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BtaE, an Adhesin That Belongs to the Trimeric Autotransporter Family, Is Required for Full Virulence and Defines a Specific Adhesive Pole of *Brucella suis*

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Brucella **is responsible for brucellosis, one of the most common zoonoses worldwide that causes important economic losses in several countries. Increasing evidence indicates that adhesion of *Brucella* spp. to host cells is an important step to establish infection. We have previously shown that the BmaC unipolar monomeric autotransporter mediates the binding of *Brucella suis* to host cells through cell-associated fibronectin. Our genome analysis shows that the *B. suis* genome encodes several additional potential adhesins. In this work, we characterized a predicted trimeric autotransporter that we named BtaE. By expressing *btaE* in a nonadherent *Escherichia coli* strain and by phenotypic characterization of a *B. suis* Δ*btaE* mutant, we showed that BtaE is involved in the binding of *B. suis* to hyaluronic acid. The *B. suis* Δ*btaE* mutant exhibited a reduction in the adhesion to HeLa and A549 epithelial cells compared with the wild-type strain, and it was outcompeted by the wild-type strain in the binding to HeLa cells. The knockout *btaE* mutant showed an attenuated phenotype in the mouse model, indicating that BtaE is required for full virulence. BtaE was immunodetected on the bacterial surface at one cell pole. Using old and new pole markers, we observed that both the BmaC and BtaE adhesins are consistently associated with the new cell pole, suggesting that, in *Brucella*, the new pole is functionally differentiated for adhesion. This is consistent with the inherent polarization of this bacterium, and its role in the invasion process.**

Brucella species are responsible for brucellosis, one of the most widespread zoonotic diseases in the world that affects a range of different mammals. Currently, there are 10 recognized species of *Brucella*, based on host preferences, pathogenicity, and other phenotypic differences. Brucellosis remains endemic in most areas of the world; several animal species with agricultural relevance are susceptible to the disease, and there are also wildlife reservoirs of *Brucella*, which makes eradication more complicated (1). In particular, *Brucella abortus*, *Brucella melitensis*, and *Brucella suis* are the most economically significant pathogens of the group. Human brucellosis is caused by accidental contamination with reproductive or mammary secretions from infected animals (2, 3) and is characterized by a strong undulating fever, which, if untreated, may lead to a chronic stage of the disease and the appearance of serious complications such as endocarditis, osteoarthritis, and neurological disorders (3).

The preferred niche of *Brucella* is intracellular; when brucellae are ingested or inhalated, they penetrate through mucosal surfaces, and subsequently are transported to the lymph nodes by macrophages. *Brucella* spp. are able to infect and survive within macrophages in a compartment derived from the endoplasmic reticulum (4–6). It was shown that *Brucella* also has the ability to infect a great variety of host cells, including epithelial cells, placental trophoblasts, neurons, and cells from male and female reproductive tissues, to name a few (6, 7). *Brucella* has a marked ability to elude some of the basic mechanisms of the host immune system, which partly explains the frequent relapse after treatment (8, 9).

It was shown that initial adhesion of several pathogens to the host cell surface is a critical step in the infection process that helps the pathogens to avoid rapid clearance from host tissues (10). Several bacterial filamentous or nonpolymeric adhesins have been shown to participate in the primary interaction with the host cells as well as with components of the extracellular matrix (ECM) (11). Cumulative evidence supports the idea that adhesion to the ECM or to host cells is also an important step for *Brucella* infection (12–16). It was proposed that binding of *Brucella* to the host is mediated by host molecules containing sialic acid and/or sulfated residues and by components of the ECM such as fibronectin, collagen, and vitronectin. By phage display, we have recently identified a unipolar fibronectin-binding protein belonging to the type I (monomeric) autotransporter family from *B. suis*, which we called BmaC (15). Autotransporters are a superfamily of proteins produced by Gram-negative bacteria that share a domain organization composed of an N-terminal functional domain named the

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"passenger" domain and a C-terminal translocator β-domain that exhibits a β-barrel structure with a hydrophilic pore. Autotransporters are transported by the Sec complex across the inner membrane, and the β-domain subsequently transports the covalently linked passenger domain across the outer membrane (17). Several sets of evidence indicated that BmaC is involved in the polar binding of single bacteria to epithelial cells (15). A comprehensive bioinformatics search allowed the identification of several additional putative adhesins or invasins. One such candidate corresponded to a protein named BtaE that is predicted to belong to the type II (trimeric) autotransporter family. In this work, we show that BtaE participates in the binding of *B. suis* to hyaluronic acid and epithelial cells and is required to achieve full infectivity in the mouse model. We also analyzed the localization of BtaE on the bacterial surface and found that it showed unipolar localization, which has not been previously reported for a trimeric autotransporter (TA). This observation, together with the previous evidence indicating that BmaC also shows unipolar localization (15), prompted us to determine whether BtaE or BmaC or both are localized at a particular pole, using markers of the new and old poles (18–20). Our observations show that BtaE and BmaC are exclusively associated with the new pole, suggesting that this pole in *Brucella* is specialized for adhesion. These data support the concept that bacterial polarity is an important feature of *Brucella* physiology.

MATERIALS AND METHODS

Bacterial strains, cell culture, and media. *Escherichia coli* strains used in this study (DH5α, K-12, and derivatives) were grown at 37°C in Luria-Bertani (LB) medium. Antibiotics were added when needed: ampicillin at 200 μg/ml, chloramphenicol (Cm) at 50 μg/ml, and tetracycline at 5 μg/ml. *Brucella suis* M1330 (ATCC 23444) and derivative strains were grown at 37°C in Bacto tryptic soy broth (TSB; Bacto). When necessary, antibiotics were added: chloramphenicol at 6 μg/ml, kanamycin (Km) at 50 μg/ml, and nalidixic acid (Nal) at 10 μg/ml. HeLa cells and lung epithelial A549 (ATCC CCL-185) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) and murine J774 macrophages in RPMI (Gibco) media; both were supplemented with 5% fetal calf serum (PAA), at 37°C in a 5% CO_2 atmosphere.

Molecular techniques. All DNA manipulations were carried out by using standard procedures. Sequencing was done by cycle sequencing performed with a BigDye kit (Applied Biosystems) in a 3130 Genetic Analyzer (Applied Biosystems). Sequence data of *B. suis* were obtained from The Institute for Genomic Research (TIGR) website (http://www.tigr.org). PCR primers containing added restriction enzyme sites were designed to amplify the flanking regions of *btaE*. Primers F1 (5′-CGGGATCCATCGAGTGAGGCAAGAGTC-3′) and R1 (5′-GGCTGCAGCATGAGATATAATCTCTTAT-3′) amplified a region of 463 bp upstream of *btaE*, and primers F2 (5′-CGCTGCAGTGGAGCATAATCCGACCGCAGC-3′) and R2 (5′-GCGCATGCGTCACACACCAATGTCCAGT-3′) amplified a region of 332 bp downstream of the gene. New restriction sites are underlined. The generated PCR fragments were purified, cleaved with the corresponding enzymes, ligated together, and cloned into the pK18mobsacB mobilizable suicide vector (Km resistant) (21), obtaining pKmobsacΔ*btaE* plasmid. This plasmid was conjugated into 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 deletion of the open reading frame (ORF) was confirmed by PCR and reverse transcription-PCR (RT-PCR).

To provide a phenotypic confirmation of the *B. suis* Δ*btaE* mutant, the entire *btaE* gene (including its own promoter) was cloned into a broad-host-range plasmid. A region of 2,839 bp containing 565 bp of the upstream region, the entire *btaE* ORF, and 54 bp corresponding to the downstream region was amplified using the following primers: FComp (5′-AGCTCGAGATGCCTGTTACCATGCGCCGCAGCG-3′) and RComp (5′-CTACTAGTGTCGGATTATGCTCCAAGACTGATCT-3′). New restriction sites are underlined. The amplified product was cloned into the pBBR1MCS-1 mid-copy-number plasmid (22), obtaining the pBBR*btaE* plasmid, which was conjugated into the *B. suis* Δ*btaE* strain. Complemented clones were selected (Cm^r) and confirmed by PCR and RT-PCR.

E. coli K-12 was transformed with pBBR*btaE* or empty pBBR1MCS vector as a control. Transformed clones were selected (Cm^r) and confirmed by PCR.

Bioinformatics. To identify putative adhesins, adhesion domains of characterized adhesins from other bacteria were compared to the entire *Brucella suis* 1330 genome using the Protein Motif Search Tool at the J. Craig Venter Institute Comprehensive Microbial Resource (JCVI CMR). Domains displayed by each candidate were analyzed with Pfam (23), BLAST (24), and daTAA (25), which is specific for trimeric autotransporters. Secretion signal was identified with the SignalP Server (26). Alignments of the proteins were made with ClustalX (1.81) (27).

Binding to ECM components. Analysis of the affinity of BtaE-expressing bacteria to extracellular matrix (ECM) components was performed as described elsewhere (28). Briefly, 96-well plates (Nunc Maxisorp) were coated overnight at 4°C with 50 μl of 100 μg/ml solutions of the ligands, dissolved in phosphate-buffered saline (PBS). Bacteria were grown overnight, washed, and resuspended in PBS to a final concentration of 1×10^9 CFU/ml (optical density [OD] = 1 for *E. coli* and OD = 0.2 for *Brucella*). Wells were washed three times with 100 μl PBS to eliminate unbound ligand. Then, 50 μl of bacterial suspensions was added to each well and incubated at 37°C for 3 h. After incubation, wells were washed three times with PBS to remove nonadherent bacteria, and then adherent bacteria were harvested with trypsin-EDTA (0.05% trypsin [Gibco]–0.5% EDTA [USB]). After incubation for 10 min at 37°C, serial dilutions were done, followed by plating on LB or TSB agar with the appropriate antibiotic, and CFU were determined.

Antibodies. Polyclonal antiserum was obtained by immunization of mice with a peptide corresponding to a portion of the BtaE passenger domain (Fig. 1, underlined region). The coding sequence was amplified using the primers FAb (5′-ATCATATGCTGCATGATATTGAAAGTGGC-3′) and RAb (5′-CTGTCGACTCACGCTTCACCAAGCG-3′) and cloned into pET28a (Novagen). New restriction sites are underlined. The recombinant peptide was purified by high-pressure liquid chromatography (HPLC) and then used for immunization of 10 C57 male mice. Animal procedures were performed in compliance with institutional as well as governmental rules and regulations. Anti-BmaC antibodies were already used in a previous publication (15).

Western blot analysis. The method used to obtain total membranes from *Brucella* strains was based on a protocol previously described (29). Briefly, 50-ml TSB cultures were harvested, resuspended in 1 ml of cold 20 mM Tris-HCl buffer (pH 8.0)–1 mM phenylmethylsulfonyl fluoride (PMSF), and disrupted using Precellys (Bertin Technologies). The homogenate was centrifuged twice at 4,700 relative centrifugal force (RCF) at 4°C for 10 min to remove unbroken cells and Precellys beads, and the supernatant was ultracentrifuged at 134,000 RCF for 90 min at 4°C. The pellet was suspended in 1% Sarkosyl (sodium lauroyl sarcosinate)–20 mM HEPES buffer and incubated for 30 min at room temperature, and then the suspension was centrifuged again at 134,000 RCF for 90 min. The pellet was resuspended in 300 μl 20 mM HEPES buffer, 700 μl of formic acid was added, and the suspension was incubated overnight at room temperature in darkness. One milliliter of distilled H_2O was added, and the sample was lyophilized. Cracking buffer was used to resuspend the resulting powder, and the suspension was heated for 10 min at 95°C and subjected to sodium dodecyl sulfate (SDS)-PAGE. Proteins resolved by SDS-PAGE were transferred to polyvinylidene difluoride (PDVF) membranes (GE Healthcare) and blocked overnight with Tris-buffered saline (TBS)–5% (wt/vol) milk powder at 4°C with gentle agitation. Blots were

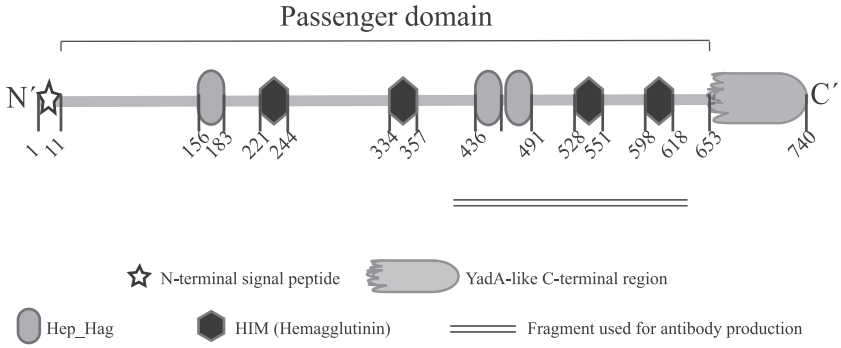

FIG 1 BtaE domain organization. A schematic representation of BtaE showing the N-terminal signal, the conserved domains, and motifs predicted by bioinformatics (SignalP, Pfam, and BLAST) is presented. Numbers indicate amino acid positions within BtaE.

probed with polyclonal mouse anti-BtaE serum (1:8,000) and a goat horseradish peroxidase (HRP)-conjugated secondary anti-mouse antibody (1:30,000; Santa Cruz) and then revealed using ECL Plus (Amersham). BtaE antiserum was preadsorbed with *E. coli* carrying empty pET28a.

Immunofluorescence analysis. *Brucella* was transformed with a derivative of pBBR1MCS-2 (30), which carries *gfp* constitutively expressed (excitation at 488 nm, emission at 509 nm; filters used a bandpass [BP] range of 505 to 530 nm) (15). The strains carrying polar markers fused to fluorescent proteins were obtained as previously described by biparental conjugation with *E. coli* S17.1 strains carrying either *pdhS-egfp* (19) or *aidB-yfp* (18).

To detect BtaE on the bacterial surface, 1 ml of a culture of fluorescent bacteria (green fluorescent protein [GFP]-tagged brucellae) or strains carrying either PdhS-enhanced GFP (PdhS-eGFP) or AidB-yellow fluorescent protein (AidB-YFP) was refreshed and grown for another hour in TSB media. The cells were then collected, washed once with PBS, and incubated with 3.7% paraformaldehyde for 15 min at 37°C. The cells were washed twice with PBS and once with PBS–1% bovine serum albumin (BSA) and then incubated 30 min with 40 μl of anti-BtaE or anti-BmaC antibodies (diluted 1:50 in PBS containing 0.5% BSA). The cells were washed again three times with PBS–1% BSA and incubated with 40 μl of IgG-CY3-conjugated donkey anti-mouse antibody preparation (Jackson ImmunoResearch) and diluted 1:250 in PBS containing 0.5% BSA for 40 min. In the case of the strains carrying the pole markers, DAPI (4′,6′-diamidino-2-phenylindole) was used to stain the DNA at a 1 μg/ml final concentration simultaneously with the secondary antibody used for the immunodetection of BtaE or BmaC. After two washes with 1 ml PBS–1% BSA and one wash with PBS, an aliquot of the cell suspension was mounted with Mowiol 4-88 (Calbiochem) on a microscope slide, or on agarose pads. Samples were covered with a coverslip and observed either with a Carl Zeiss LSM 5 Pascal laser scanning microscope, using a Plan-Aprochromat 100×/1.4 oil differential interference contrast (DIC) objective, or, in the case of fluorescent markers, with a Carl Zeiss LSM 510 Meta laser scanning microscope, using a Plan-Apochromat 60×/1.4 oil DIC objective. All incubations were carried out at room temperature.

Cell infection assays. HeLa or A549 (ATCC CCL 185) cells were seeded in 24-well plates (5×10^4 cells per well) and inoculated with the different strains (multiplicity of infection [MOI], 100:1). Plates were centrifuged for 10 min at 170 RCF and taken to a 5% CO_2 atmosphere at 37°C for 1 h. To determine the total number of bacteria associated to the cells, wells were washed three times with PBS and treated for 10 min at 37°C with 0.1% Triton X-100 (in deionized sterile water). Serial dilutions of the obtained lysates were done in PBS and plated on TSA with the appropriate antibiotics, and CFU were determined. To determine the number of intracellular viable bacteria, a standard gentamicin protection assay was performed: 1 h postinfection (p.i.), the culture medium was changed to a fresh one containing 20 μg/ml gentamicin to eliminate remaining extracellular bacteria. After 1 h (5% CO_2 atmosphere at 37°C), wells were washed three times with PBS and treated with 0.1% Triton X-100 as previously described. Lysates were serially diluted and plated on TSA with the appropriate antibiotics, and CFU were determined. The number of adherent bacteria was calculated as the difference between the total bacteria associated to the cells and the intracellular bacteria. All the results were expressed in reference to the wild-type strain, defined as 100%. For the inhibition experiment with hyaluronic acid, wild-type or mutant bacteria were preincubated with different concentrations of hyaluronic acid for 30 min at 37°C and bacterial adhesion to HeLa cells was determined as described above.

In the competition assay with the wild-type and mutant strains, the final MOI was maintained; for that, equal quantities of CFU of each strain were inoculated (MOI, 50:1 each). In order to determine the number of intracellular and total bacteria corresponding to each strain, the wild-type and mutant strains were labeled with different antibiotic resistances. The wild-type strain was transformed with a plasmid derivative of pBBR1MCS-2 that confers resistance to kanamycin (Km) (30). On the other hand, the gentamicin resistance (Gm^r) gene from the miniTn7(Gm)$P_{A1/04/03}$ *HcRed-a* plasmid (Gm^r Cm^r) (31) was removed by deletion using the PstI and ApaI restrictions sites, due to international restrictions with respect to antibiotic use for *Brucella*. The mini-Tn7 *HcRed* transposon (now Cm^r, Gm^s) was inserted in the genome of the *btaE* mutant, generating a Cm^r strain (31). Cellular lysates that included the bacteria were plated on TSA with either Cm or Km to determine the CFU of each strain. Both the wild-type and Δ*btaE B.suis* strains, carrying the pBBR1MCS-2 (Km^r) and the miniTn7 (Cm^r), respectively, were shown to grow normally in liquid or semisolid TSB media.

Virulence in BALB/c mice. Six-to-8-week-old female BALB/c mice were purchased from the animal facility at Leloir Institute, Argentina. They were randomly distributed in experimental groups at least 1 week before being inoculated. The animals were housed in filter-ventilated containment in the Laboratory Animal Facility of the Facultad de Ciencias Veterinarias (Tandil, Argentina), receiving water and food *ad libitum*. All experimental protocols were performed according to the premise of minimizing the suffering to which animals are exposed and using the minimum number of experimental animals to ensure statistically significant results. Animal procedures and management protocols were approved by the local Institutional Animal Welfare Committee according to the Animal Welfare Policy (Act 087/02) of the Faculty of Veterinary Medicine (Universidad Nacional del Centro de la Provincia de Buenos Aires [U.N.C.P.B.A.], Tandil, Argentina; http://www.vet.unicen.edu.ar). BALB/c mice (10 per group) were inoculated by intragastric delivery of wild-type *B. suis* 1330 (1.3×10^8 CFU/mouse), its isogenic Δ*btaE* mutant (1.3×10^8 CFU/mouse), or the complemented mutant (1.48×10^8 CFU/mouse) suspended in 300 μl of 10% sodium bicarbonate by use of a plastic feeding tube introduced through the mouth. Five mice from each group

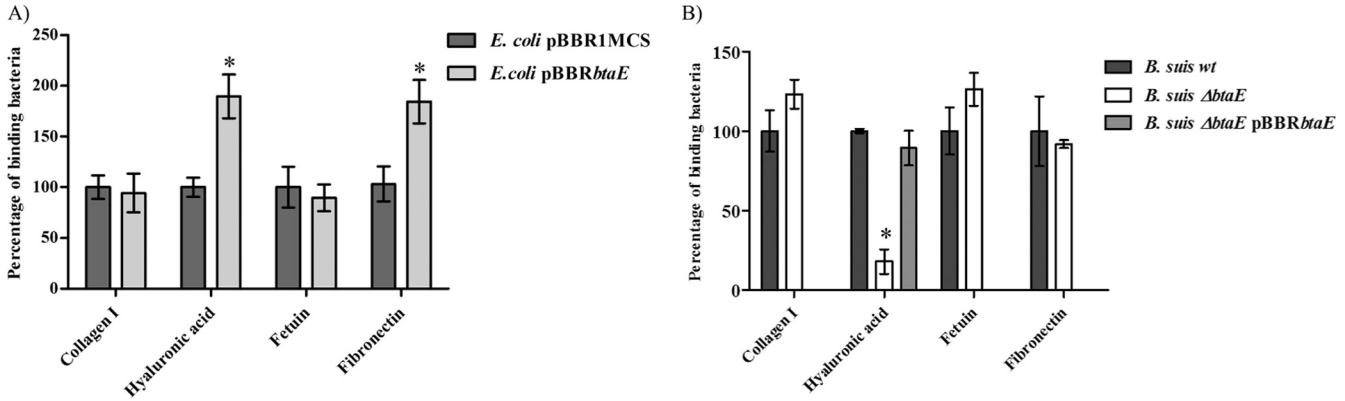

FIG 2 Binding of BtaE-expressing bacteria to ECM components. Plastic wells were coated with collagen type I, hyaluronic acid, fetuin, and fibronectin and subsequently incubated with the different strains. After being washed, bound bacteria were harvested with trypsin-EDTA; then, serial dilutions were done and the bacteria were plated on appropriate media. (A) *E. coli* pBBR*btaE* and *E. coli* pBBR1MCS (control strain) were assayed. (B) The wild-type (*wt*) strain of *B. suis* and the *B. suis* Δ*btaE* and Δ*btaE* pBBR*btaE* isogenic strains were analyzed. Values correspond to the percentages of total bacteria recovered from the wells after incubation with reference to the control strain (*E. coli* pBBR1MCS in panel A and *B. suis wt* in panel B), to which a value of 100% was assigned. Data represent the means ± standard deviations (SD) of the results of a representative experiment done in triplicate. Three independent experiments were performed with similar results. Data were analyzed by Student's *t* test and by one-way ANOVA followed by a Tukey *a posteriori* test. *, significantly different from control ($P < 0.05$), with 95% confidence.

were sacrificed at 7 and 30 days postinfection (p.i.), and spleens were removed. Dilutions of spleen homogenates were plated in duplicate on TSA. After 4 days of incubation, CFU were counted and expressed as the $\log_{10}$ value per spleen. The CFU data were normalized by log transformation and evaluated by analysis of variance (ANOVA) followed by Dunnett's *post hoc* test (Prism 5.0; GraphPad Software, Inc.). The experiment was repeated twice with similar results.

RESULTS

BtaE: a putative adhesin from the trimeric autotransporter family. Database searches within the NCBI genomic BLAST search engine showed the presence in the *B. suis* 1330 genome of 11 predicted ORFs that exhibit one or more conserved domains associated with adhesion, including BmaC (15). One of these candidates (BR0072) is annotated in the *B. suis* 1330 genome (32) as an "outer membrane protein" and has a predicted molecular mass of 75.45 kDa (740 amino acids). *In silico* analysis indicated that BR0072 displays an N-terminal signal peptide (26), a YadA-like C-terminal region (23), and a sequence of 642 amino acids between the two regions. The YadA-like and N-terminal regions (together with a central region or "passenger domain") are the characteristic precursor domains of type II trimeric autotransporter (TA) proteins (17, 33, 34); therefore, we named BR0072 BtaE (for *B*rucella *t*rimeric *a*utotransporter). As mentioned earlier, TAs represent a subfamily of autotransporter proteins, which are defined by the presence of a distinctive C-terminal translocator domain that forms a highly stable trimer in the outer membrane (33). The characterized members of the TA subfamily have been found to mediate bacterial adherence to eukaryotic cells and components of the extracellular matrix (ECM) (33–35), as YadA of *Yersinia enterocolitica* (36) and UspA1 and UspA2 of *Moraxella catarrhalis* (37).

TAs have been found to exhibit similar architectural structures, consisting of a globular head and a stalk (both corresponding to the passenger domain) and an anchor domain (corresponding to the C-terminal region), similar to a lollipop-shaped structure (38). To analyze the modular organization, the entire BtaE sequence was entered into the Domain Annotation of Trimeric Autotransporter Adhesins (daTAA) (25). Three regions corresponding to the head (amino acids 154 to 222, 254 to 335, and 434 to 529) and four corresponding to the neck (amino acids 223 to 244, 336 to 357, 530 to 551, and 600 to 618) were identified. Interestingly, *in silico* analysis using the Pfam protein database (23) showed that two of the three regions predicted to be part of the head contain three Hep_Hag repeats, and the neck portions overlap four noncontiguous HIM repeat motifs (Fig. 1), which represent potential adhesion motifs. In addition, an anchor domain and connectors were also identified. As expected, the anchor domain matches the YadA-like C-terminal region (Fig. 1).

***In vitro* binding activities: BtaE is involved in adhesion to hyaluronic acid.** It has been shown that *Brucella* spp. bind to components of the ECM, such as fibronectin and collagen. Indeed, as mentioned earlier, we have recently shown that the type I autotransporter BmaC protein is involved in the binding of *B. suis* to fibronectin (15). On the other hand, there are some examples of TAs mediating adherence to ECM components (34). In order to assess the affinity of BtaE for ECM components, we first performed a heterologous approach. The *btaE* gene was cloned with its own promoter into the pBBR1MCS vector, and the resulting plasmid (pBBR*btaE*) was transferred into a nonadherent, noninvasive *E. coli* K-12-derivative strain. Binding of *E. coli* carrying pBBR*btaE* plasmid or the empty vector to different immobilized ligands was evaluated. Expression of the BtaE protein in the heterologous host was confirmed by Western blotting (see below). *E. coli* pBBR*btaE* was able to bind more efficiently to hyaluronic acid (190% ± 21%) and fibronectin (185% ± 22%) than the control strain, which was considered 100% (Fig. 2A). No differences were observed in the adhesion of the heterologous host expressing BtaE to collagen or fetuin (a sialic acid-rich protein) (Fig. 2A).

Autotransporters have been shown to mediate a variety of other functions. For example, YadA of *Yersinia enterocolitica* participates in the binding to hydrophobic abiotic surfaces, in cellular autoagglutination, and in serum resistance, in addition to adhesion to and invasion of host cells (36). *E. coli* expressing *btaE* did not show any differential phenotype regarding attachment to

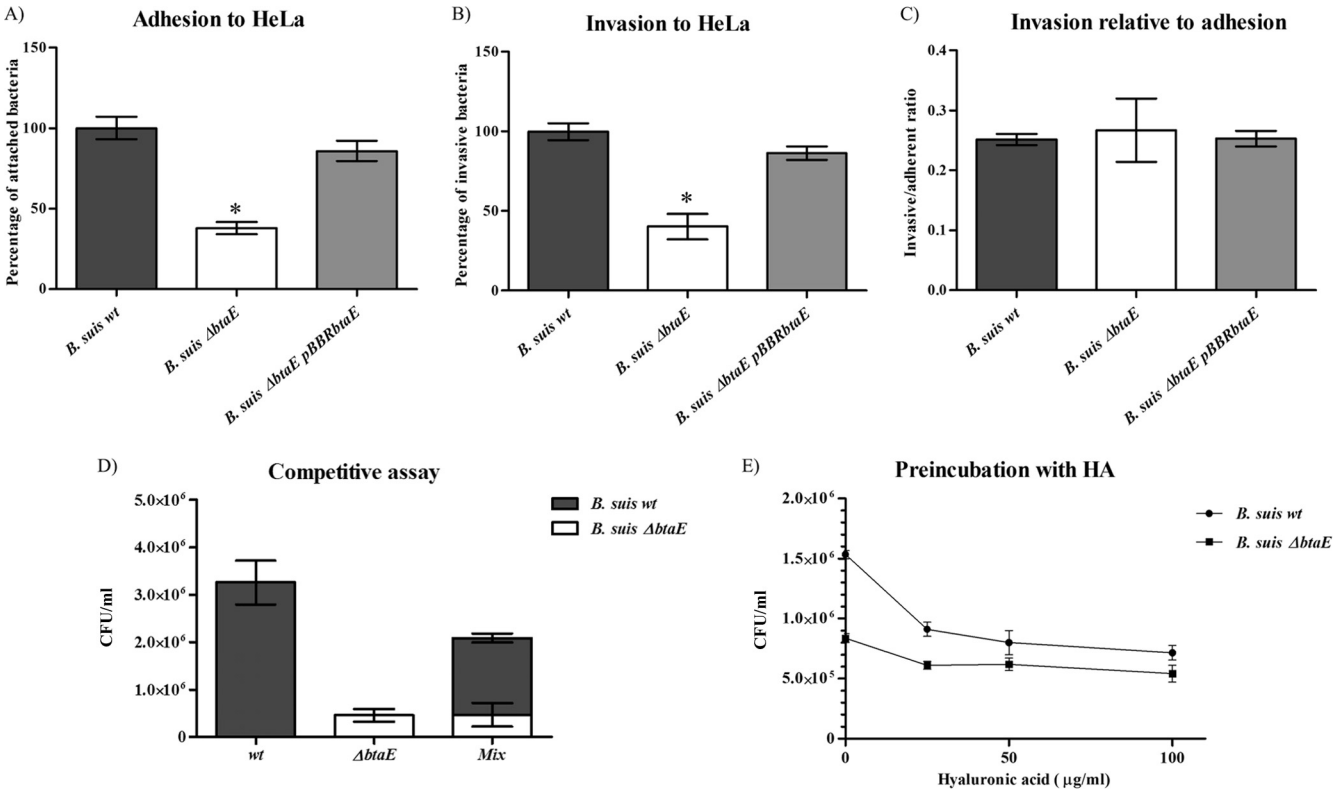

FIG 3 Adhesion to and invasion of HeLa cells. Approximately 5×10^6 bacteria were used to challenge 5×10^4 HeLa cells (MOI, 100:1). Total numbers of adherent bacteria (A) and intracellular or invasive bacteria (B) were determined, and the invasive cell/adherent cell ratio was calculated (C). Total numbers of adherent and intracellular bacteria are expressed relative to wild-type *B. suis* 1330, defined as 100%. (D) A competitive assay was carried out in which HeLa cells were coinoculated with wild-type and *B. suis* Δ*btaE* bacteria. The number of adherent bacteria was expressed as CFU/ml. (E) Inhibition of bacterial binding to HeLa cells by preincubation with hyaluronic acid (HA); data are expressed as total CFU/ml. Values represent the means ± SD of the results of an experiment representative of three independent assays done in triplicate or quadruplicate. Data were analyzed by one-way ANOVA followed by a Tukey *a posteriori* test. *, significantly different from the wild type ($P < 0.05$).

polystyrene or autoagglutination or even resistance to 8% serum (with complement activity) compared with the control strain (data not shown).

We next investigated the effect of a *btaE* deletion on the binding of *B. suis* to the immobilized ligands. No significant differences between the wild-type and mutant strains were observed in the binding to collagen, fetuin, or fibronectin. However, the *B. suis* Δ*btaE* mutant showed a significant decrease in its ability to bind to hyaluronic acid (18% ± 8%) relative to the wild type (considered 100%). Wild-type binding levels were restored in the mutant strain complemented with the pBBR*btaE* plasmid (Fig. 2B). Thus, although a contribution of BtaE to the interaction of *B. suis* with fibronectin cannot be ruled out, our observations support a role of BtaE in the binding of *B. suis* to hyaluronic acid.

The *B. suis* Δ*btaE* mutant was not impaired in attachment to abiotic surfaces or in its ability to autoaggregate in liquid cultures, and was not more susceptible to 10% or 50% sera (both bovine and porcine, with complement activity) than the wild-type strain (data not shown). Hence, taken together, these observations suggest that, unlike other trimeric autotransporters, BtaE is not involved in any of these activities.

BtaE participates in the adhesion of *B. suis* to host cells and is required for full virulence in mice. To analyze the contribution of BtaE to the interaction of *B. suis* with host cells, the ability of the *B. suis* Δ*btaE* mutant to adhere to and invade HeLa cells (human epithelial carcinoma cell line) was compared with that of the wild-type strain. Cells were seeded in multiwell plates and inoculated with the different strains for 1 h. To determine the total number of cell-associated bacteria, cells were lysed with a nonionic detergent and CFU were determined by plating serial dilutions of cell lysates. To determine the number of intracellular viable bacteria, a standard gentamicin protection assay was performed. The number of adherent bacteria was calculated as the difference between total cell-associated and intracellular bacteria. *B. suis* Δ*btaE* showed a significant reduction (by 63% ± 7%) in the ability to adhere to HeLa cells compared to the wild-type strain (Fig. 3A) as well as a decrease (by 70% ± 14%) in the ability to invade these cells (Fig. 3B). Both adhesion to and invasion of HeLa cells were restored by pBBR*btaE*, confirming that the absence of *btaE* was responsible for the observed *in vivo* phenotypes. In order to determine if the diminished invasion was a consequence of the reduction in adherence or, alternatively, was due to a reduced invasion aptitude, the invasive cell/adherent cell ratio was calculated. As shown in Fig. 3C, the wild-type and mutant strains showed similar efficiencies of invasion, suggesting that BtaE is involved in the initial attachment of *B. suis* to HeLa cells rather than in host cell internalization. To further investigate the ability of the *B. suis* Δ*btaE* mutant to attach to HeLa cells, a competitive infection assay was carried out by coinoculating equal amounts of *B. suis* wild-type and Δ*btaE* bacteria into the HeLa cell monolayer.

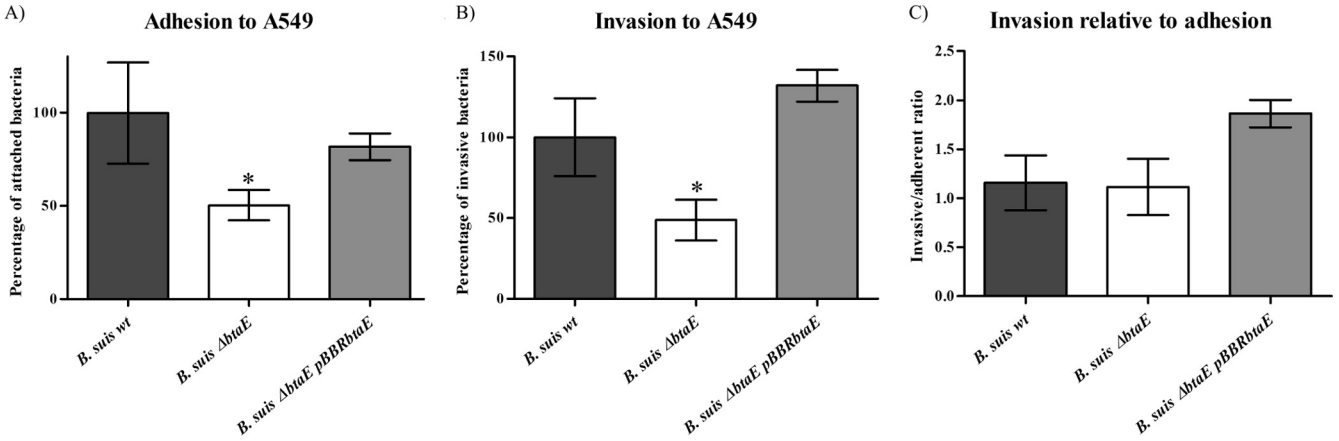

FIG 4 Adhesion to and invasion of A549 lung epithelial cells. Approximately 5×10^6 bacteria were used to challenge 5×10^4 A549 cells (MOI, 100:1). Total numbers of adherent bacteria (A) and intracellular bacteria (B) were determined, and the invasive cell/adherent cell ratio was calculated (C). Total numbers of adherent and intracellular bacteria are expressed relative to the adhesion and invasion values of the wild type, defined as 100%. Values represent the means ± SD of the results of an experiment representative of three independent assays done in triplicate. Data were analyzed by one-way ANOVA and a Tukey *a posteriori* test. *, significantly different from the wild type ($P < 0.05$).

The BtaE-defective mutant was strongly outcompeted by the wild-type strain for adhesion to HeLa (Fig. 3D), thus reinforcing the idea that BtaE participates in the binding of *B. suis* to these cells.

We also investigated whether hyaluronic acid is able to compete the binding of *B. suis* to HeLa cells. We found that preincubation of wild-type bacteria with 25 μg/ml hyaluronic acid inhibited the binding to these cells by about 60%. In contrast, the binding of the *btaE* mutant was inhibited only slightly by hyaluronic acid (Fig. 3E). This observation provides further evidence that BtaE is involved in the binding of *Brucella* to hyaluronic acid.

To further investigate the *in vivo* adhesive phenotype of the *btaE* mutant, the A549 epithelial cell line from human lung was used as an alternative cellular model in the binding assay (39). *B. suis* Δ*btaE* showed a significant reduction in the ability to adhere to (by 50% ± 8%) and invade (by 51% ± 13%) A549 cells compared with the wild type (considered 100%) (Fig. 4A and B). As predicted, the pBBR*btaE* plasmid restored adhesion and invasion capacity to wild-type levels in the *B. suis* Δ*btaE* mutant. Similar to the phenotypes in HeLa cells, our data indicate that the decrease in the invasion capacity of *B. suis* Δ*btaE* was a consequence of a lower adhesion to A549 cells (Fig. 4C).

To test the possibility that BtaE could participate in later steps of host cell infection, such as intracellular survival, a classical intracellular proliferation assay was carried out, using murine J774 macrophages and HeLa cells. In both cell models, the wild-type and mutant strains showed the same kinetics of intracellular replication (data not shown), indicating that the absence of BtaE does not affect later stages of cellular infection.

To evaluate the contribution of BtaE to the virulence of *B. suis*, we used the mouse infection model. Since it is possible that BtaE is involved in the initial stages of infection, we decided to inoculate mice through the intragastric route (40) and evaluate splenic infection at different times postinfection (p.i.). It was previously reported that the bacterial load in spleens and other organs of mice infected with *B. abortus* through the digestive track significantly increases at 7 days p.i. and stabilizes at later times (41). Therefore, we analyzed the spleen load after 7 and 30 days p.i. The *B. suis* Δ*btaE* mutant and the complemented (*B. suis* Δ*btaE* pBBR*btaE*) and parental strains were administered intragastrically to mice, and CFU counts in spleens were determined. At 7 and 30 days p.i., the splenic infection by the *B. suis* Δ*btaE* mutant was significantly reduced by 0.76 log and 1.24 log, respectively, compared with infection by the parental wild-type strain (Fig. 5A and B). As predicted, the complemented strain harboring the pBBR*btaE* plasmid showed a splenic CFU count similar to that of the wild type. These results show that BtaE is required for full virulence of *B. suis* in mice infected through the oral route.

BtaE has a surface-exposed unipolar localization. While the passenger domains of monomeric autotransporters may be cleaved after secretion, the passenger domains of characterized TAs remain intact after secretion (33). A recombinant peptide corresponding to a portion of the passenger domain (see Fig. 1, underlined region) was cloned, expressed in *E. coli*, and purified to generate polyclonal anti-BtaE antibodies in mice. To evaluate the membrane association of BtaE, Western blot analysis of whole membranes from the *B. suis* wild-type or Δ*btaE* mutant strain obtained from exponential-phase cells was performed. Similar to other TAs (42, 43), we noted that BtaE is difficult to denature. It was suggested that the C-terminal domains of TAs form heat- and sodium dodecyl sulfate (SDS)-resistant trimers. Hence, the samples were submitted to a strong denaturing process in the presence of formic acid and resolved by SDS-PAGE. Western blot analysis using the anti-BtaE antibodies showed the presence of an ~75-kDa protein band corresponding to monomeric BtaE, which was absent in the membrane fraction of the *B. suis* Δ*btaE* strain (Fig. 6A).

To assess the surface presentation of BtaE, late-exponential-phase cultures of the *B. suis* wild-type or Δ*btaE* strain labeled with green fluorescent protein (GFP) were refreshed for 1 h in TSB medium and then fixed without permeabilization, incubated with anti-BtaE antibodies, and observed by confocal microscopy. Under the conditions assayed, we found that BtaE was detected in 1% to 3% of bacteria examined, but in all cases ($n = 50$), the red signal corresponding to BtaE exhibited unipolar localization (Fig. 6B). As expected, no red signal was observed on the surface of the *B. suis* Δ*btaE* mutant but the unipolar signal was restored in the complemented strain harboring the pBBR*btaE* plasmid (data not

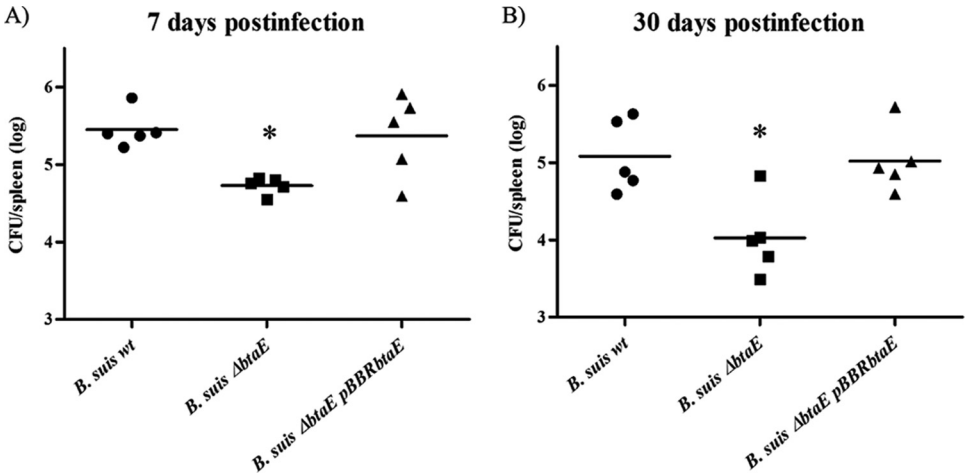

FIG 5 Role of BtaE in the virulence of *B. suis* in BALB/c mice. BALB/c mice (10 mice per group) were inoculated by intragastric delivery of the *B. suis* wild type, *B. suis* Δ*btaE*, or the complemented strains. Five mice from each group were sacrificed at 7 (A) and 30 (B) days postinfection, and then spleens were removed. Dilutions of spleen homogenates were plated in duplicate, and CFU were counted and expressed as the $\log_{10}$ value per spleen. The CFU data were normalized by log transformation and evaluated by one-way ANOVA followed by Dunnett's *a posteriori* test. The experiment was repeated twice with similar results. *, significantly different from the wild type ($P < 0.05$).

shown). To our knowledge, this is the first description of a TA with unipolar localization.

We also evaluated the BtaE localization in the *E. coli* heterologous host, carrying the pBBR*btaE* plasmid. Western blot analysis indicated the presence of the 75-kDa protein corresponding to BtaE in the total membrane fraction of *E. coli* pBBR*btaE*, which was absent in the control strain (Fig. 6C). Interestingly, immunofluorescence analysis of GFP-tagged *E. coli* carrying pBBR*btaE* showed BtaE foci at one bacterial pole in 5% of bacteria examined (Fig. 6D), while no signal was observed in the control strain (data not shown). This observation suggests that the mechanism involved in polar targeting of the BtaE trimeric autotransporter is conserved in *E. coli*.

***Brucella*'s new pole is functionally differentiated for adhesion.** Brucellae are proposed to be polarized bacteria, exhibiting asymmetric division (44) and unipolar growth, as seen with several other *Rhizobiales* (45). Moreover, proteins specifically recruited to the old pole or to the new pole have been reported. The PdhS histidine kinase is recruited to the old pole (19, 20), and the putative DNA repair protein AidB is recruited to the new pole (18). Since both the BmaC and BtaE adhesins showed unipolar localization, the question arises as whether these adhesins are localized at a specific pole and, in that case, if they are localized at the same pole. Therefore, to assess the relative localizations of BmaC and BtaE, we used the PdhS and AidB proteins as markers of the old and new poles, respectively.

Since the PdhS and AidB pole markers were identified in *B. abortus*, we first evaluated the localization of the BmaC homologue of *B. abortus*, which is 99.6% identical to BmaC from *B. suis*. By immunofluorescence, it was confirmed that in *B. abortus* the BmaC homologue consistently displays unipolar localization (data not shown). Next, we used the *B. abortus* strains carrying the

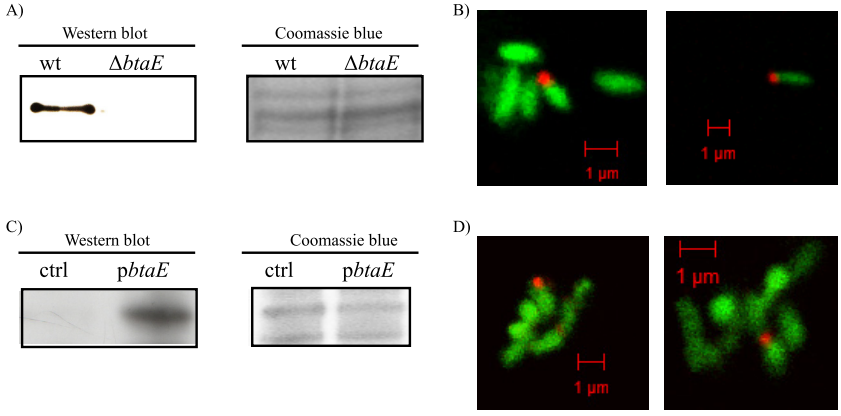

FIG 6 BtaE localization. (A and C) Western blot analysis of whole-membrane fractions from *B. suis* (A) and *E. coli* pBBR*btaE* (C) was carried out. Membrane samples were submitted to a strong denaturing process. Samples were electrophoresed by SDS-PAGE and transferred to a PDVF membrane. Blots were incubated with anti-BtaE antisera and with anti-mouse HRP-conjugated secondary antibody and finally revealed using ECL Plus. On the right, a Coomassie blue pattern is shown as a loading control (ctrl). (B and D) Detection of BtaE (in red) on the *B. suis* surface (B) and on the *E. coli* pBBR*btaE* surface (D) by immunofluorescence of GFP-tagged bacteria. Cultures of GFP-labeled strains were fixed, incubated with anti-BtaE antibodies, and then probed with a CY3-conjugated donkey anti-mouse antibody preparation. Samples were observed with a Plan-Aprochromat 100×/1.4 oil DIC objective on a Zeiss LSM 5 Pascal confocal microscope.

fusion proteins encoded by either *pdhS-egfp* (19) or *aidB-yfp* (18) to evaluate by immunofluorescence the relative localization of BmaC. DAPI staining and phase contrast were used to distinguish the bacterial shapes. Analysis by confocal microscopy showed that every time BmaC was detected (red signal) on the surface of *B. abortus* carrying PdhS-eGFP protein (green), it was at the pole opposite PdhS-eGFP (29 occurrences) (Fig. 7A). It was never observed that BmaC and PdhS-eGFP colocalized. Conversely, every time BmaC was detected in the strain carrying the AidB-YFP protein (yellow), it was either at the same pole as the AidB-YFP marker (31 occurrences) or in cells with diffuse markers, but never at the opposite pole (Fig. 7B). As expected, a *bmaC* mutant of *B. abortus* did not show any red signal (data not shown).

The *pdhS-egfp* and *aidB-yfp* fusions were introduced into the wild-type strain of *B. suis* and also into the isogenic *B. suis* Δ*bmaC* mutant as a negative control. The same analysis described above was carried out with these strains, leading to the same conclusion; i.e., BmaC is localized at the new pole of *B. suis* (data not shown). We next analyzed the relative localization of BtaE in *B. suis*. We found that every time BtaE was detected on bacteria expressing the old pole marker (PdhS-eGFP), it was at the opposite pole (30 occurrences) (Fig. 7C), but it was never observed at the same pole as PdhS-eGFP. Instead, in all the cases where BtaE was detected in bacteria carrying the *aidB-yfp* fusion, it was observed either at the same pole as the AidB-YFP fusion (17 occurrences) or in bacteria in which this marker was diffuse, but on no occasion was the BtaE fluorescent focus at the opposite pole (Fig. 7D). Taken together, our results show that under the conditions tested here both the BmaC and BtaE adhesins are localized at the new pole, suggesting that this pole is functionally differentiated for adhesion.

DISCUSSION

In microbial pathogens, adhesion to host cells is a critical step in the infection process. The importance of adhesion is reflected by the finding that several bacterial pathogens display multiple adhesins, which can act synergistically, thereby enhancing their adhesive capacity. Despite the relevance of adhesion in the interaction with the host, the impact of this step in *Brucella* pathogenesis was little explored. We presented evidence indicating that the monomeric BmaC autotransporter is required for an efficient adhesion of *B. suis* to host cells (15). In this work, we aimed to explore the role of BtaE, a protein that is predicted to belong to the TA family, in the interaction of *B. suis* with the host. Since it is expected that pathogens harbor several adhesins, probably exhibiting overlapping functions, we initially assessed the function of BtaE using a heterologous approach and found that this protein was able to increase the affinity of a "nonadherent" *E. coli* strain to immobilized hyaluronic acid and fibronectin. Analysis of the knockout *btaE* mutant confirmed the role of BtaE in the binding of *B. suis* to one of these ECM components, the hyaluronic acid glycosaminoglycan. The possibility exists that a fibronectin-binding-defective phenotype can be masked by other fibronectin-binding proteins, such as BmaC (15). The ability of BtaE to mediate the binding of *Brucella* to hyaluronic acid may be relevant at different stages of the infection. For example, it could facilitate initial bacterial adhesion to epithelial cells. It was previously described that hyaluronic acid is involved in adhesion of other pathogens such as *Mycobacterium tuberculosis* to the human lung epithelial cell line A549 (46), a cell type to which *Brucella* also adheres (47). Indeed, binding assays demonstrated that BtaE contributes to the adhesion of *B. suis* to both A549 and HeLa cells. Interestingly, addition of hyaluronic acid was able to compete the binding of the wild-type strain to HeLa cells, while adhesion of the *btaE* mutant was inhibited only slightly by hyaluronic acid. This observation provides further evidence that BtaE mediates the binding of *Brucella* to hyaluronic acid. Furthermore, taken together, our observations are consistent with the possibility that BtaE mediates adhesion to cells, at least in part, through hyaluronic acid. As hyaluronic acid is a component of the ECM of several tissues, BtaE could also be involved in *Brucella* dissemination to different target tissues such as cartilage, heart, and bone, which may result in serious complications of brucellosis.

The role of BtaE in the virulence of *B. suis* was studied by inoculating the wild type, the *btaE* deletion mutant, and the complementing strain in mice through the oral route. At 7 and 30 days p.i., splenic infection by *B. suis* Δ*btaE* was significantly reduced, compared to infection by the wild type, while the complemented strain showed a restored phenotype. This result strongly suggests that the BtaE adhesin might be considered a virulence factor and highlights the importance of bacterial adhesion to host components during the *Brucella* infection process.

Immunofluorescence analysis showed that BtaE exhibits unipolar localization. To the best of our knowledge, this is the first report of unipolar localization for a TA. Yet several monomeric (type I) autotransporters from rod-shaped pathogens, including the BmaC adhesin of *B. suis* (15), the IcsA protein from *Shigella flexneri* (48), and the AIDA-I monomeric autotransporter that mediates adhesion to human cells by pathogenic diffusely adherent *E. coli* (DAEC) strains (49), were shown to localize at one bacterial pole. It was reported that IcsA localizes to the pole at which actin assembly occurs, which is the old pole (48). Remarkably, some of the paradigmatic type I autotransporters, including AIDA-I and IcsA, are polar in the cytoplasm prior to secretion, suggesting that secretion occurs at the pole (49, 50). Intriguingly, it was shown that NalP, a monomeric autotransporter of spherically shaped *Neisseria meningitidis*, contains the molecular information to localize to the pole of *E. coli*. In fact, the BtaE TA also showed unipolar localization in the *E. coli* heterologous host, supporting the notion that the polar targeting mechanism of proteins from the autotransporter families is conserved in several bacteria.

The observation that both BtaE and BmaC show unipolar localization prompted us to explore whether they are associated with a specific (new or old) pole and, if this is the case, whether they are at the same or a different one. Evidence presented by others and us indicates that the individual *Brucella* bacterium interacts with the host cell surface through one of its poles (15, 51). Furthermore, it seems that the pole where BmaC is located on single bacteria is the one that interacts with the cell surface (15). Therefore, it was also interesting to address the issue as to which could be the "adhesive" pole. To determine the pole where the adhesins localize, we used the PdhS-eGFP fusion as a marker of the old pole (19), and the AidB-YFP fusion as a marker of the new pole (18). We observed that every time BmaC or BtaE was detected, it localized to the pole opposite the pole where PdhS-eGFP was located. In contrast, on every occasion that the BmaC or BtaE fluorescent foci were observed in bacteria expressing the new pole fusion, they were found at the same pole as AidB-YFP. We conclude that under the conditions tested here, both BmaC and BtaE localize to the new pole generated after cell division. Accordingly,

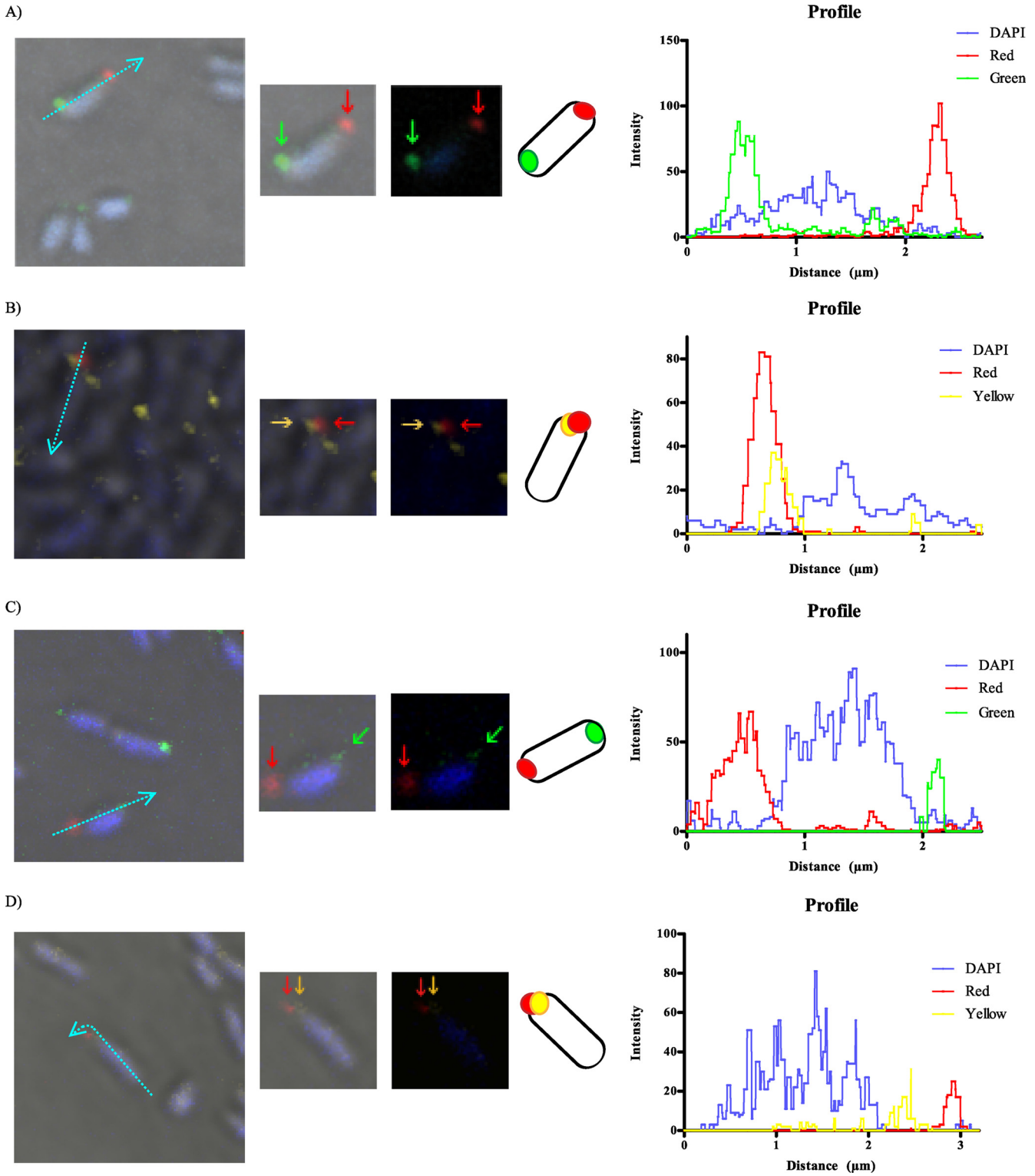

FIG 7 BtaE and BmaC are localized to the new bacterial pole. (A and B) Immunofluorescence microscopy using anti-BmaC antibodies was carried out with fixed cultures of *B. abortus* strains expressing PdhS-eGFP (A) and AidB-YFP (B) as old and new pole markers, respectively. DAPI staining and phase contrast (PC) were used to visualize the bacterial DNA content and shape, respectively. Samples were observed with a confocal LSM 510 Meta microscope using a Plan-Apochromat 60×/1.4 oil DIC objective. Representative images are shown. BmaC, PdhS-eGFP, and AidB-YFP are indicated with red, green, and yellow arrows, respectively. A schematic representation is also displayed. An intensity profile of the channels (constructed through the cyan dotted arrow) is presented, expressed in arbitrary units. (C and D) The same analysis was carried out for BtaE in *B. suis* with both PdhS-eGFP (C) and AidB-YFP (D).

we propose that the new pole is functionally differentiated for adhesion.

BtaE was detected in a low proportion of cells. A similar observation was previously made for the unipolar BmaC adhesin (15). At present, we do not know the reason for these observations. One possibility is that the level of *btaE* or *bmaC* gene transcription *in vitro* is not high enough to allow detection of fluorescent foci in most cells. If this is the case, an increase in *btaE* or *bmaC* expression is predicted to occur within the host environment before bacterial internalization. Since the anti-BtaE and anti-BmaC antibodies used in the immunofluorescence assays recognize a peptide of the passenger domains, another nonexclusive explanation is that these peptides are not very accessible to the antibodies in the native β-roll structure exposed on the bacterial surface (17). Finally, another interesting hypothesis is that BtaE and BmaC are expressed only in a bacterial subpopulation. This possibility is supported by the finding that several cell types generated by asymmetric division and differentiation coexist in a culture of *B. abortus* (19, 20, 44). Furthermore, relatively low expression of *btaE* or *bmaC* in a small subpopulation of bacteria might be sufficient for bacterial internalization and a successful (but limited) infection. Further studies are required to explore these possibilities.

Several surface virulence factors (including adhesive structures) were found to exhibit polar localization in other alphaproteobacteria closely related to *Brucella*. It was reported that all *Agrobacterium tumefaciens* VirB proteins are polarly localized (52). Furthermore, *A. tumefaciens* attaches efficiently to plant and abiotic surfaces through one of its poles in a process mediated by several structures, including a unipolar polysaccharide (UPP) (53). Very interestingly, evidence was presented indicating that cells of *A. tumefaciens* preferentially attach to surfaces via the old cell pole (45). Evidence presented in this work and other previously reported evidence (15) support the hypothesis that the unipolar BmaC and BtaE adhesins mediate *Brucella* binding to host cells at its new pole. It is possible that the characteristics of the polar asymmetry depend on the bacterial life style. In the case of *A. tumefaciens*, a sessile or attached mode of life would require that adhesion to abiotic or biotic surfaces is mediated by adhesins localized at the relatively stable old pole (53); in *Brucella*, initial binding of single bacteria to host cells prior to cellular internalization would be mediated by adhesins localized at the new pole of a subpopulation of infective bacteria.

In addition to BmaC and BtaE, other putative adhesins were shown to participate in the interaction of *Brucella* with the host. Recently, it was reported that a pathogenicity island of four genes contributes to the efficiency of attachment of *B. abortus* to both HeLa and J774 macrophage cells. One of the proteins encoded by the gene cluster was a hypothetical protein (Bab1_2009) that harbors an immunoglobulin-like BID_1 adhesion domain, found in adhesins such as the *E. coli* intimin (13). In addition, it was previously reported that a predicted adhesin from the monomeric autotransporter family from *B. suis* (OmaA) would be important during the chronic phase of infection (54). It will be interesting to determine whether Bab1_2009, OmaA, BtaE, and BmaC play redundant, synergistic, or complementary roles in the interaction of *Brucella* with different cell types and/or host tissues. Furthermore, different adhesins could exert their roles at different infection stages. Studies on gene regulation could give a hint regarding these possibilities. Through a transcriptomic analysis, it was previously shown that the expression of the *btaE* orthologue from *B. melitensis* (BMEI1873) is reduced in a mutant defective in the LuxR homologue VjbR regulator (55), which modulates the expression of transcripts required for intracellular survival (56). Besides, by microarray analysis it was found that transcription of the *bmaC* orthologue of *B. abortus* was downregulated by the BvrR/BvrS two-component system. This system plays a role in the adjustment of *Brucella* physiology to the shift expected to occur during the transition from the extracellular to the intracellular niche (57). Thus, one plausible hypothesis is that *btaE* expression and *bmaC* expression are induced under different stimulus conditions. Future studies will focus on the regulatory elements that control the expression of the autoransporter adhesins during the onset of cell infection.

Not only surface proteins were shown to participate in the adhesive properties of *Brucella*. Overexpression of an acyl homoserine-lactone (AHL) acylase (that degrades AHLs) in *B. melitensis* induced clumping and increased adhesion to both polystyrene and HeLa cells, probably due to the production of both some polymeric substances and outer membrane vesicles (16). It was argued that adhesion of the quorum-sensing-defective strain was somehow reminiscent of the adherence of localized bacterial colonies of *B. abortus* to epithelial cells (12). Thus, it is possible that initial and polar attachment of single bacteria to the host cell is mediated by proteinaceous adhesins, such as BtaE or BmaC and that, eventually, subsequent formation of bacterial microcolonies would be promoted by the production of other polymeric substances induced in contact with the cell surface.

Finally, host tropism is a very important feature of the *Brucella* genus, for which the concept of species is controversial, due to the high degree of similarity between the *Brucella* genomes (58). For example, comparison of the *B. melitensis* 16 M and *B. suis* 1330 genomes revealed extensive gene similarity and synteny, with the majority (>90%) of genes sharing 98% to 100% identity (32, 59). It is striking that most of the genes that were more variable were genes encoding surface-exposed proteins, leading to the hypothesis that they could contribute to differences in host preference and disease manifestation (32). In addition to *B. suis* and *B. melitensis*, we also found BtaE orthologues in *B. abortus* 2308 (BAB1_0069), *B. canis* ATCC 23365 (BCAN_A0073), *B. ovis* 25810 (BOV_0071), and *B. microti* CCM4915 (BMI_I75). Interestingly, *in silico* analysis indicated that there are significant differences either in protein lengths or in the number of repetitive motifs between the different ortologues. Therefore, variations found in BtaE and other adhesins such as BmaC (15) may well contribute to host preference or tissue tropism. It will be interesting, in the future, to assess this hypothesis.

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