A Brucella abortus cstA mutant is defective for association with endoplasmic reticulum exit sites and displays altered trafficking in HeLa cells

Marie de Barsy, Aurélie Mirabella, Jean-Jacques Letesson and Xavier De Bolle

Members of the genus Brucella are facultative intracellular pathogenic bacteria able to control maturation of their vacuoles. In several cell types, Brucella is able to reach a proliferation compartment derived from the endoplasmic reticulum (ER). Since ER exit site (ERES) functions are required for Brucella proliferation, we performed a yeast two-hybrid screen between human ERES-associated proteins and the predicted brucella proteome. This screening led to the identification of CstA, a conserved protein that specifically interacts with Sec24A, a component of the ERES. We found that a tagged CstA is secreted in Brucella abortus culture medium. This secretion is independent of the type IV secretion system VirB and the flagellum, suggesting that CstA is secreted through another system. We also discovered that a B. abortus cstA mutant is impaired for its association with the Sec23 ERES marker. The B. abortus cstA mutant displayed peculiar trafficking, with reduced association with LAMP1 and Calnexin 12 h post-infection in HeLa cells. However, its intracellular proliferation kinetics was not affected. The data reported here suggest that CstA could be directly or indirectly involved in the control of B. abortus intracellular trafficking in HeLa cells.

INTRODUCTION

Many intracellular pathogenic bacteria are able to proliferate in a membrane-bound compartment. These bacteria can adopt different strategies to survive after phagocytosis. Some bacteria, such as Coxiella burnetii, survive and proliferate in an acidic compartment (Hackett & Williams, 1981; Heinzen et al., 1996). Others avoid lysosome fusion by blocking phagosome maturation, such as Mycobacterium tuberculosis (Vergne et al., 2004), or by hijacking the eukaryotic secretory pathway, such as Legionella pneumophila (Ninio & Roy, 2007), which specifically targets human proteins such as Arf1, Rab1 and Sec22b to ultimately create a proliferative organelle, by translocating effector proteins targeting these eukaryotic proteins (Isberg et al., 2009).

Brucella species are intracellular facultative bacteria responsible for a worldwide zoonosis called brucellosis that affects a variety of mammals including humans. Members of the genus Brucella invade professional and non-professional phagocytes and reside in membrane-bound compartments called Brucella-containing vacuoles (BCVs). BCVs segregate from the endocytic pathway to finally mature in endoplasmic reticulum (ER)-derived compartments that are permissive for proliferation (Celli et al., 2003). The early secretory pathway is involved in the biogenesis of ER-derived compartments since (i) BCVs interact with ER exit sites (ERESs) (Celli et al., 2005) involved in the generation of COPII vesicles that transit from the ER to Golgi apparatus and (ii) blockade of Sar1 activity, which disrupts ERES, perturbs Brucella proliferation (Celli et al., 2005). Moreover, mutants of the virB operon, encoding the type IV secretion system (T4SS), are unable to sustain interaction with the ER, suggesting that the translocation of effector proteins is involved in this specific step of the trafficking (Celli et al., 2003). Until now, little has been known about the nature and the molecular functions of Brucella effectors. Several proteins were identified as T4SS substrates, but their functions remain unknown (de Jong et al., 2008; Marchesini et al., 2011). Recently, the RicA Brucella effector involved in Rab2 recruitment on the BCVs was identified using a yeast two-hybrid (Y2H) screen (de Barsy et al., 2011). Using a Y2H screen between a set of 15 human ERES-associated proteins and proteins encoded by the Brucella ORFeome (Dricot et al., 2004), we identified CstA, a secreted Brucella protein that specifically interacts with human Sec24A. A Brucella abortus cstA mutant is impaired for its association with the ERES and displays altered trafficking in HeLa cells.

Abbreviations: BCV, Brucella-containing vacuole; ER, endoplasmic reticulum; ERES, ER exit site; T4SS, type IV secretion system; Y2H, yeast two-hybrid [screen].

A supplementary table is available with the online version of this paper.
METHODS

**Bacterial and yeast strains.** All brucella strains used in this study were derived from *B. abortus* 2308 NaR (spontaneous nalidixic acid resistant mutant) and were routinely cultivated in 2YT rich medium (1 % yeast extract, 1.6 % peptone, 0.5 % NaCl). The *Escherichia coli* strains used for molecular cloning experiments were DH10B and DB3.1 (both Invitrogen Life Technologies). All *E. coli* strains were grown in LB broth. Antibiotics were used at the following concentrations when appropriate: nalidixic acid, 25 μg ml⁻¹; kanamycin, 20 μg ml⁻¹; gentamicin, 50 μg ml⁻¹; carbenicillin 50 μg ml⁻¹. The plasmids (Table 1) were mobilized from *E. coli* strain S17-1 (Simon et al., 1983) to *B. abortus* 2308 NaR. The yeast growth media and genetic techniques were previously described by Sherman (1991).

**Antibodies.** The primary antibodies used for immunofluorescence were rabbit polyclonal anti-Sec23 (Thermo Scientific) diluted 1000-fold, rabbit polyclonal anti-Calnexin (Stressgen) diluted 200-fold, a mouse monoclonal anti-LAMP-1 clone H4A3 (developed by J. T. Augustand E. K. Hildreth, obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by the University of Iowa) diluted 200-fold, a mouse mAb against the O antigen of *Brucella* A76-12G12 (undiluted hybridoma culture supernatant) (Cloeckaert et al., 1993b), a mouse monoclonal anti-Flag M2 (Stratagene) diluted 500-fold, homemade rabbit polyclonal antibodies against *Brucella* diluted 2000-fold. The secondary antibodies used for immunofluorescence were goat anti-mouse IgG antibodies coupled to Alexa 546 (Invitrogen), donkey anti-rabbit IgG coupled to Alexa 488 (Invitrogen), sheep anti-mouse IgG coupled to horseradish peroxidase (HRP) (GE Healthcare), and donkey anti-rabbit IgG coupled to HRP (GE Healthcare). All secondary antibodies were diluted 500-fold. The cells were fixed and permeabilized with PBS containing 0.1 % Triton. The primary and secondary antibodies were diluted in PBS containing 0.1 % Triton X-100 and 5 % BSA. TOPRO-3 (Invitrogen) diluted 100-fold was used to stain nuclei. The primary antibodies used for dot blot and Western blot were a mouse mAb against the O antigen of *Brucella* A76-12G12 (undiluted

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**Table 1. Plasmids and bacterial and yeast strains used**

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<tr>
<th>Strain or plasmid</th>
<th>Description</th>
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<tr>
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<td>Wild-type</td>
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<td>ΔvirB in 2308 NaR</td>
<td>de Barsy et al. (2011)</td>
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<td>This study</td>
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*Y9390 yeast strains expressing DB-ORF fusions. The strain names represent the plate number and the plate position in the human ORFeome (Lamesch et al., 2007; Rual et al., 2004).*
hybridoma culture supernatant) (Clockaert et al., 1993b), a mouse mAb against LPS core A68-24G12 and homemade rabbit polyclonal anti-PrlR antibodies. The secondary antibodies were sheep anti-mouse IgG, HRP-linked whole antibodies (Amersham no. NA931, 1:5000 dilution) and donkey anti-rabbit IgG, HRP-linked whole antibodies (Amersham no. NA934, 1:5000 dilution).

**Y2H screen.** The Y2H screen was performed as described previously (de Barsy et al., 2011) with some modifications. We used Y8800 MATa and Y8930 MATα yeast strains harbouring HIS3 and ADE2 reporter genes (James et al., 1996). Strain Y8800 was transformed with 71 AD-ORF mini-pools of expression plasmids. More than 2000 colonies were obtained for each mini-pool. Y8930 DB-ORF strains were kindly provided by J.-C. Twizere (FUSAGx, Gembloux, Belgium). Each Y8930 DB-ORF strain was mated against all 71 Y8800 AD-ORF mini-pools on YPAD (10 g yeast extract l\(^{-1}\), 20 g peptone l\(^{-1}\), 20 g glucose l\(^{-1}\) and 0.32 mM adenine) solid medium. After overnight growth at 30 °C, colonies were transferred to Sc-L-W-H plates (lacking leucine, tryptophan, histidine) and to Sc-L-H-CHX plates (lacking leucine, histidine and containing 1 mg cycloheximide ml\(^{-1}\)) to identify autoactivators that can arise during Y2H screens (Vidalain et al., 2004). Synthetic complete (SC) medium was as described by Walhout & Vidal (2001). The plates were cleaned by replication with sterilevelts and incubated overnight at 30 °C (Walhout & Vidal, 2001). This cleaning was performed to dilute out the yeasts present in each spot, in order to eliminate non-specific growth. The plates were then incubated at 30 °C for 3–5 days. This first phenotypic screen generated a collection of positive colonies for the HIS3 reporter. A second round of screening was performed with these first positive clones by spotting them on four different media (Sc-L-W-H, Sc-L-H-CHX, Sc-L-W-A, Sc-L-A-CHX, where ‘A’ indicates the absence of adenine). This yielded a second generation of positives clones for ADE2 and HIS3 reporters. A third screening was performed for clones that were only positive for one reporter in the second round of screening, yielding a third generation of positive clones for both reporters. The ORFs of the positive clones from the second and third generations were amplified by PCR and sequenced.

**Construction of the B. abortus ΔcstA mutant.** Molecular cloning and gel electrophoresis were performed as described by Sambrook & Russell (2001). To construct the deletion mutant of cstA in B. abortus, the upstream and downstream regions flanking cstA were amplified by PCR using B. abortus 2308 genomic DNA as the template, and the following primer pairs: (i) cstAup1 (5’-atgtcatcg-9’-aggccatgaaacatattcagccaaatg-3’); (ii) cstAdown1 (5’-agggctaccaaatattcagaaagt-3’), and cstAdown2 (5’-cggttgaccagcaacctg-3’). A PCR using cstAup1 and cstAdown2 primers and the purified products of the two previous ampiclons was performed to amplify a deletion allele for cstA that was cloned and sequenced, and then used for allelic replacement in the B. abortus 2308 NaL\(^{+}\) genome, as previously described for other gene deletions (Migneot et al., 2010).

**Cell culture, infection and transfection.** HeLa cells were cultured at 37 °C with 5% CO\(_2\) in DMEM supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids and 1% sodium pyruvate. RAW264.7 macrophages were cultured in DMEM supplemented with 4 mM L-glutamine, 4.5 g glucose l\(^{-1}\), 1.5 g sodium bicarbonate l\(^{-1}\) and 10% FBS. RAW264.7 or HeLa cells were inoculated at an m.o.i. of 300 bacteria per cell for the microscopy studies and c.f.u. counting. Briefly, bacteria were centrifuged onto cells at 400 g for 10 min at 4 °C and then incubated for 1 h at 37 °C with 5% CO\(_2\). The cells were washed twice with medium and then incubated for 1 h in DMEM supplemented with 50 μg gentamicin ml\(^{-1}\) to kill extracellular bacteria. Thereafter, the culture medium was eliminated and replaced by DMEM with 10 μg gentamicin ml\(^{-1}\). To monitor Brucella intracellular survival, infected cells were washed twice with PBS and lysed with 0.1% (v/v) Triton X-100 in PBS. Serial dilutions in PBS were plated onto 2YT agar to count the c.f.u. For immunofluorescence staining, cells were fixed in 2% paraformaldehyde, pH 7.4, at 37 °C for 15 min. The number of BCVs positive or negative for each marker (Sec23, LAMP1 or Calnexin) was counted for each independent immunofluorescence, and the values obtained for the wild-type and ΔcstA strains were compared using a statistical homogeneity test (χ² test). Thus, each immunofluorescence experiment is characterized by the total number of BCVs counted (n) and the P-value of the χ² test; n = 364 (P<0.001) and n = 160 (P<0.001) for Sec23; n = 215 (P<0.05) and n = 169 (P<0.05) for LAMP1; n = 421 (P<0.001), n = 256 (P<0.05) and n = 244 (P<0.05) for Calnexin. Representative experiments are shown in Results for the acquisition of Sec23, LAMP1 and Calnexin markers, respectively.

**Mice infection.** Mice were injected intra-peritoneally with 4×10⁴ c.f.u. B. abortus in 500 μl RPMI. The infection doses were validated by plating serial dilutions of the inoculum on 2YT medium. Ten days post-infection, the mice were sacrificed by cervical dislocation. The spleens were harvested and homogenized in PBS 0.1% Triton X-100 (Sigma-Aldrich). Successive serial dilutions in PBS were performed and plated onto 2YT medium. C.f.u. were counted after 3 days of culture at 37 °C. The animal handling and procedures in this study were in accordance with the current European legislation (directive 86/609/EEC) and in agreement with the corresponding Belgian law “Arrêté royal relatif à la protection des animaux d’expérience du 6 avril 2010 publié le 14 mai 2010”. The complete protocol was reviewed and approved by the Animal Welfare Committee of the University of Namur (FUNDP, Belgium) (permit number: 05-558).

**Secretion test in bacteriological medium and translocation assay.** The secretion test and sample preparation for Western blotting were performed as described previously (de Barsy et al., 2011).

**SDS/proteaseinase K extraction of LPS and total extraction.** Exponentially growing B. abortus cells cultured in 2YT were used to inoculate 100 ml 2YT at an initial OD\(_{600}\) 0.1. B. abortus strains were then grown for 48 h. Bacteria were killed by incubation with 0.5% phenol for 72 h. Bacteria were washed in PBS and pellets were used for SDS/proteaseinase K extraction of LPS, as described previously (Garin-Bastuji et al., 1990). After the third isoprenol precipitation, pellets containing LPS were resuspended in 500 μl water and frozen. Laemmli buffer was added to the samples that were heated for 10 min at 100 °C before migration on an SDS-PAGE gel containing 18% polyacrylamide. The gels were then silver stained or transferred to nitrocellulose membranes for Western blotting. Total extracts were prepared from B. abortus strains cultured in 2YT. Bacteria were killed by incubation at 80 °C for 1 h. Samples were concentrated to obtain an OD\(_{600}\) of 10.0. Serial dilutions were made and 2 μl each dilution was spotted on nitrocellulose membranes saturated by overnight incubation in PBS with 5% non-fat dried milk. Immunodetection was performed using the same protocol as for Western blotting.

**Western blotting.** Membranes were blocked overnight in PBS with 5% non-fat dried milk as the blocking agent, then incubated for 1 h with the primary antibody (diluted in PBS/1% blocking agent), washed three times for 10 min in PBS/0.05% Tween 20, incubated for 1 h with the secondary antibody (diluted in PBS/1% blocking agent) and finally washed three times for 10 min in PBS/0.05% Tween 20. The membranes were revealed with ECL (100 mM Tris/HCl pH 8.3, 0.009% H\(_2\)O\(_2\), 0.2 mM coumaric acid and 1.25 mM luminol).
RESULTS

Identification of the interaction between CstA and Sec24A, an ERES-associated protein

A genome-wide Y2H screen was performed between ERES-associated proteins and all cloned predicted coding sequences for brucella proteins. We tested 15 ORFs for ERES-associated proteins available in the human ORFeome (Table 1) (Lamesch et al., 2007; Rual et al., 2004), against the 3091 ORFs cloned in the Brucella ORFeome (Dricot et al., 2004). Five interactions were identified (Table S1, available with the online version of this paper), among which three were considered as specific, since each partner was detected only once or twice in the Y2H screens recently performed in our laboratory using the Brucella ORFeome (Sec24A-BMEI10900, Sec16L-BMEI1081, Sec16L-BMEI0938). We focused on Sec24A which was found only once in the Y2H screen performed with ERES proteins and its interaction with CstA (conserved Sec24A-targeted protein A, BMEI0900 ORF in the Brucella melitensis genome) was confirmed when retested individually. The Sec24–Sec23 protein complex forms the inner layer of COPII vesicles that bud from ER exit sites (Mancias & Goldberg, 2005). Sec24 is involved in cargo recognition and human cells could express four isoforms of Sec24, named Sec24A, Sec24B, Sec24C and Sec24D (Wendeler et al., 2007).

CstA is secreted in bacterial culture

To determine whether CstA is secreted in bacterial culture, translational fusions with the 3Flag tag at the N or C terminus of CstA were constructed. Strains carrying these fusions were grown in a defined medium and the presence of fusion proteins in pellets and concentrated culture supernatants was detected by Western blot using the M2 anti-Flag antibody. 3Flag–CstA and CstA–3Flag fusion proteins were detected in the pellets at the expected molecular mass, thus suggesting that these fusions are produced in this culture condition. The 3Flag–CstA fusion protein was also detected in concentrated culture supernatants, whereas PrlR, a transcriptional regulator of the two-component family already used as a cytoplasmic control (de Barsy et al., 2011), was only detected in pellets (Fig. 1). These results suggest that the presence of the 3Flag–CstA protein in culture supernatants is due to secretion and not bacterial lysis. Moreover, we also tested the secretion of three other proteins fused to 3Flag, and none of them was secreted (data not shown), suggesting that secretion of CstA is due to a specific export and not to bacterial lysis. The C-terminal fusion with 3Flag was not secreted (data not shown), which would be expected if the 3Flag tag alters secretion signal recognition at this position. In order to find out whether the 3Flag–CstA secretion depends on T4SS VirB or flagellum, we performed the secretion test with B. abortus ΔvirB and ΔfliF strains. FliF encodes the basal body of the flagellum. It was shown for Yersinia enterocolitica that the flagellum can function as a protein secretion system (Young et al., 1999). We showed that 3Flag–CstA secretion is maintained in these two mutant strains, demonstrating that the T4SS and the flagellum are not required for CstA secretion in bacterial culture (Fig. 1). It is therefore likely that another secretion system for 3Flag–CstA is involved under these conditions.

A B. abortus cstA mutant is altered for its association with ERES

As CstA interacts with Sec24A in Y2H assays, we were interested in evaluating the role of CstA in a cellular infection and we therefore constructed an in-frame deletion mutant for cstA in the B. abortus 2308 NalR strain. Since CstA is homologous to ManA and ManC enzymes involved in GDP-mannose biosynthesis (see Discussion), we checked that the ΔcstA mutant was smooth and had a normal core. Indeed, it was stained with the anti-O-chain A76-12G12 mAb and the expected distances of migration were detected in concentrated culture supernatants (left) or bacterial pellets (right), using either Anti-Flag antibody. 3Flag–CstA and CstA–3Flag fusion proteins were grown in a defined medium and the presence of fusion proteins in pellets and concentrated culture supernatants was detected by Western blot using the M2 anti-Flag mAb (anti-Flag, upper) or polyclonal anti-PrlR antibodies (anti-PrlR, lower). PrlR detection was used as a lysis control and should be negative in the concentrated supernatant if there is no detectable lysis. The expected distances of migration for 3Flag–CstA and PrlR are indicated. The difference in the abundance of 3Flag–CstA in the ΔfliF strain compared to the other strains was not reproducible (data not shown).

Fig. 1. VirB- and flagellum-independent secretion of 3Flag–CstA by B. abortus. Western blots were carried out on concentrated culture supernatants (left) or bacterial pellets (right), using either Anti-Flag or Anti-PrlR antibodies (anti-Flag, upper). Anti-PrlR antibodies (anti-PrlR, lower). PrlR detection was used as a lysis control and should be negative in the concentrated supernatant if there is no detectable lysis. The expected distances of migration for 3Flag–CstA and PrlR are indicated. The difference in the abundance of 3Flag–CstA in the ΔfliF strain compared to the other strains was not reproducible (data not shown).
Since strains affected for intracellular trafficking are not necessarily attenuated (de Barsy et al., 2011), we tested the co-localization of the ΔcstA strain with some typical markers of intracellular trafficking in HeLa cells. We first tested the ability of the ΔcstA strain to interact with ERES by localization of Sec23 and B. abortus during HeLa cell infection. As a control, we used a B. abortus 2308 ΔvirB strain that is unable to interact with the ER in a sustained manner (Celli et al., 2003). HeLa cells were infected with either wild-type, ΔcstA, ΔvirB or a ΔcstA strain carrying a rescue plasmid containing the 3Flag–cstA fusion (complemented strain). After 12 h of infection, the cells were fixed and immunostained to detect bacteria and Sec23. As shown in Fig. 4(a), 17 % of the vacuoles containing the ΔcstA strain were positive for Sec23 staining (n=220) compared with 38 % for the wild-type strain (n=144; P<0.001 for a χ² statistical analysis; representative images for the wild-type strain are shown in Fig. 4b). Only 16 % of vacuoles containing the ΔvirB strain were Sec23 positive (n=136), consistent with previously reported data (P<0.001 for a χ² statistical analysis) (Celli et al., 2005). Interestingly, 31 % of the vacuoles containing the complemented ΔcstA strain were positive for Sec23 (n=231), therefore showing partial complementation. These results suggest that cstA deletion results in an impaired association of B. abortus with ERES.

Intracellular trafficking of the B. abortus cstA mutant is altered

We showed that the ΔcstA strain is impaired for its association with ERES despite the fact that its intracellular proliferation is very similar to that of the wild-type strain in HeLa cells. One possible hypothesis would be that the ΔcstA mutant reaches its proliferation niche by an alternative trafficking route. To test if the B. abortus ΔcstA mutant is trafficking like the wild-type strain, we performed co-localization of B. abortus with

**Fig. 2.** The ΔcstA mutant strain does not lack O antigen and has no detectable core defect. (a) A dot blot was performed with a mAb against the O antigen (A76-12G12) on total extracts of the B. abortus wild-type strain, ΔcstA strain and, as the negative control, the B. melitensis B3B2 rough mutant (Godfroid et al., 1998). The A76-12G12 antibody equally recognizes B. abortus and B. melitensis strains (Cloeckaert et al., 1993a). (b) SDS/protease K LPS extracts were used for migration on an SDS-PAGE gel containing 18 % polyacrylamide. Gels were then silver stained or transferred to nitrocellulose membranes for Western blotting with a mAb against the LPS core (A68-24G12).

**Fig. 3.** The cstA deletion does not significantly affect the number of c.f.u. of B. abortus extracted from infected HeLa cells (a), RAW264.7 macrophages (b) and mouse spleens (c). (a) and (b) ●, Wild-type strain; □, ΔcstA strain. Values are means ± SD calculated from three replicates. (c) Medians are indicated for each sample (7 or 9 mice). A u test (Mann–Whitney) statistical analysis was performed and showed no significant difference (ns) between the wild-type and the ΔcstA strains (P>0.05).
LAMP1, a late endosome/lysosome marker, and an ER marker, Calnexin, at 12 and 24 h post-infection using fluorescence confocal microscopy after immunostaining. At 12 h post-infection, the percentage of vacuoles containing the ΔcstA strain positive for LAMP1 (n=108) was significantly lower than in the wild-type strain (n=107; 18% for ΔcstA strain, 30% for wild-type strain, P<0.05 for a χ² test) (Fig. 5a). The percentage of vacuoles positive for the Calnexin marker was also significantly lower for the ΔcstA strain (n=241) compared with the wild-type control (n=180), with 33% for the ΔcstA strain and 56% for the wild-type strain (P<0.001 for a χ² test; Fig. 6a) (representative pictures of Calnexin- and LAMP1-positive vacuoles containing the wild-type strain at 12 h post-infection are shown in Figs 5b and 6b). At 12 h post-infection, 32 and 59% of the vacuoles containing the ΔcstA strain carrying a rescue plasmid were positive for the LAMP1 (n=117) and Calnexin (n=121) markers, respectively, showing full complementation. At 24 h post-infection, the ΔcstA, complemented ΔcstA and wild-type strains proliferated in a Calnexin-positive and LAMP1-negative compartment (n>400 for each strain and each marker; Figs 5a and 6a). These results suggest that the ΔcstA strain has altered trafficking at 12 h post-infection, but finally reaches an ER-derived compartment for proliferation at 24 h post-infection.

**DISCUSSION**

In this report, we identified CstA, a conserved protein specifically interacting with the ERES-associated protein Sec24A, using a Y2H screen. As ERES function is required for the maturation of BCVs in a proliferative compartment (Celli et al., 2005), we tested if a *B. abortus* ΔcstA mutant is able to proliferate in HeLa and RAW264.7 cells (Fig. 2). Our data strongly suggest that the *B. abortus* ΔcstA strains are not dramatically attenuated, and do not present a strong delay in the c.f.u. profile during HeLa or RAW264.7 cell infection. Similarly, the *B. abortus* ΔcstA mutant is not attenuated during infection of mice.

At 12 h post-infection in HeLa cells, the *B. abortus* ΔcstA mutant strain is impaired for co-localization with Sec23, an ERES-associated protein. This means that a (partial) defect in association with ERES could not be sufficient to prevent or delay trafficking to an intracellular niche compatible with bacterial proliferation. Of course, the molecular mechanism involving association of BCV with ERES could involve the direct interaction between CstA and Sec24A, but currently available data do not sufficiently support this hypothesis, and more characterization of CstA translocation and function would be needed.

Interestingly, at 12 h post-infection, the *B. abortus* ΔcstA mutant colocalized less frequently with LAMP1 and was less frequently associated with Calnexin, compared with the wild-type control. If LAMP1 and Calnexin label distinct BCVs, it means that 50% of the *B. abortus* ΔcstA bacteria are located in compartments that are not labelled by LAMP1 or Calnexin. Several hypotheses may explain this observation, including exclusion of markers and lysis of the vacuole. However, one simple hypothesis would be that the ΔcstA mutant is stalled in an intermediate compartment, usually transient for the wild-type strain or even never reached by the wild-type strain. This intermediate compartment would allow intracellular proliferation for the ΔcstA mutant since c.f.u. counting showed no difference with the wild-type strain. This is not very surprising since previous data reported proliferation outside ER-derived compartments (Arenas et al., 2000; Bellaire et al., 2005). The nature of the hypothetical compartment is unknown, but it could be ER–Golgi Intermediate Compartments.

![Graph and images](http://mic.sgmjournals.org 2615)
(ERGIC) or autophagosomes, as proposed previously (Pizarro-Cerdá et al., 1998). ERGIC would be an interesting candidate since it was already shown that (i) Rab2, a Rab GTPase associated with ERGIC, is recruited on the BCVs and that (ii) the GAPDH/COPI/Rab2/PKCi/lcomplex, involved in retrograde transport between the ER and Golgi apparatus, is required for bacterial proliferation (Fugier et al., 2009). Preliminary data indicate that although the wild-type strain mainly colocalizes with an ERGIC marker (ERGIC53) at 12 h post-infection, though markedly less at 20 h post-infection, the ΔcstA mutant is not more frequently associated with this marker (data not shown), suggesting that ΔcstA does not accumulate and proliferate in ERGIC.

We also reported that the 3Flag–CstA fusion is secreted in bacterial culture supernatants independently of the T4SS and flagellum, suggesting that another secretion system is involved in this condition. This secretion system remains to be identified. Conversely, the CstA–3Flag fusion is not secreted, suggesting that a tag at the C-terminal region of CstA impaired secretion signal recognition, or that a C-terminal 3Flag tag destabilizes the fusion protein. Recently, a new Brucella T4SS substrate, BPE123, was identified (Marchesini et al., 2011). It was shown that its 25 aa N-terminal region, corresponding to the Sec secretion signal, is essential for its translocation to the host cell cytosol. CstA does not harbour a Sec secretion signal and a C-terminal T4SS secretion signal, which is consistent with a secretion independent of the T4SS and rules out a two-step secretory pathway. Brucella genome analysis suggests the presence of several predicted membrane fusion proteins, which could be part of type I secretion systems (T1SSs). Therefore the involvement of a T1SS in CstA secretion cannot be excluded.

The translocation of CstA in host cells is required for its interaction with Sec24A, and further experiments using different translocation reporters such as TEM-β-lactamase or CyaA’ and different cell lines will be needed.

Fig. 5. The B. abortus ΔcstA mutant strain is altered for LAMP1 acquisition. (a) HeLa cells were infected with the B. abortus wild-type, ΔcstA or complemented ΔcstA strain (dark, mid and light grey, respectively) and were fixed at 12 or 24 h post-infection. The percentage of BCVs that harbour LAMP1, a marker of late endosomes and lysosomes, was scored using immunofluorescence confocal microscopy. A χ² statistical analysis was performed and showed a significant difference between the wild-type and the ΔcstA strains at 12 h post-infection for LAMP1 (P<0.05). (b) Representative confocal microscopy images of vacuoles containing the wild-type strain at 12 h post-infection. Fixed infected cells stained with anti-LAMP1 antibody (red) and bacteria (green) detected by a rabbit polyclonal antibody against Brucella. Arrowheads indicate BCVs positive for LAMP1 and full arrows indicate BCVs negative for LAMP1 (bars, 10 μm).

Fig. 6. The B. abortus ΔcstA mutant strain is altered for Calnexin acquisition. (a) HeLa cells were infected with the B. abortus wild-type, ΔcstA or complemented ΔcstA strain (dark, mid and light grey, respectively) and were fixed at 12 or 24 h post-infection. The percentage of BCVs that harbour the ER marker Calnexin was scored using immunofluorescence confocal microscopy. A χ² statistical analysis was performed and showed a significant difference between the wild-type and the ΔcstA strains at 12 h post-infection for Calnexin (P<0.001). (b) Representative confocal microscopy images of vacuoles containing the wild-type strain at 12 h post-infection. Fixed infected cells were stained with anti-Calnexin antibody (green) and bacteria (red) detected by an anti-O antigen antibody (A76-12G12). Arrowhead indicates BCVs positive for Calnexin and full arrow indicates BCVs negative for Calnexin (bars, 10 μm).
The CstA coding sequence (BAB2_0856) is apparently in the operon with the manBcore coding sequence (BAB2_0855). A manBcore mutant lacks the O antigen and is defective in the LPS core biosynthesis (González et al., 2008; Monreal et al., 2003). Sequence analysis suggests that CstA could have two different enzymic activities, mannose-6-phosphate isomerase and mannose-1-phosphate guanylyltransferase. Interestingly, these two activities are also predicted to be carried by two distinct proteins, ManA and ManC, respectively, encoded in the wbk region (Haag et al., 2010). A third protein (BAB1_2103) contains a nucleotidyltransferase domain that is able to transfer a nucleotide onto a phosphosugar. This nucleotidyltransferase family includes x-D-glucose-1-phosphate cytidylyltransferase, mannose-1-phosphate guanylyltransferase, and glucose-1-phosphate thymidylyltransferase. Since the Δcsta mutation removes the complete coding sequence, the apparently normal O antigen and LPS core of the Δcsta mutant suggest that either CstA does not code for the predicted enzymic activities or these activities are redundant with those encoded by the wbk locus and/or BAB1_2103. One attractive hypothesis would be that functional redundancy between CstA and ManA and/or ManC would have allowed for the acquisition of new functions for CstA during evolution, for the control of bacterial trafficking inside host cells. It is also possible that the enzymic activity of CstA is required for normal intracellular trafficking, e.g. by indirectly modifying surface polysaccharides.

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