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Pathogens and Disease, 75, 2017, ftx047

doi: 10.1093/femspd/ftx047
Advance Access Publication Date: 25 April 2017
Research Article

RESEARCH ARTICLE

The *Coxiella Burnetii* type IVB secretion system (T4BSS) component DotA is released/secreted during infection of host cells and during *in vitro* growth in a T4BSS-dependent manner

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One sentence summary: The T4BSS component protein DotA of *Coxiella burnetii* is released during infection of host cells and localizes to the membrane of the parasitophorous vacuole in a T4BSS-dependent manner.

Editor: Patrik Bavoil

ABSTRACT

Coxiella burnetii is a Gram-negative intracellular pathogen and is the causative agent of the zoonotic disease Q fever. To cause disease, *C. burnetii* requires a functional type IVB secretion system (T4BSS) to transfer effector proteins required for the establishment and maintenance of a membrane-bound parasitophorous vacuole (PV) and further modulation of host cell process. However, it is not clear how the T4BSS interacts with the PV membrane since neither a secretion pilus nor an extracellular pore forming apparatus has not been described. To address this, we used the acidified citrate cysteine medium (ACCM) along with cell culture infection and immunological techniques to identify the cellular and extracellular localization of T4BSS components. Interestingly, we found that DotA and IcmX were secreted/released in a T4BSS-dependent manner into the ACCM. Analysis of *C. burnetii*-infected cell lines revealed that DotA colocalized with the host cell marker CD63 (LAMP3) at the PV membrane. In the absence of bacterial protein synthesis, DotA also became depleted from the PV membrane. These data are the first to identify the release/secretion of *C. burnetii* T4BSS components during axenic growth and the interaction of a T4BSS component with the PV membrane during infection of host cells.

Keywords: Q fever; type 4B secretion system; DotA, *Coxiella*; intracellular pathogenesis; ACCM

INTRODUCTION

Coxiella burnetii is a Gram-negative intracellular pathogen and is the causative agent of acute Q fever as well as chronic diseases. Transmission of *C. burnetii* is predominantly through the aerosol route and one to ten organisms is sufficient to cause disease (Marrie 2004). Post inhalation, *C. burnetii* targets alveolar macrophages to establish an intravacuolar replicative niche termed the parasitophorous vacuole (PV), which has many of the attributes of a mature phagolysosome (Voth and Heinzen 2007). During the ∼6-day infectious cycle, *C. burnetii* alternates between the environmentally stable, metabolically quiescent small cell variant (SCV) and the replicative large cell variant

Received: 2 December 2016; **Accepted:** 20 April 2017
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(LCV) (Heinzen, Hackstadt and Samuel 1999). This cycle is defined by a morphogenesis of the SCV into the LCV by 6–8 h post infection (hpi) and then at 6 days the LCV asynchronously converts back to the environmentally stable SCV form (Coleman *et al.* 2004). After cellular uptake and entering the endocytic pathway, infectious *C. burnetii* delay the trafficking of the early PV for ~2 hpi (Howe and Mallavia 2000). During this time, the PV gains the host markers early endosome antigen 1, Rab5 and LC3 (Beron *et al.* 2002; Romano *et al.* 2007). As the PV matures the early markers dissociate and Rab7 (Beron *et al.* 2002), lysosome-associated membrane protein (LAMP) 1, LAMP 2 and LAMP 3 (CD63) (Voth and Heinzen 2007), vacuolar H^+ ATPase (Heinzen *et al.* 1996), and flotillin 1 and 2 (Howe and Heinzen 2006) decorate the PV membrane. The development of the PV is essential for the replication and subsequent pathogenesis of *C. burnetii* and is mediated by the type 4B secretion system (T4BSS) (Howe *et al.* 2003b; Beare *et al.* 2011).

From genomic sequence analysis, it was determined that *C. burnetii* possesses homologs to 23 of the 26 *Legionella pneumophila* T4BSS components (Seshadri *et al.* 2003). *Legionella pneumophila* utilizes the T4BSS as an essential virulence factor for the translocation of effector proteins into the cytoplasm of the host cell (Segal and Shuman 1997; Bardill, Miller and Vogel 2005). *Legionella pneumophila* has been used as a surrogate to identify T4BSS-dependent secretion of several putative *C. burnetii* effector proteins (Pan *et al.* 2008; Chen *et al.* 2010). This indicates a degree of functional similarity between the T4BSSs of each organism, and is further supported by the ability of *C. burnetii dotB*, *icmS*, *icmT* and *icmW* genes to complement the corresponding *L. pneumophila* mutants although complementation was not observed for *dotL*, *dotM*, *dotN*, *dotO*, *icmQ* and *icmX*-deficient mutants (Zamboni *et al.* 2003; Zusman, Yerushalmi and Segal 2003). The ability to grow *C. burnetii* axenically and generate site-specific mutants has shown that the cellular pathogenesis of *C. burnetii* is dependent on a functional T4BSS (Omsland and Heinzen 2011; Beare *et al.* 2012). Deletion of *C. burnetii dotA* and *dotB* (Beare *et al.* 2012) and transposon mutagenesis of *dotE, dotF, dotG, dotL1, dotH, dotK, dotM, dotN, icmD, icmV and icmX* have been shown to prevent the development of a replicative PV during infection of cells (Beare *et al.* 2011; Carey *et al.* 2011; Martinez *et al.* 2014). These findings demonstrate the crucial role that the *C. burnetii* T4BSS plays in infection and replication within host cells. However, unlike the model T4ASS of *Agrobacterium tumefaciens,* a pilus for the *C. burnetii* and *L. pneumophila* T4BSS has not been demonstrated (Vincent and Vogel 2008) despite the core structure of the *L. pneumophila* T4BSS being similar in architecture and likely secretion mechanism as the T4ASS (Kubori *et al.* 2014; Ghosal *et al.* 2017). This lack of a secretion pilus in the T4BSS further confounds how *C. burnetii* and *L. pneumophila*, which are contained in a membrane-bound vacuole during infection, engages the PV membrane for the transfer of effector proteins into the host cell cytoplasm.

Interestingly, it was observed that during growth in axenic media *L. pneumophila* secretes, or releases, DotA and IcmX in a T4BSS-dependent manner (Matthews and Roy 2000; Nagai and Roy 2001). Although predicted to be a polytopic inner membrane protein, DotA from *L. pneumophila* was found to form hollow ring structures when harvested from the growth medium and observed by transmission electron microscopy (TEM). It was hypothesized that this structure might interact with the host or vacuolar membrane to mediate the transfer of effector molecules into the host cytoplasm during infection, but direct evidence of DotA interacting with the vacuolar membrane during infection was not achievable (Nagai and Roy 2001). In addition, IcmX from *L. pneumophila* is also released into the axenic media during growth although it is primarily localized to the periplasmic space (Matthews and Roy 2000) and requires a functional type II secretion system for subsequent T4BSS-dependent secretion (DebRoy *et al.* 2006). The significance and function of IcmX in the periplasm or away from the cell and the release of DotA are unclear, yet both proteins are essential for *L. pneumophila* pathogenesis.

Here, we sought to identify *C. burnetii* T4BSS components with secretion signals that are potentially secreted or released from the bacteria during growth in the axenic acidified citrate cysteine medium (ACCM) and subsequently to characterize their localization in the host cell during infection. Our hypothesis is that T4BSS components found secreted in the ACCM will localize extracellularly in an infectious setting. Previously, we have shown that the T4BSS localizes to the pole(s) of *C. burnetii* and that the bacterium can often be observed in direct contact with the PV (Morgan, Luedtke and Shaw 2010). This suggests that an intimate interaction of the T4BSS with the PV membrane occurs, but it is not clear how effector molecules are translocated without clear evidence of a secretion pilus. In this study, we demonstrate that *C. burnetii* DotA and IcmX are secreted, or released, into the ACCM during *C. burnetii* growth by a mechanism that is dependent on an intact T4BSS. During infection, we have identified DotA colocalizing with the PV membrane.

MATERIALS AND METHODS

Bacterial cultivation and storage

Avirulent *Coxiella burnetii* Nine Mile phase II RSA 439 (NMII) and virulent *C. burnetii* Nine Mile phase I RSA 493 (NMI) were cultivated and the SCV form purified as previously described (Coleman *et al.* 2004; Morgan, Luedtke and Shaw 2010). All work with NMI was performed in a CDC approved biosafety level-3 (BSL-3) with proper personal protection. NMII and NMI were stored at –80°C in SPG buffer (0.7 M sucrose, 3.7 mM KH_2PO_4, 6.0 mM K_2HPO_4, 0.15 M KCl, 5.0 mM glutamic acid, pH 7.4). In addition, *C. burnetii* NMII and *C. burnetii* NMII Δ*dotA*, Δ*dotB* and Δ*icmD* mutants (kindly provided by Dr Robert Heinzen) (Beare *et al.* 2011, 2012) were propagated using ACCM and stored at –80°C as previously described (Omsland *et al.* 2009).

Cell culture and infection

Vero, HeLa and RK13 (rabbit kidney) cell lines were propagated in antibiotic free RPMI 1640 medium supplemented with 5% FBS and incubated in a humidified chamber at 37°C with 5% CO_2. Vero and HeLa cells were infected with NMII using centrifugation at 600 g for 15 min at 25°C (termed spinoculation) (Wike *et al.* 1972). After spinoculation, unphagocytosed bacteria were removed using three washes of pre-warmed RPMI 1640 medium. NMI infection of RK13 cells was accomplished without spinoculation by inoculating the cell culture with NMI and incubating for 2 h at 37°C, then washing cells with pre-warmed RPMI 1640 three times to remove extracellular bacteria. Infected cells were cultured at 37°C, 5% CO_2 atmosphere in a humidified chamber for the desired length of hpi with time zero being immediately post washing.

Antibody production

Full-length *C. burnetii icmX*, *dotH*, *dotN*, *com1* and nucleotides corresponding to amino acids 177–298 of DotA were

amplified by PCR for cloning into the pET200/D-TOPO vector as described by the manufacturer (Invitrogen, Carlsbad, CA, USA). Expression and isolation of recombinant protein as well as subsequent polyclonal antibody production was performed as described (Morgan, Luedtke and Shaw 2010). Protein A cross-linked beads (Pierce, Rockford, IL Rockford, IL, USA) were used to enrich IgG from the serum, and the antibody was absorbed against *Escherichia coli* BL21 to eliminate non-specific reactivity (Morgan, Luedtke and Shaw 2010). Rabbit antibodies against *C. burnetii* IcmD were kindly provided by Dr Robert Heinzen (Beare *et al.* 2011). Rabbit polyclonal antibodies against *C. burnetii* DotB and DotC were raised using the synthetic peptides KSQRYP-NEPSRFEPK, TLTRRELSNAETSDL and HYEIRPNRNERFRYR and LNSLPPGSGQINNIR, LNLADDDTIRTADKT and TAQSALQPDSSH-WNP, respectively. Polyclonal antibodies against whole, heat killed sucrose gradient purified *C. burnetii* NMII were produced in guinea pigs using a custom antibody production service (Rockland Immunochemicals, Gilbertsville, PA, USA). The specificity of each antibody against the T4BSS homologs IcmX, DotB, DotC, DotH, DotN and the major outer membrane protein Com1 was determined by immunoblot against the respective recombinant protein and compared to NMII lysate. Recombinant protein was subsequently produced for DotB and DotC in full length to analyze the specificity of the antibodies against the synthetic peptides. The specificity of antibody against DotA was addressed by comparing NMII and NMII Δ*dotA* lysates by immunoblot and by absorbing the antibody with recombinant DotA (rDotA). In addition, the DotA and DotH antibodies were utilized as previously described (Beare *et al.* 2014)

Reverse transcriptase-PCR

Reverse transcriptase-PCR (RT-PCR) was performed on total RNA harvested from NMII cultures grown in ACCM for 4 and 7 days post inoculation as previously described (Morgan *et al.* 2010). Briefly, nucleic acids were isolated using Tri-Reagent® (Ambion, Carlsbad, CA, USA), and DNA was removed using RQ1 DNase (Promega, Madison, WI, USA). Primer sets for RT-PCR of *dotH* (forward 5′-ATTGGGGCCAGTATCATTCC-3′, reverse 5′-ATGGAGTGTGCGGATTTGAT-3′), *icmT* (forward 5′-AT GAAATCTCTCGATGAGG-3′, reverse 5′-GTTATCCCACCATGCTAT GG-3′), *icmV* (forward 5′-ATGATTCTTTTGGAGTCTTCC-3′, reverse 5′-TTGTTTGGACCCCTTAAAGGTG-3′) and *icmW* (forward 5′-ATG CCAGATCTGTCGC-3′, reverse 5′-TAAACCACCTTCCTCAAGAG-3′) were internal of the open reading frames. To confirm samples contained no DNA, SuperScript® III RT (ThermoFisher, Carlsbad, CA, USA) was not included in paired reactions. The ability of primer sets to amplify DNA was validated using *C. burnetii* genomic DNA.

Immunoblot analysis

NMII cultures were grown in ACCM for 96 and 168 hpi and were harvested by centrifugation at 13 000 g for 15 min at 4°C. The supernatant (media) was carefully removed and Halt protease inhibitor cocktail (Pierce, Rockford, IL, USA) was added prior to storage at –80°C. The resulting NMII pellet was washed with sterile PBS pH 7.4, pelleted again as above and resuspended in protein running buffer containing Halt protease inhibitor cocktail (Pierce) and stored at –80°C. To analyze the ACCM for *C. burnetii* secreted/released proteins, the medium was concentrated using I-Con 9kDa spin concentrators (Pierce). Halt protease inhibitor cocktail (Pierce) was added to samples and stored at –80°C. Total protein was not boiled prior to being separated using 12% SDS-PAGE to prevent the insolubility or aggregation of T4BSS membrane proteins. After SDS-PAGE, total proteins were immobilized on a nitrocellulose membrane (Whatman, Dassel, Germany). Immunoblot analysis was carried out with overnight incubation at 4°C with the respective primary antibody all at 1:3000 and followed by HRP-conjugated secondary antibody at 1:20 000 for 1 h. The labeled proteins were detected using the Pico Western Chemiluminescence Kit (Pierce) following the manufacturer's directions. Digital images were captured using a FluorChem HD2 Imaging System (Alpha Innotech Corporation, Leandro, CA). All micrographs were processed equally and analyzed using ImageJ version 1.46h (Rasband 1997–2012).

Immunoflurocescent microscopy

Uninfected cell lines were seeded on 12-mm glass coverslips in 24-well tissue culture plates at 10^5 cells/well. Cells were allowed at least 16 h to adhere to the coverslip before infecting. Infections were carried out as described above. At various hpi, cells were fixed for 5 min using 100% ice-cold methanol. In some cases, the protein synthesis of *C. burnetii* was inhibited using chloramphenicol (10 μg/mL) beginning at 72 hpi. Antigen was stained using the polyclonal antibodies developed against the $DotA_{177\text{-}298}$ epitope (1:500), IcmX (1:250) and whole *C. burnetii* (1:2500) as previously described here. Monoclonal antibody against the host protein CD63 was purchased commercially (BD Biosciences, San Diego, CA). Host cell lysate, for the respective cell line, was added at a 1:100 dilution to the diluted primary antibodies and allowed to incubate at 25°C for 1 h before applying to the fixed cells. The primary antibodies were detected using antibody conjugated with either Alexa Fluor® 488, 555 or 633 all at a 1:2000 dilution and previously absorbed as described for the primary antibodies (Molecular Probes®, Carlsbad, CA). Total nucleic acid was stained with 4′,6-diamidino-2-phenylindole (DAPI). Images were captured using a Nikon Eclipse TE 2000 SE microscope with a Nikon DS FI1 camera or a Leica DMIRB2 SP2 laser scanning confocal microscope. All images were processed equally using ImageJ version 1.46h (Rasband 1997–2012), and the Coloc 2 plugin was used to determine areas of protein colocalization (Schindelin *et al.* 2012).

Immunoelectron microscopy

Vero cells were seeded on sterilized ACLAR film (Ted Pella, Redding, CA) in six-well tissue culture plates and allowed to adhere at least 16 h prior to infection. The cells were infected using spinoculation as described above. At 96 hpi, all cells were fixed using a periodate-lysine-paraformaldehyde (PLP) fixative as previously described, with slight modifications (McLean and Nakane 1974; Brown and Farquhar 1989; Bannantine, Rockey and Hackstadt 1998). Briefly, media was removed from cells and 0.01M periodate, 0.075M lysine, 0.075M sodium phosphate, 1% paraformaldehyde and 0.05% glutaraldehyde were applied for 3 h at room temperature and then washed with PBS. For immunoelectron microscopy, the cells on ACLAR were infiltrated overnight in the cryoprotectant 2.3M sucrose/20% polyvinyl pyrrolidone in PIPES/$MgCl_2$ at 4°C. To permeabilize cells for antibody labeling, cells on the ACLAR were plunge frozen in liquid nitrogen and subsequently thawed in PBS at room temperature. Samples were probed with the rabbit anti-DotA (1:500) followed by FluoroNanogold anti-rabbit Fab (1:250) (Nanoprobes, Yaphank, NY, USA) and silver enhancement for 2 to 5 min (HQ silver enhancement kit, Nanoprobes). Samples were then processed for standard resin embedding. Prelabeling

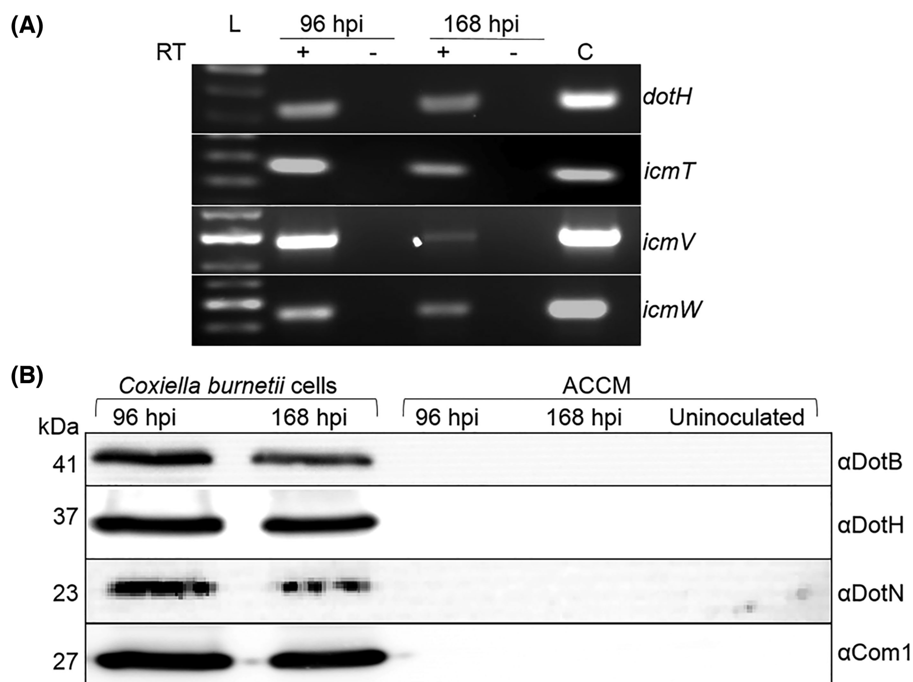

Figure 1. The *C. burnetii* T4BSS is expressed during growth in ACCM. **(A)** Total *C. burnetii* RNA was harvested from ACCM cultures at 96 and 168 hpi. Transcripts for *dotH*, *icmT*, *icmV* and *icmW* are detectable only when reverse transcriptase (RT) is added to the 96 and 168 hpi RT-PCR reactions. **(C)** Represents a chromosomal DNA PCR-positive control. **(B)** Total *C. burnetii* protein was harvested from the 96 and 168 hpi cultures. Equal volumes of the uninoculated and spent media (ACCM) from the respective cultures were retained and concentrated to equal volumes. The loading volumes of the 96 and 168 hpi cell samples were normalized using the membrane protein Com1. Predicted molecular weights (kDa) are indicated on the left. Immunoblot was used to detect the predicted cytoplasmic protein DotB, the predicted inner membrane protein DotN and the predicted outer membrane protein DotH. These proteins were detectable in all of the *C. burnetii* lysates and could not be detected in the ACCM.

experiments were conducted in parallel with controls omitting the primary antibody. These controls were consistently negative at the concentration of Nanoprobes-conjugated secondary.

RESULTS

The *Coxiella burnetii* T4BSS is expressed at the mRNA and protein levels during growth in ACCM

With little existing analysis of *C. burnetii* T4BSS gene expression during growth in ACCM, we sought to further confirm/define the expression of select homologs, which were previously confirmed using tissue culture infection (Morgan *et al*. 2010), in the axenic medium. *Coxiella burnetii* was grown to and harvested at 96 and 168 h post inoculation, which represents the mid log and stationary growth phases, respectively. These time points were selected to represent the metabolically active LCVs at 96 h post inoculation and the mixed SCV/LCV population in the 168 h post inoculation (Omsland *et al*. 2009), in which T4BSS transcripts and protein is most likely to be at a detectable level. Using total RNA harvested from ACCM cultures grown for 96 and 168 h post inoculation, non-quantitative RT-PCR analysis revealed that the *C. burnetii* T4BSS is transcribed during axenic growth (Fig. 1A). Transcripts of *dotH*, *icmT, icmV* and *icmW* are amplified in the plus-RT lanes and absent from the minus–RT controls (Fig. 1A). The detection of *C. burnetii* T4BSS proteins during ACCM growth was performed by immunoblot analysis of whole *C. burnetii* harvested at 96 and 168 h post inoculation. Analysis of antibody specificity showed that each of the antibodies respectively detected IcmX, DotA, DotB, DotC, DotH, DotN and Com1 at the expected molecular weight and were specific to the corresponding T4BSS protein in the NMII lysate when compared to the recombinant protein (data not shown). Using antibodies against *C. burnetii* T4BSS homologs shown by Vincent *et al*. (2006) to reside in the cytoplasm (DotB), the inner membrane (DotN) and the outer membrane (DotH), all of these proteins were found to be expressed at both time points during ACCM growth. Com1, which was previously found to be equally present for SCVs and LCVs during axenic growth, was used to normalize the loading volume between *C. burnetii* lysates (Omsland *et al*. 2008). In addition, the ACCM did not contain detectable amounts of DotB, DotH, DotN and Com1, which represents each of the cellular fractions (Fig. 1B). These results demonstrate that *C. burnetii* T4BSS proteins are expressed during bacterial growth in ACCM, and the absence of DotB, DotH, DotN and Com1 in the ACCM from 96 and 168 h post inoculation suggests that during growth in ACCM and preparation of the samples that the cellular structure of the bacteria is not compromised. (Fig. 1B).

Coxiella burnetii T4BSS homologs contain secretion sequences and DotA and IcmX are secreted/released into the ACCM during growth in a T4BSS-dependent manner

With the observation of *C. burnetii* T4BSS transcripts and proteins being synthesized during growth in ACCM, the conditioned growth medium at 96 and 168 h post inoculation was investigated for the secretion or release of T4BSS proteins. We hypothesized that *C. burnetii* T4BSS components with an N-terminal secretion signal sequence could be secreted into the ACCM. *In silico* analysis (using SignalP 4.0) of the 23 *C. burnetii* T4BSS proteins revealed 6 proteins with predicted secretion sequences (Petersen *et al*. 2011). *Coxiella burnetii* T4BSS proteins with a predicted

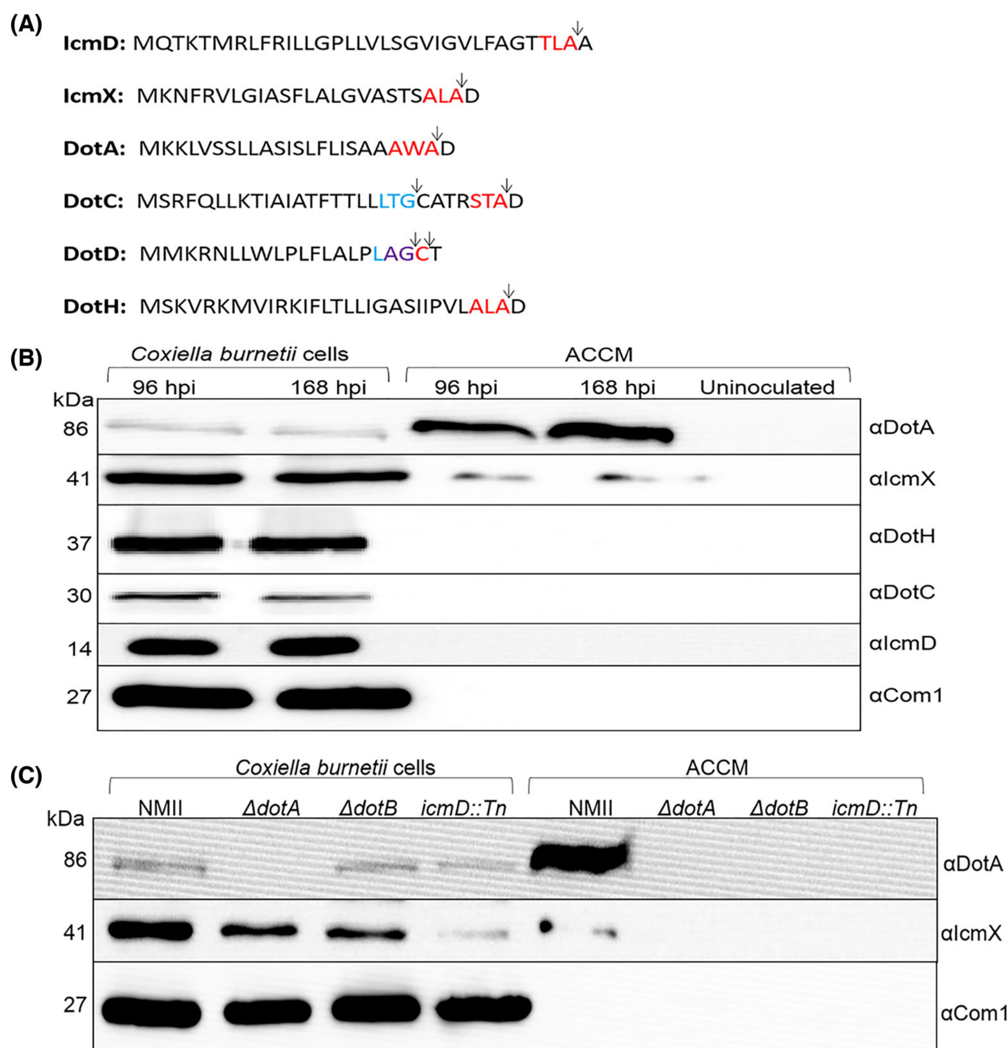

Figure 2. Prediction of T4BSS secretion signal sequences, detection of T4BSS components in the ACCM during growth of *C. burnetii* and dependence of a functional T4BSS secretion system for the release of DotA and IcmX. (**A**) SignalP 4.0 was used to identify N-terminal secretion signals amongst the 23 T4BSS proteins (Petersen *et al.* 2011). IcmD, IcmX, DotA, DotC, DotD and DotH contained a secretion signal for type I peptidase (red text). In addition, DotC and DotD also contained a secretion signal for type II peptidase (blue text). In DotD, the type I and type II signals overlapped (purple text). Arrows designate predicted cleavage location. (**B**) *Coxiella burnetii* grown in ACCM were harvested at 96 and 168 hpi and the ACCM from these cultures was retained. T4BSS components with predicted secretion signals were independently probed for by immunoblot using antibodies against DotA, IcmX, DotH, DotC and IcmD. All T4BSS components with a predicted secretion signal are detectable in the *C. burnetii* lysate and only DotA and IcmX are released into the ACCM. The amount of total *C. burnetii* protein loaded was normalized using Com1. Equal starting volumes of an uninoculated ACCM (negative control) and the 96 and 168 hpi ACCM samples were concentrated to an equal volume and equal volumes loaded. Predicted molecular weights (kDa) are indicated on the left. (**C**) ACCM cultures of *C. burnetii* NMII and the *C. burnetii* NMII T4BSS Δ*dotA*, Δ*dotB* and *icmD*::Tn mutants were grown to 168 h post inoculation and the ACCM from the respective cultures retained. *Coxiella burnetii* lysates were normalized to Com1 and equal volumes of the conditioned ACCM were concentrated to an equivalent volume. Proteins were detected by immunoblot using antibodies against DotA and IcmX. Predicted molecular weights (kDa) are indicated on the left. DotA and IcmX were not detectable in the ACCM from mutant strains and are only found in ACCM from the *C. burnetii* NMII culture and the *C. burnetii* cell lysates.

secretion signal sequence for type I signal peptidase cleavage included DotA, DotC, DotD, DotH, IcmD and IcmX while DotC and DotD had an additional secretion signal sequence for type II signal peptidase, which is associated with lipoproteins (Fig. 2A). Immunoblot analysis of these proteins, with the exception of DotD (no existing antibody), shows that IcmX and DotA are released into the ACCM during growth while DotC, DotH and IcmD could not be detected in the ACCM but were found associated with the *C. burnetii* lysates (Fig. 2B). To determine whether the secretion/release of *C. burnetii* IcmX and DotA requires a functional T4BSS, *C. burnetii* mutants deficient for T4B secretion were grown in ACCM and the medium was probed for IcmX and DotA. IcmX and DotA from secretion competent *C. burnetii* were consistently found in the ACCM, while in Δ*dotA*, Δ*dotB* and *icmD::Tn C. burnetii* strains (Beare *et al.* 2011, 2012) neither IcmX nor DotA could be detected in the media (Fig. 2C). These results demonstrate that the secretion/release of these proteins is dependent on a functional *C. burnetii* T4BSS.

Coxiella burnetii DotA localizes to the host cell PV membrane and cytoplasmic vesicles during infection

With the finding that *C. burnetii* IcmX and DotA are released into the conditioned ACCM, we sought to determine whether the release of IcmX and DotA occurred during *C. burnetii* infection of

host cells. Using polyclonal antibodies against *C. burnetii*, IcmX and DotA, and monoclonal antibody against the established host cell PV marker, CD63, multiplex IFAs were performed on NMII-infected HeLa cells over the course of a 120-h infection. In infected cells, IcmX was not found to localize to areas away from the bacteria (data not shown). However, in the infected cells, the fluorescent signal indicating the presence of DotA localized to the PV membrane and colocalized with CD63 as early as 48 hpi and remained through 120 hpi (Fig. 3A). This colocalization was observed in ∼72%, 79%, 90% and 91% of infected cells for times 48, 72, 96 and 120 hpi, respectively (Fig. 3B). In addition, the specificity of the antibodies against DotA was observed by absorbing the reactive antibodies against rDotA. The rDotA effectively neutralized the staining of DotA and no fluorescence was detectable at the PV membrane or on the *C. burnetii* (Fig. 3C). To determine whether DotA secretion/release occurs during infection with virulent *C. burnetii*, RK13 cells were infected with NMI and IFA analysis was performed. In agreement with the *C. burnetii* NMII observations, DotA staining localized to the PV membrane of the infected RK13 cells (Fig. 3D). In addition, the use of RK13 cells infected with NMI demonstrates that the localization of DotA to the PV membrane is not a cell line-specific observation. To further confirm our IFA observations and gain a more detailed visualization of DotA localization in an infected host cell, IEM was utilized. Using IEM to observe DotA localization in Vero cells infected for 96 h with NMII, it was found that DotA localizes to the PV membrane and to host cell cytoplasmic structures, which are devoid of *C. burnetii*, that resemble multivesciular bodies (MVBs) (Fig. 4).

Chloramphenicol treatment of *Coxiella burnetii*-infected cells depletes DotA from the PV membrane and cytoplasmic vacuoles

It is known that *C. burnetii* protein synthesis is required for the fusogenicity of the PV with host cell vesicles, but vesicle trafficking or recycling of membrane originating from the PV has not been observed (Howe *et al.* 2003b). However, it is presumed to occur due to the decrease in PV size when infected cells are treated with antimicrobial agents (Howe *et al.* 2003b; Mahapatra, Ayoubi and Shaw 2010). Using DotA as a marker for PV-derived membrane, NMII-infected cells at 72 hpi were mock treated or treated with a bacteriostatic concentration (10 μg/mL) of chloramphenicol. By 8 h post treatment, DotA signal in both cytoplasmic vesicles and the PV was still observable regardless of treatment (Fig. 5); however, by 24 h post treatment with chloramphenicol, DotA no longer appeared to be associated with CD63-positive structures in the host cell cytoplasm and the PV membrane had reduced DotA staining (Fig. 5). Interestingly, DotA was found in the lumen of the collapsed PVs. The CD63 within the cytoplasm of the host cell returned to pre-infection distribution, resembling what has been previously reported for uninfected cells (Larson and Heinzen 2017) (Fig. 5).

DISCUSSION

The T4BSS has been shown to be an essential virulence mechanism for *Legionella pneumophila* and *Coxiella burnetii* pathogenesis (Andrews, Vogel and Isberg 1998; Beare *et al.* 2011; Carey *et al.* 2011). However, the details of assembly, functionality and structure of the T4BSS are limited when compared to what is known for T4ASSs. The knowledge gap between the two systems is primarily due to (i) a lack of significant homology between the two systems, (ii) the T4BSS is composed of twice as many protein components and (iii) the difficulties associated with culturing intracellular bacteria when compared to the genetically malleable model systems historically available for T4ASS studies (Christie *et al.* 2005; Nagai and Kubori 2011). The analysis of the *L. pneumophila* T4BSS has revealed clues to the functionality of some of the T4BSS proteins and their localization within the bacterial cell. Vincent *et al.* (2006) was the first to begin to detail the subcellular localization of the *L. pneumophila* T4BSS components and provided predictions of protein interactions thought to be involved in the formation of subcomplexes. A model outlining the predicted assembly and localization of the subcomplexes and proteins was drawn from these studies. In Vincent's model, a pilus structure was not predicted and has not been identified, leaving a question as to how effector proteins are translocated through the bacterial membranes, and across the host membrane to reach the host cell cytoplasm. To examine this question, we sought to utilize axenic cultivation of *C. burnetii* as a tool to analyze the extracellular localization of *C. burnetii* T4BSS components.

The development of an axenic growth medium for the propagation of *C. burnetii* has created an opportunity to understand and gain insight into the physiology and pathogenesis of this unique bacterium (Omsland *et al.* 2009, 2011). However, our understanding of the expression of the T4BSS components at the transcriptional and translational level during growth in ACCM is limited. Our detection of transcripts for T4BSS genes and proteins suggests that the *C. burnetii* T4BSS is produced during growth in ACCM. However, the functionality of the *C. burnetii* T4BSS during growth in ACCM remains unclear as the detection of known T4BSS effector proteins has not been reported during growth in ACCM (Carey *et al.* 2011) while one effector protein, which the authors suspect to be a false positive, was found in ACCM2 (Stead *et al.* 2013). The lack of detectable effector proteins further suggests that a host cell or environmental signal is required for the maturation and competence of the secretion system as it is in other bacterial virulence secretion systems (van der Goot *et al.* 2004; Epler *et al.* 2009; Jimenez-Soto *et al.* 2009). Moreover, the identification of PmrA, a part of a two-component regulatory system, as a modulator of T4BSS and effector protein expression in *L. pneumophila* and *C. burnetii* further suggests that an environmental signal(s) are involved in the development of the T4BSS (Beare *et al.* 2014). Indeed, multiple approximate 20 nm in length 'needle-like structures' have been observed by TEM that appear to extend from a *C. burnetii* cell towards what appears to be the PV membrane (Larson *et al.* 2016).

Our observation of DotA and IcmX being secreted by a T4BSS-dependent mechanism agrees with observations of this process occurring in liquid cultures of *L. pneumophila* (Matthews and Roy 2000; Nagai and Roy 2001). However, the functional significance of DotA secretion by *L. pneumophila* was debated by Vincent and Vogel (2008) who claimed that the secretion of a polytopic membrane protein from a bacterial secretion system has no precedent and that it is unclear how DotA could function as both an inner membrane protein and a T4B secretion substrate. Interestingly, our *in silico* detection of a conventional type I signal peptidase signal suggests that DotA could be targeted for transport across the inner membrane, and our observation of DotA being secreted into ACCM by a T4BSS-dependent mechanism agrees with observations of this process occurring in liquid cultures of *L. pneumophila* (Nagai and Roy 2001). With the detection of DotA in the ACCM, which was absent of detectable amounts of protein from the cytoplasm (DotB and DotN), inner membrane (DotN and IcmD), and outer membrane (DotC, DotH and Com1)

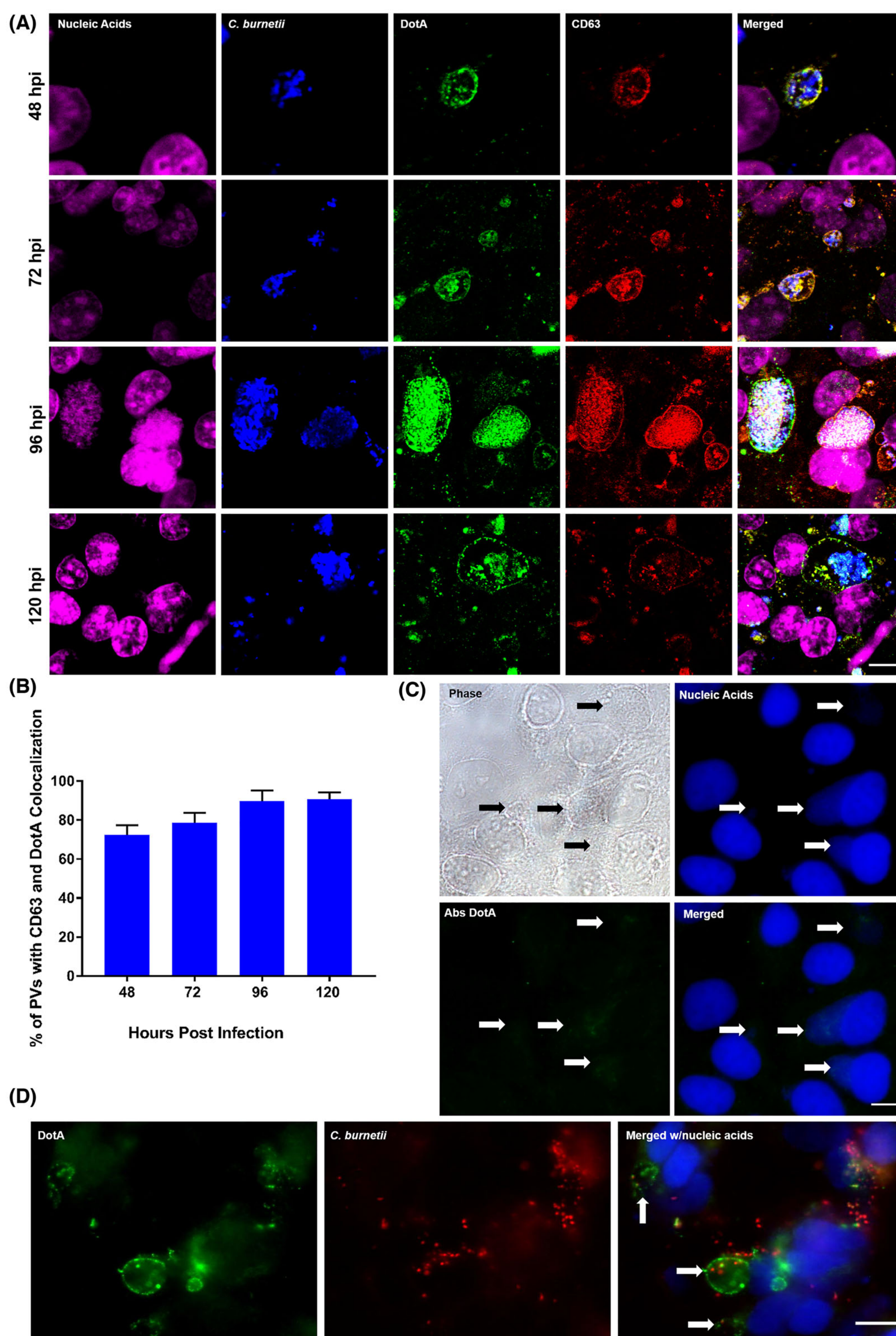

Figure 3. Temporal analysis of DotA localization during infection of host cells, specificity of DotA antibody and DotA staining using virulent *C. burnetii*. **(A)** Representative fluorescence micrographs of HeLa cells infected with *C. burnetii* over the course of infection. Infections were initiated with *C. burnetii* SCVs and fixed at 48, 72, 96 and 120 hpi. Fixed cells were labeled for total nucleic acids (pseudo-colored pink), *C. burnetii* (pseudo-colored blue), DotA (green) and CD63 (red). DotA is detectable by 48 hpi and in the merged images remains colocalized with CD63 through the course of infection. Scale bar equals 10 μm. **(B)** The average number of infected cells with PVs that were positive for CD63 and DotA colocalization enumerated from 100 infected cells from three separate replicates at 48, 72, 96, and 120 hpi. The percentage of PVs with CD63 and DotA colocalization remains consistent through the course of infection. **(C)** Representative phase contrast and fluorescent micrograph of infected Vero cells. Vero cells were fixed at 96 hpi and probed with antibodies against DotA that were previously absorbed with rDotA (Abs DotA). Arrows indicate the PV within the infected cells. No DotA staining is observed in the infected cells. Scale bar equals 10 μm. **(D)** Representative fluorescence micrographs of RK13 cells infected with *C. burnetii* NMI. RK13 cells were fixed at 72 hpi and labeled for DotA (green), *C. burnetii* (red) and total nucleic acids (blue). DotA staining appears at the PV membrane (arrows) as observed with the avirulent *C. burnetii* NMII-infected HeLa and Vero cell lines. Scale bar equals 10 μm.

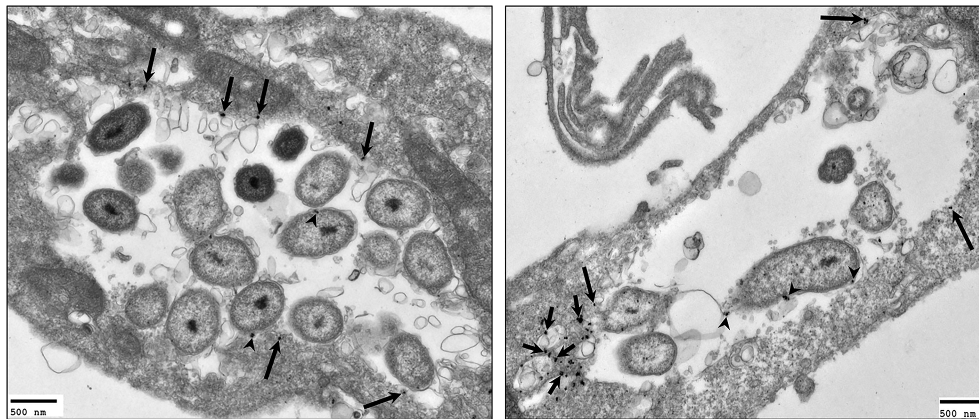

Figure 4. Localization of DotA using immunoelectron microscopy. Infected Vero cells were fixed using a modified version of the PLP method at 96 hpi for immunoelectron microscopy. Fixed cells were probed with antibodies against DotA and counterstained with gold-conjugated secondary antibodies. The gold particles (arrows) were observed localizing to the PV membrane (long arrows), multivesicular-like bodies (short arrows) and *C. burnetii* (arrow heads). Scale bars equal 500 nm.

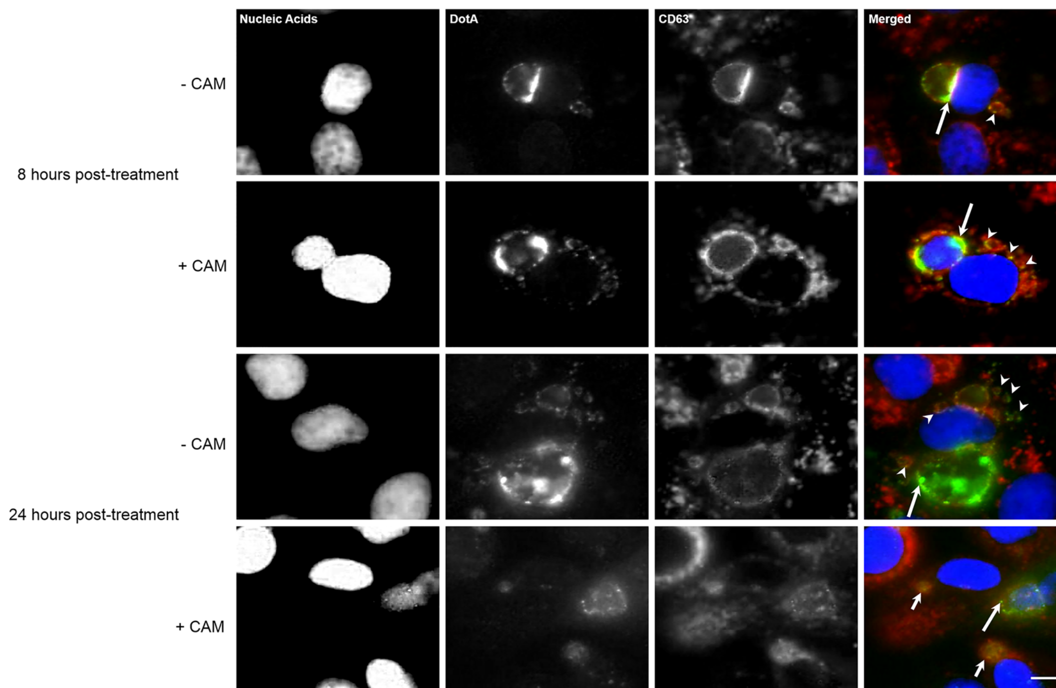

Figure 5. DotA–host cell membrane association is dependent on bacterial protein synthesis. Representative monochrome and respective merged fluorescence micrographs of Vero cells infected with *C. burnetii* and either mock treated (–CAM) or treated with 10 μg/mL chloramphenicol (+CAM) at 72 hpi. Top group: Infected cells were fixed 8 h after the mock treatment and chloramphenicol treatment. Nucleic acids (blue), DotA (green) and CD63 (red) were fluorescently labeled. At 8 h post treatment (hpt), DotA is visible in the PV membrane (long arrows) and cytoplasmic vesicles (arrowheads) for the both the mock and chloramphenicol-treated cells. Bottom group: Infected cells were fixed 24 h after the mock treatment and chloramphenicol treatment. Nucleic acids (blue), DotA (green) and CD63 (red) were fluorescently labeled. By 24 hpi, the +CAM cells had DotA associated only with PVs that are considered spacious (long arrow) and absent from collapsed PVs (short arrows) and also is no longer observed within the host cell cytoplasm while in the –CAM cells had DotA present in the PV (long arrow) and the cytoplasmic vesicles (arrowheads). Scale bars equal 10 μm.

(Figs 1 and 2), in combination with the observation of DotA associating with CD63-positive membrane supports the mobility of DotA beyond the inner membrane and is likely not an artifact from growth in axenic media or from sample preparation. In addition, it is unlikely that DotA is inadvertently released into the ACCM via outer membrane vesicles (OMVs) since DotA was not detected in ACCM2 by a highly sensitive method such as mass spectrometry despite the EM observation of OMVs at the *C. burnetii* cell surface during ACCM2 growth. (Stead *et al.* 2013). Moreover, OMVs were not found to be associated with the release of DotA in *L. pneumophila* (Nagai and Roy 2001) and the release of DotA has been used as an indicator of T4BSS function (Kuroda *et al.* 2015).

Additional support for the secretion of DotA in an infectious setting, by Nagai and Roy (2001), was absent, but they speculated that DotA could serve as a pore in the *L. pneumophila*-containing vacuole membrane for the translocation of effector proteins into the host cytoplasm. With the drastic difference in infectious cycle rates between *L. pneumophila* and *C. burnetii*, it is possible that cells infected with *L. pneumophila* would not have detectable

levels of DotA in the vacuolar membrane prior to host cell lysis as we were not able to clearly detect DotA in the PV membrane until 48 hpi (Alli *et al.* 2000). In addition, the cellular fixation method used by Nagai and Roy could have masked the anti-DotA immunoreactive epitopes (Kakuk *et al.* 2006). We found that using a standard paraformaldehyde with detergent fixation method (Morgan, Luedtke and Shaw 2010) did not provide a distinct fluorescent signal, which suggests an inability of the anti-DotA antibodies to find the corresponding eptiopes (data not shown). It is reasonable to believe that the use of methanol to fix the infected cells for IFA would extract lipids from the PV membrane and MVBs and denature proteins, which would expose the immunoreactive epitopes (DiDonato and Brasaemle 2003). Moreover, for TEM, the best DotA detection was done using the alternative PLP fixation method, which can be used to cross-link proteins bound in membrane (Brown and Farquhar 1989) and was also found to provide the best results for staining the *Chlamydia psittaci* IncC protein that associates with the inclusion membrane (Bannantine, Rockey and Hackstadt 1998). IcmX was also found to be secreted within tissue culture cells, but the detection of the protein in a localized area of the cell could not be elucidated (Matthews and Roy 2000). We were also unable to detect IcmX that was not localized to *C. burnetii* during infection. This could be due to a proteolysis of IcmX in the PV or insufficient localized antigen to produce a detectable signal. Further research is required to understand the importance of the secretion of IcmX in relation to T4BSS functionality and pathogenesis.

These data provide evidence that a component of the T4BSS interacts at the PV membrane during infection. The observation of DotA colocalizing with CD63 at the PV membrane suggests that DotA engages the membrane and maybe capable of membrane insertion. However, at this time it is not clear if the interaction of DotA with the PV membrane is dependent on *C. burnetii* associating directly with the PV membrane. Indeed, some T4SSs are dependent on contact with a host cell for the structural alteration of the secretion complex (Hayes, Aoki and Low 2010). With the previous visualization of DotA as a hollow ring structure (Nagai and Roy 2001), it could be hypothesized that DotA and IcmX form a T4BSS translocon to allow the transfer of effector proteins across the PV membrane.

The host cell process of trafficking endocytic and autophagic vesicles is important for degrading foreign particles as well as damaged or unnecessary cellular components for recycling within the cell. Some intracellular parasites have evolved mechanisms to alter vesicle trafficking as a way to form a replicative niche and/or acquire essential nutrients (Hackstadt 2000; Wang, Weiss and Orlofsky 2009). During infection, the *C. burnetii* PV is highly fusogenic with autophagic and endocytic vesicles; however, the trafficking of PV membrane away from the PV has not been described (Howe *et al.* 2003a; Romano *et al.* 2007). Our observation of DotA colocalization with CD63-positive vesicles, which do not contain *C. burnetii*, in the host cytoplasm by 72 hpi suggests that membrane and/or components from the PV lumen are entering a host cell membrane recycling pathway as transferrin receptor and clathrin associate with the PV (Latomanski *et al.* 2016; Larson and Heinzen 2017). Moreover, this could also explain the previous observation of increased CD63-positive vesicles in the host cell cytoplasm during infection (Larson and Heinzen 2017). However, it is not clear which host protein(s) or *C. burnetii* protein(s) are involved in this process, but it is likely that this process is *C. burnetii* driven as after the 24-h treatment with a bacteriostatic concentration of chloramphenicol, the infected cells had no detectable DotA colocalization with CD63-positive vesicles in the cytoplasm, and agrees with previous studies showing *C. burnetii* alters the normal host cell vesicle trafficking, recycling, and fusion for PV development and maintenance (Larson *et al.* 2013, 2015; Latomanski *et al.* 2016; Larson and Heinzen 2017).

The T4BSS-dependent development and maintenance of the PV is essential for *C. burnetii* pathogenesis. Our use of ACCM and tissue culture to characterize the localization of the *C. burnetii* T4BSS has provided data that demonstrates an array of *C. burnetii* T4BSS genes are expressed at the mRNA and protein level during growth in ACCM. The analysis of *C. burnetii* T4BSS homologs containing a predicted secretion signal determined that *C. burnetii* IcmX and DotA are released into the ACCM in a manner that is dependent on a functional T4BSS. Here we showed *C. burnetii* T4BSS proteins being released during growth in liquid media as well as during infection of host cells. Moreover, we demonstrate that a T4BSS protein colocalizes with the host cell/PV membrane during infection. In addition, we show that DotA can be found associated with MVBs/vesicles that are devoid of *C. burnetii* and located away from the PV. This finding may allow DotA to be used as a marker of PV-derived membrane for trafficking/recycling studies in infected host cells and could provide a better understanding of how *C. burnetii* interacts with the host cell from within the membrane-bound PV.

ACKNOWLEDGEMENTS

We wish to thank Dr Anders Omsland, Rocky Mountain Laboratories, Dr Dan Voth, University of Arkansas College of Medicine, for technical expertise and advice on the *in vitro* growth of *C. burnetii*, and Dr Wandy Beatty Washington University in St. Louis School of Medicine, for expertise and processing of samples for immunoelectron microscopy.

FUNDING

This research was supported by National Institutes of Health grant A1072710 (EIS).

Conflict of interest. None declared.

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