TOPO vector, into the *B. suis* ∆*bmaC* mutant and simple recombinants kanamycin resistant were selected. Successful restoration of the ORF in the knock-in strain was confirmed by PCR.

Library construction

The phage library was constructed as described previously (Jacobsson *et al*., 2003). The display system is based in in-frame fusions to the pVIII M13 protein. Briefly, genomic DNA of *B. suis* was purified and sheared by sonication; the genomic DNA fragments were blunt ended by using T4 DNA polymerase (New England Biolabs). Two micrograms of SnaBI-digested and dephosphorylated pG8SAET vector (Jacobsson *et al*., 2003) were ligated to 6 μg of *B. suis* sonicated genomic DNA (fragments between 0.7 and 5 kb). Ready-To-Go T4 ligase (Amersham Pharmacia Biotech) was used in the ligation reaction. The ligated material was phenol- and chloroform-extracted, ethanol-precipitated and resuspended in 2 μl of H_2O. One microlitre of the ligation mix was electrotransformed into high efficiency *E. coli* TG1, and the transformed cells were grown overnight (ON) in 10 ml of LB ampicillin medium. A 0.5 ml aliquot of the ON culture was infected with helper phage (HP) R408 (Promega, moi 100:1), mixed with 5 ml of 0.5% soft agar and poured onto Luria agar (LA) ampicillin plates. Phages were eluted from the agar by incubating the soft agar in LB medium with shaking. Remaining bacteria were eliminated by sterile filtration (0.45 μM filter). Electrotransformation of the ligation mix into *E. coli* TG1 yielded 9×10^5 ampicillin-resistant clones and approximately 85% of the clones contained foreign inserts. The titre of phagemid particles in the final library was 6×10^{11} cfu ml^{-1}.

Panning procedures

Panning steps were performed as described previously (Jacobsson *et al*., 2003). Briefly, multiwell plates (MaxiSorp, Nunc) were coated for 2 h at room temperature with a solution of the ligand (concentration 100 μg ml^{-1}). The wells were blocked with 150 μl of 1% bovine serum albumin (BSA) in PBS for 2 h at room temperature. A portion of the library (200 μl) was added to the wells and incubated for 4 h to overnight. After extensive washing (25–30) with PBS-T (PBS with 0.05% Tween 20), bound phages were eluted by decreasing pH followed by neutralization, or by adding free ligand. The eluted phage were amplified by *E. coli* TG1 infection: the eluate was used for infection of *E. coli* TG1 cells (ON culture), which were plated on LA supplied with ampicillin. The clones obtained were resuspended in LB and were infected with HP (moi 100:1), for production of a phage stock for repanning. The second and third enrichment cycles (repanning) were made according to the same protocol but with a 2-h-long panning. Human fibronectin was from Sigma-Aldrich.

Preparation and titration of phage stocks

Stocks of phages were prepared by infecting 2 ml of mid-exponential-phase *E. coli* TG1 containing the recombinant phagemids with the HP R408 at a multiplicity of infection of 100. After 30 min of incubation at 37°C, the infected cells were added to 13 ml of LB containing ampicillin and grown ON at 37°C. The phage particles were recovered from the culture supernatant and sterilized by filtration using a 0.45-μm-pore-size membrane. The number of phages was determined as cfu after infecting *E. coli* TG1 grown to mid-exponential phase with the phage suspension. After 60 min of incubation at 37°C, cells were plated onto LB containing ampicillin. Resistant colonies were counted for titre determination.

Phage ELISA

Phage stocks were used in phage ELISAs. Maxisorp immunoplates were coated with fibronectin, or control ligands, blocked for 1 h at room temperature with 1% BSA in PBS and washed five times with PBS, 0.05% Tween 20. Phage particles were diluted serially into ELISA buffer (PBS, 1% BSA, 0.1% Tween 20) and 150 μl aliquots were transferred to coated wells. After 1 h, plates were washed 15 times with PBS, 0.05% Tween 20, incubated with 100 μl of 1:1000 horseradish peroxidase anti-M13 antibody conjugate (Sigma) in ELISA buffer for 30 min, and then washed five times with PBS, 0.05% Tween 20 and twice with PBS. Plates were developed using an OPD peroxidase substrate system (OPD 2 mg ml^{-1}, H_2O_2 0.03%). The reaction was stopped with 50 μl of 4N H_2SO_4 and absorbance at 485 nm was measured in a Beckman-Coulter plate reader.

Binding activity of recombinant phage determined by panning

Wells of Nunc Maxisorp microtitre plates were coated for 2 h at room temperature with 0.2 ml aliquots of 100 μg ml^{-1} solutions of the ligands dissolved in PBS. Each well was blocked using 0.1 ml of PBS, 1% Tween 20 for 1 h. Afterwards, the wells were rinsed three times with PBS containing 0.05% Tween 20 and then with PBS. An aliquot of 0.1 ml of a 1×10^{10} cfu ml^{-1} phage suspension in PBS was later added to each well and incubated for 2 h. Unbound phages were removed by five washes with 250 μl of PBS containing 0.05% Tween 20, followed by five washes with 0.2 ml of PBS. The bound phages were eluted with 200 μl of

50 mM Na-citrate and 140 mM NaCl (pH 2.0) for 10 min and then neutralized with 50 μl of 2 M Tris-HCl buffer (pH 9.0). The eluate was used for infection of *E. coli* TG1 cells as described above. The number of cfu obtained for each ligand was determined by triplicate.

Inhibition of Brucella *binding to HeLa cells by recombinant phages*

HeLa cells were seeded in 24-well plates (5×10^4 cells per well) as described before and inoculated alternatively with the *B. suis* wild-type strain (moi 50:1) or a mix of the bacteria and each recombinant phage (moi 50:1, phage titre 1×10^{10} cfu ml^{-1}); HP was used as control phages (1×10^{10} cfu ml^{-1}). Multiwell plates were placed in a 5% CO_2 atmosphere at 37°C. After 1–4 h, wells were washed three times with PBS and the number of total cell-associated *B. suis* bacteria was determined by serial dilution and plate counting on TS agar with the appropriate antibiotics.

Immunofluorescence microscopy

An EcoRI-1041 bp fragment from the pHC60 plasmid, containing the GFP gene was cloned under the constitutive *tac* promoter into the EcoRI site of the pBBR1MCS-2 polylinker (Km^R) (Kovach *et al*., 1995) to generate pBBR1MCS-2-GFP. For detection of BmaC on the surface of *B. suis*, 1 ml of TSB cultures of *B. suis* M1330 harbouring the pBBR1MCS-2-GFP plasmid was collected, washed once with PBS and incubated 30 min with a paraformaldehyde 3.7% solution for fixing. The cells were washed three times with PBS and incubated 2 h with anti-BmaC antibodies (diluted 1:50 in PBS containing 0.5% BSA). Then the cells were washed again and incubated with CY3-conjugated donkey anti-mouse antibody preparation (Jackson ImmunoResearch, diluted 1:250 in PBS containing 0.5% BSA) for 40 min. After two washes with 1 ml of PBS-BSA and one with PBS alone, an aliquot of the cell suspension was mounted with Mowiol 4-88 (Calbiochem).

For detection of BmaC displaying phages in cell cultures, HeLa cells were seeded on coverslips in 24-well plates (1×10^4 cells per well) and inoculated alternatively with the GFP-marked wild-type strain (*Bs*GFP, moi 50:1), phages displaying BmaC (1×10^{10} cfu ml^{-1}), HP as control phages (1×10^{10} cfu ml^{-1}) or a mix of wild-type bacteria and the BmaC phages. Multiwell plates were placed in a 5% CO_2 atmosphere at 37°C. After 1–4 h, wells were washed three times with PBS and covered with a paraformaldehyde 3.7% solution for fixing. The cells were washed three times with PBS and incubated 2 h with mouse anti-M13 antibodies (Sigma, diluted 1:100 in PBS containing 0.5% BSA). Then the cells were washed again and incubated with CY3-conjugated donkey anti-mouse antibody preparation (Jackson ImmunoResearch, diluted 1:250 in PBS containing 0.5% BSA) for 40 min. After two washes with 1 ml of PBS-BSA and PBS, coverslips were mounted with Mowiol.

To analyse polar binding of *B. suis* to HeLa cells and for immunodetection of polar BmaC protein in cell cultures, HeLa cells were inoculated with GFP-labelled bacteria (moi 500:1) and incubated for 20 min as previously described. Immunofluorescence protocol was performed on fixed samples. Anti-BmaC antibodies (diluted 1:50 in PBS containing 0.5% BSA) and CY3-conjugated donkey anti-mouse antibody preparation (Jackson ImmunoResearch, diluted 1:250 in PBS containing 0.5% BSA) were used as described before. Samples were observed in a Pascal CSLM. All incubations were carried out at room temperature.

Reverse transcription (RT)-PCR analysis of bmaC *expression*

Overnight cultures of *B. suis* and derived mutant grown in TSB or HeLa conditioned media were harvested by centrifugation and total cellular RNA was extracted by using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The purified RNA was treated with RQ1 Rnase-Free DNase (Promega), and cDNA was prepared from 1 μg of RNA by using random hexamers and Superscript III (Invitrogen). PCR detection of the *bmaC* gene was performed by using 1 μl of the cDNA and the oligonucleotides 5′-AACTTGGCGCACAACTAACC-3′ and 5′-ATAGGTGCTGTCGGAATTGG-3′, which were designed to amplify a 97 bp region of *bmaC*. The products of the RT-PCR were analysed by agarose gel electrophoresis. HeLa conditioned medium was obtained by incubating comercial DMEM medium complemented with 5% fetal bovine serum with a monolayer of semiconfluent HeLa cells during 16 h. Conditioned medium was then filtered through a 0.2 μm millipore filter and inoculated for bacterial growth.

BmaC detection by Dot blot

Total membranes from *B. suis* wild-type strain and *ΔbmaC* mutant were obtained by mechanical disruption using Precellys and ultracentrifugation of 50 ml of TSB cultures. Membrane vesicles were resuspended in 1 M Tris-HCl buffer (pH 8.0). The membrane fractions were spotted on nitrocellulose filters and blocked by incubating in PBS containing 3% BSA for 1 h and then incubated for 2 h with a 1:2000 dilution of anti-BmaC mouse polyclonal antibody. The nitrocellulose filters were washed five times with PBS containing 1% BSA and then incubated with HRP-conjugated goat anti-mouse (Sigma) at a 1:1000 dilution. The filters were washed as described above and the ECL-plus (Amersham) detection reagents were added; signal detection was performed in a STORM imager 840 optical scanner (Amersham Pharmacia Biotech).

BmaC detection by Western blot

Whole extracts of the wild type, Δ*bmaC* mutant and complemented strain were resuspended in 0.5 ml of loading buffer. Samples were heated for 10 min at 95°C and subjected to 7% SDS-PAGE electrophoresis. Proteins resolved by SDS-PAGE were transferred to PDVF membranes (GE Healthcare) and blocked overnight with PBS 3% milk powder (w/v) at 4°C with gentle agitation. Blots were probed with the polyclonal mice anti-BmaC antiserum (1:3000) and a goat HRP-conjugated secondary anti-mouse antibody (1:30.000 Santa Cruz), and then revealed using ECL Plus (Amersham).

DNA sequencing and sequence analysis

All DNA manipulations were carried out by using standard procedures. Phagemid DNA was extracted with a QIAprep maxiprep

kit (Qiagen). The inserted DNA was amplified and sequenced with the oligonucleotides 5′-TATCTGGTGGCGTAACACCT GCT-3′ (Fwd) and 5′-GATCGTCACCCTCGGATCCCTAGG-3′ (Rev), by cycle sequencing performed with a BigDye terminator kit (ABI Perkin-Elmer, Warrington, UK). The sequencing reactions were performed with an ABI 310 genetic analyser. Homology searches were carried out against sequence databases by using the BLAST algorithm with either the nucleotide sequence of the *B. suis* insert DNA or the predicted amino acid sequence in frame with the open reading frame encoded by the gene VIII of the phagemid. Sequence data for *B. suis* was obtained from The Institute for Genomic Research (TIGR) website (http://cmr.jcvi.org). Sequence analysis of the insert DNA supported the prediction that the sequence corresponding to the candidates should be expressed on the phage surface, since cloned sequences were in frame with the phage coat protein pVIII and the E-tag epitope.

Antibodies

Antiserum against BmaC was generated by mouse immunization with the portion (113-amino-acid fragment) of the BmaC passenger domain that was in frame with the M13 pVIII protein in the recombinant phage isolated by phage display. BmaC-phages were purified from culture supernatants of *E. coli* cells carrying pG8SAET-*bmaC* as described above. Culture supernatants containing the BmaC phages were filtered, concentrated to achieve a final concentration of 40 μg/200 μl and used for the immunization of five C57 male mice. Immune serum was collected and the specificity of the antibody was verified by ELISA against BmaC phages and by Dot blot against membranes purified from wild-type *B. suis* and the *bmaC* deletion mutant.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington DC: 2010) and those from the Principles for Biomedical Research involving animals developed by the Council for International Organizations of Medical Sciences. The protocol AZ16/09 was approved by the Institutional Animal Care and Use Committee (IACUC) of the Fundación Instituto Leloir (OLAW assurance number A5168-01). All efforts were made to minimize suffering.

Inhibition of Brucella *binding to HeLa cells by antibodies*

HeLa cells were seeded in 24-well plates (5×10^4 cells per well) as described before and inoculated alternatively with the *B. suis* wild type, the *bmaC* mutant strain (moi 50:1) or a mix of the bacteria and serially diluted antibodies. Monoclonal anti-cellular fibronectin (clone FN-3E2, Sigma prod. number F6140) or polyclonal anti-BmaC antibodies were used in the assay (dilutions 1:10 to 1:1000 and 1:2 to 1:1000 respectively). Multiwell plates were placed in a 5% CO_2 atmosphere at 37°C. After 1 h, wells were washed three times with PBS and the number of total cell-associated *B. suis* bacteria was determined by serial dilution and plate counting on TS agar with the appropriate antibiotics.

Brucella *binding to fibronectin*

Bacteria were grown overnight, washed and resuspended in PBS to a final concentration of 1×10^9 cfu ml^{-1}. Multiwell plates (Maxisorp, Nunc) were coated for 2 h at room temperature with a solution of the ligand (100 μl, fibronectin from Sigma, dissolved in PBS, concentration 100 μg ml^{-1}). Wells were washed three times with 100 μl of PBS, to eliminate unbound ligand. Then 50 μl of bacterial suspensions or a mix of bacteria and recombinant phage were added to each well and incubated at 37°C for 2 h. After incubation, wells were washed five times with PBS to remove non-adherent bacteria, and adherent bacteria were harvested with trypsin-EDTA [0.05% trypsin (Gibco)–0.5% EDTA (USB)]. After incubating for 10 min at 37°C, serial dilutions were plated on TSB agar with the appropriate antibiotic, and cfu were determined. Phage titre 1×10^{11} cfu ml^{-1}.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. E-tag expression. Recombinant phages (ϕ) are E-tag positive, indicating that fusion proteins are expressed in-frame with pVIII protein on the phage surface.
Fig. S2. ELISA assay with purified recombinant phage stocks. Binding of each candidate phage to Fn is plotted. HP was used as a control phage. Each point represents the mean ± standard deviation of values from a representative experiment. Experiments were made in triplicate.
Fig. S3. Immunofluorescence microscopy. Fixed Δ*bmaC* mutant expressing GFP were incubated with mouse anti-BmaC antisera and revealed with anti-mouse Cy-3-conjugated secondary antibody. There is no detectable signal on the cell surface.
Fig. S4. Affinity of ϕ-BmaC to different immobilized ECM components and to sialic acid-rich molecules. Panning with a purified phage stock of ϕ-BmaC showed significant affinity to type I collagen, in addition to fibronectin. Affinity to other ligands was comparable to the negative control ligand BSA.
Fig. S5. A. Western blot. Whole extracts of the wild type, the Δ*bmaC* mutant and the complemented strain were resuspended in loading buffer and electrophoresed in 7% SDS-PAGE. Proteins were transferred to a PVDF membrane, incubated with anti-ϕ-BmaC antisera and revealed with anti-mouse HRP-conjugated secondary antibody. A large band, corresponding to the BmaC protein (340 kDa), is only present in the wild type and in the KI strains.
B. Dot blot. Membrane fractions were purified from the wild-type and the Δ*bmaC* mutant strains, immobilized on a nitrocellulose filter, incubated with anti-ϕ-BmaC antisera and revealed with anti-mouse HRP-conjugated secondary antibody. A positive signal is visible in the wild-type strain (that expresses BmaC) and absent in the deletion mutant.
Fig. S6. Survival in J774 macrophages and HeLa cells by the different *B. suis* strains.
A. J774 macrophages. Viable brucellae recovered from macrophages over a 48 h course experiment showed a similar biphasic curve, proving there are no differences in invasion or intracellular replication between the strains.
B. HeLa cells. Significant differences in cfu were observed at short times (1 and 4 h p.i.); however, no differences between the wild-type and the mutant strains were found at longer times, indicating that intracellular replication is not affected by the *bmaC* mutation.
Table S1. Recombinant phages obtained after panning.
Table S2. Features of candidates isolated by phage display.

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