IAI Journals.ASM.org

# *Coxiella burnetii* Effector Proteins That Localize to the Parasitophorous Vacuole Membrane Promote Intracellular Replication

**Charles L. Larson,[a] Paul A. Beare,[a] Daniel E. Voth,[b] Dale Howe,[a] Diane C. Cockrell,[a] Robert J. Bastidas,[c] Raphael H. Valdivia,[c] Robert A. Heinzen[a]**

*Coxiella* Pathogenesis Section, Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana, USA[a]; Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA[b]; Department of Molecular Genetics and Microbiology, Center for Microbial Pathogenesis, Duke University, Durham, North Carolina, USA[c]

**The intracellular bacterial pathogen *Coxiella burnetii* directs biogenesis of a parasitophorous vacuole (PV) that acquires host endolysosomal components. Formation of a PV that supports *C. burnetii* replication requires a Dot/Icm type 4B secretion system (T4BSS) that delivers bacterial effector proteins into the host cell cytosol. Thus, a subset of T4BSS effectors are presumed to direct PV biogenesis. Recently, the PV-localized effector protein CvpA was found to promote *C. burnetii* intracellular growth and PV expansion. We predict additional *C. burnetii* effectors localize to the PV membrane and regulate eukaryotic vesicle trafficking events that promote pathogen growth. To identify these vacuolar effector proteins, a list of predicted *C. burnetii* T4BSS substrates was compiled using bioinformatic criteria, such as the presence of eukaryote-like coiled-coil domains. Adenylate cyclase translocation assays revealed 13 proteins were secreted in a Dot/Icm-dependent fashion by *C. burnetii* during infection of human THP-1 macrophages. Four of the Dot/Icm substrates, termed *Coxiella* vacuolar protein B (CvpB), CvpC, CvpD, and CvpE, labeled the PV membrane and LAMP1-positive vesicles when ectopically expressed as fluorescently tagged fusion proteins. *C. burnetii* Δ*cvpB*, Δ*cvpC*, Δ*cvpD*, and Δ*cvpE* mutants exhibited significant defects in intracellular replication and PV formation. Genetic complementation of the Δ*cvpD* and Δ*cvpE* mutants rescued intracellular growth and PV generation, whereas the growth of *C. burnetii* Δ*cvpB* and Δ*cvpC* was rescued upon cohabitation with wild-type bacteria in a common PV. Collectively, these data indicate *C. burnetii* encodes multiple effector proteins that target the PV membrane and benefit pathogen replication in human macrophages.**

*Coxiella burnetii* is an intracellular pathogen and the etiological agent of human Q fever. This highly infectious Gram-negative bacterium is capable of colonizing mammalian, avian, and arthropod host organisms (1). The pathogen is shed in high numbers by infected livestock and easily disseminated via aerosols (1). *C. burnetii* exhibits a biphasic developmental cycle in which the bacterium transitions between small cell variant (SCV) and large cell variant (LCV) forms (2–4). SCVs are 0.2 to 0.5 μm in size with densely packed chromatin and low metabolic activity. Once internalized within a host cell, SCVs differentiate into replicative LCVs of ~1 μm in size with dispersed chromatin. The compact structure of SCVs correlates with resistance to osmotic stress, sonic disruption, and high pressure (3, 5). Therefore, the SCV is presumed to be the environmentally stable form of *C. burnetii* that facilitates disease transmission (3).

Successful intracellular replication of *C. burnetii* in mononuclear phagocytes, such as alveolar macrophages, is required for development of human Q fever, a disease that typically manifests as an acute flu-like illness (6). *C. burnetii* replicates within a specialized parasitophorous vacuole (PV) with characteristics of a phagolysosome (6). After internalization by a host cell, *C. burnetii* is sequestered within a nascent phagosome that traffics canonically through the endolysosomal system to ultimately acquire late endosomal and lysosomal markers such as Rab7, lysosome-associated membrane protein 1 (LAMP1), and cathepsin D (7). PV acquisition of acid hydrolases correlates with pronounced degradative activity that *C. burnetii*, by unknown mechanisms, is able to resist (7). In response to vacuole acidification, *C. burnetii* becomes metabolically active, resulting in the synthesis of bacterial proteins required for PV maturation (8, 9). PV biogenesis involves fusion of the vacuole with vesicles originating from endocytic, autophagic, and secretory pathways through processes regulated by multiple host factors, including Rab GTPases and soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (10–15).

Translocation by *C. burnetii* of proteins directly into the host cell cytosol by a specialized type 4B secretion system (T4BSS) is required for PV formation (16–18). The *C. burnetii* T4BSS is homologous to the virulence-associated T4BSS of *Legionella pneumophila*, encoded by defect in organelle trafficking (*dot*) and intracellular replication (*icm*) genes (19). *C. burnetii* strains harboring *Himar1* transposon (Tn) insertions in *icmL* or *icmD* (16, 17), or deletions in *dotA* or *dotB* (20), fail to secrete effector proteins and have severe defects in intracellular growth. Interestingly, a *C. burnetii icmD*::Tn mutant can replicate intracellularly if se-

**Received** 8 October 2014 **Returned for modification** 3 November 2014
**Accepted** 17 November 2014

**Accepted manuscript posted online** 24 November 2014

**Citation** Larson CL, Beare PA, Voth DE, Howe D, Cockrell DC, Bastidas RJ, Valdivia RH, Heinzen RA. 2015. *Coxiella burnetii* effector proteins that localize to the parasitophorous vacuole membrane promote intracellular replication. Infect Immun 83:661–670. doi:10.1128/IAI.02763-14.

**Editor:** C. R. Roy

Address correspondence to Robert A. Heinzen, rheinzen@niaid.nih.gov.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/IAI.02763-14.



doi:10.1128/IAI.02763-14

questered in a vacuole with wild-type *C. burnetii* that provide Dot/Icm functions in *trans* (16). Collectively, these data confirm that Dot/Icm T4BSS function is essential for the productive infection by *C. burnetii*.

Based on similar function of *L. pneumophila* and *C. burnetii* Dot/Icm T4BSSs, investigators have extensively used *L. pneumophila* to screen candidate *C. burnetii* effectors for Dot/Icm-dependent secretion using either adenylate cyclase (CyaA) or β-lactamase (BlaM) translocation assays (17, 18, 21–25). *C. burnetii* proteins are typically selected as candidate effectors using bioinformatic criteria that include the presence of eukaryote-like motifs (21, 23, 24), a C terminus enriched in acidic residues (22, 25–27), and/or a PmrA regulatory element upstream of the encoding gene promoter (21, 25, 28). Screening of candidate effectors has resulted in the identification of approximately 130 *C. burnetii* proteins that are secreted in a Dot/Icm-dependent fashion (17, 18, 21–25, 29). Recently, several groups have used new genetic tools to express proteins in *C. burnetii* and validate that substrates recognized by the *L. pneumophila* Dot/Icm system are also exported during *C. burnetii* infection of host cells, including six Dot/Icm substrates encoded by the QpH1 cryptic plasmid (18) and 21 substrates encoded by chromosomal genes (14, 17, 21, 25).

*C. burnetii* Dot/Icm effectors are predicted to remodel host endomembrane compartments for generation of the replication-permissive PV, but information is lacking on specific effector functions that promote this process. However, inactivation of *C. burnetii* genes is now possible, allowing direct assessment of effector requirements during *C. burnetii* infection of mammalian cells. From a *Himar1* Tn mutant library, Weber et al. (25) identified 20 *C. burnetii* mutants where a Tn insertion disrupts a gene encoding a known T4BSS substrate. Five of these mutants exhibit impaired growth in J774A.1 mouse macrophages and, consequently, the affected genes are named *cir* genes for *C*oxiella effectors for *i*ntracellular *r*eplication. Martinez et al. (30) recently identified 12 *C. burnetii Himar1* Tn mutants with significant growth defects in Vero epithelial cells where the Tn insertion disrupts a gene encoding a known Dot/Icm substrate. The functions of these Tn-disrupted effector-encoding genes are currently unknown (25, 30).

Recently, we functionally characterized a new T4BSS effector protein, termed CvpA for *C*oxiella *v*acuolar *p*rotein *A*, that was identified based on the presence of a eukaryote-like leucine-rich repeat and multiple endocytic sorting motifs (14). CvpA localizes to the PV membrane when ectopically expressed as a fusion to mCherry red fluorescent protein. A *C. burnetii* Δ*cvpA* mutant displays severe defects in intracellular replication and PV biogenesis that are rescuable by genetic complementation. In uninfected cells, mCherry-CvpA labels the membrane of endocytic recycling vesicles, an interaction mediated by the multiple endocytic sorting motifs within CvpA that bind the heterotetrameric clathrin adaptor protein complex 2 (AP2) (14, 31). Depletion of cellular AP2 or clathrin with small interfering RNA significantly inhibits *C. burnetii* replication. In addition, a mutated form of CvpA lacking endocytic motifs does not rescue growth of the Δ*cvpA* mutant. Collectively, these data suggest CvpA modulates clathrin-mediated vesicle transport events on the PV membrane that promote vacuole biogenesis (14). We predict additional *C. burnetii* effectors on the PV membrane regulate vesicle fusion events required for vacuole maturation and pathogen growth.

In the present study, bioinformatic criteria were used to compile a list of candidate *C. burnetii* T4BSS substrates that were then screened for Dot/Icm-dependent translocation during *C. burnetii* infection of mammalian cells. To gain insight into potential effector functions, confirmed T4BSS substrates were ectopically expressed in eukaryotic cells to identify those that traffic to the PV membrane. Four effectors localized to the PV in *Coxiella*-infected cells, and strains harboring targeted deletions in the encoding genes of each effector displayed significant defects in intracellular replication and PV formation. These results indicate multiple *C. burnetii* Dot/Icm effectors localize to the PV membrane that promote *C. burnetii* replication. Importantly, these findings are based on assessments of Dot/Icm substrate recognition and effector function performed directly within *C. burnetii*.

## MATERIALS AND METHODS

**Bacterial and mammalian cell culture.** *C. burnetii* Nine Mile RSA439 (phase II, clone 4) was cultivated axenically in ACCM-2 as previously described (32). HeLa (CCL-2; American Type Culture Collection [ATCC]) human cervical epithelial cells were cultured in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Invitrogen) at 37°C and 5% $CO_2$. THP-1 (TIB-202; ATCC) human monocytic leukemia cells and Vero (CCL-81; ATCC) African green monkey kidney cells were maintained in RPMI 1640 medium (Invitrogen) containing 10% FBS at 37°C and 5% $CO_2$. THP-1 monocytes were differentiated into macrophage-like cells by overnight treatment with 200 nM phorbol-12-myristate-13-acetate (PMA) and then washed twice with phosphate-buffered saline (PBS; 1.05 mM $KH_2PO_4$, 155 mM NaCl, 2.96 mM $Na_2HPO_4$ [pH 7.2]) prior to infection.

**Construction of plasmids for *C. burnetii* transformation.** The bacterial strains and plasmids utilized in the present study are listed in Table S1 in the supplemental material, and the primers used in plasmid construction are listed in Table S2 in the supplemental material. *C. burnetii* genes were amplified with Accuprime *Pfx* DNA polymerase (Invitrogen) from genomic DNA extracted from the Nine Mile (RSA439), Dugway (5J108-111), and K (Q154) strains of *C. burnetii* using gene-specific primers (Integrated DNA Technologies, Coralville, IA). For the construction of plasmids used in targeted deletion of *C. burnetii* genes, the 5′ and 3′ flanking sequences of *cbu0021*, *cbu1556*, *cbu1818*, and *cbu1863* were PCR amplified using the respective primer pairs listed in Table S2 in the supplemental material. Using the In-Fusion PCR cloning system (BD Clontech, Mountain View, CA), the 5′ and 3′ amplicons were inserted into pJC-CAT linearized by BamHI/SalI digestion to generate pJC-CAT::CBU0021-5′3′, pJC-CAT::CBU1556-5′3′, pJC-CAT::CBU1818-5′3′, and pJC-CAT::CBU1863-5′3′. The kanamycin cassette was amplified from pJB-Kan (32) using either P1169-Kan-NdeI-KO-F and P1169-Kan-NdeI-KO-R or P1169-Kan-AgeI-KO-F and P1169-Kan-AgeI-KO-R and then inserted into the NdeI or AgeI site located between the 5′ and 3′ sequences within the pJC-CAT constructs to produce pJC-CAT::CBU0021-5′3′-Kan, pJC-CAT::CBU1556-5′3′-Kan, pJC-CAT::CBU1818-5′3′-Kan, and pJC-CAT::CBU1863-5′3′-Kan, which were then used in targeted gene deletion (20). For construction of Tn*7* complementation plasmids, *cbu1818* or *cbu1863*, and their upstream promoter regions were PCR amplified from genomic DNA by using the primer pairs CBU1818comp-F and CBU1818comp-R or CBU1863comp-F and CBU1863comp-R. PCR products were inserted into the EcoRI site of pMini-Tn*7*T-CAT (20) with In-Fusion to create pMini-Tn*7*T-CAT::CBU1818comp and pMini-Tn*7*T-CAT::CBU1863comp.

Genes conferring resistance to chloramphenicol, kanamycin, or ampicillin are approved for *C. burnetii* genetic transformation studies by the Rocky Mountain Laboratories Institutional Biosafety Committee and the Centers for Disease Control and Prevention, Division of Select Agents and Toxins Program.

***C. burnetii* transformation.** Transformation and selection of *C. burnetii* Δ*cvp* mutants was conducted as described previously (20). Briefly,

pJC-CAT::CBU0021-5′3′-Kan, pJC-CAT::CBU1556-5′3′-Kan, pJC-CAT::CBU1818-5′3′-Kan, and pJC-CAT::CBU1863-5′3′-Kan chromosomal integrants were selected by culturing the bacteria in ACCM-2 containing 350 μg/ml kanamycin and 3 μg/ml chloramphenicol and then subculturing the bacteria for 4 days in ACCM-2 supplemented with 1% sucrose and kanamycin to select for cointegrants that had excised the *sacB*-containing plasmid backbone. *C. burnetii* Δ*cvpB*, Δ*cvpC*, Δ*cvpD*, and Δ*cvpE* clones were isolated by limiting dilution in ACCM-2 and gene deletions confirmed by PCR (20). Transformation and selection of *C. burnetii* Δ*cvp* complement strains was accomplished as previously described (20).

**Construction of plasmids for expression of *C. burnetii* proteins in eukaryotic cells.** *C. burnetii* effector-coding genes were amplified by PCR using the oligonucleotide primers listed in Table S2 in the supplemental material and cloned into pENTR/D-TOPO (Invitrogen). The plasmids were transformed into *E. coli* Top10 (Invitrogen), purified, and then cloned genes transferred to Gateway compatible pT-Rex-DEST30/N-mCherry or pT-Rex-DEST30/N-GFP that allow anhydrotetracycline (aTet)-inducible expression of N-terminal mCherry or green fluorescent protein (GFP) fusion proteins. Plasmids were purified from *E. coli* TOP10 using the GenElute HP Plasmid Midiprep kit (Sigma, St. Louis, MO) and sequence confirmed.

**Adenylate cyclase translocation assays.** Adenylate cyclase assays were conducted as previously described (14). Briefly, PMA-differentiated THP-1 macrophages were plated in 24-well plates ($5 \times 10^5$ cells/well) and infected at a multiplicity of infection (MOI) of 50 with *C. burnetii* strains expressing each of the candidate proteins N-terminally fused to CyaA. After a 48 h of incubation, the cells were washed with PBS and then lysed with 200 μl of lysis buffer containing 50 mM HCl and 0.1% Triton X-100. Samples were boiled for 5 min, and 400 μl of 95% ethanol was added. The samples were dried under vacuum and resuspended in 400 μl of assay buffer (0.5 M sodium acetate [pH 6.0], 0.002% [wt/vol] bovine serum albumin). The amount of cyclic AMP (cAMP) in the samples was determined with the cAMP Biotrak enzyme immunoassay system (GE Healthcare, Piscataway, NJ) according to the nonacetylation procedure. Samples were measured in duplicate for each independent experiment ($n = 3$). Values are reported as the fold change in cAMP concentration versus the empty vector control (CyaA only). Proteins were deemed T4BSS substrates if the fold change in cAMP concentration was significantly greater ($P > 0.05$) than the CyaA only control, as judged by one-way analysis of variance (ANOVA).

**Ectopic expression and immunofluorescence microscopy.** HeLa cells infected with *C. burnetii* for 24 h on 12-mm coverslips in a 24-well plate were transfected with 500 ng of the pT-REx-DEST30/N-GFP or pT-Rex-DEST30/N-mCherry constructs described above and 250 ng of pcDNA 6/TR (Invitrogen) using FuGENE HD (Promega, Madison, WI) as previously described (14). The cells were incubated 24 h; fresh growth medium containing 1 μg/ml aTc (Sigma) was then added to induce protein expression. The cells were fixed 24 h later with 4% paraformaldehyde and permeabilized with PBS containing 0.05% saponin and 5% FBS. For immunostaining, rabbit anti-LAMP1 polyclonal antibody (Abcam, Cambridge, England) and guinea pig anti-*Coxiella* serum (14) were used as primary antibodies, followed by goat anti-rabbit Alexa Fluor 594 (Invitrogen) and goat anti-guinea pig Alexa Fluor 647 (Invitrogen). Coverslips were mounted using Prolong Gold with DAPI (4′,6′-diamidino-2-phenylindole; Invitrogen) and imaged. For subcellular localization of ectopically expressed pT-Rex-DEST30/N-mCherry-CBU1556, cells were stained with a mouse antibody against transferrin receptor (Life Technologies) and goat anti-mouse Alexa Fluor 488 (Invitrogen). Imaging was conducted with a LSM710 confocal laser scanning microscope (Carl Zeiss Micro Imaging, Thornwood, NY) or a Nikon Eclipse Ti-E inverted microscope.

**Quantification of *C. burnetii* growth and PV morphology.** THP-1 cells seeded at $5 \times 10^5$ per well in a 24-well plate were infected at an MOI of 0.5 with *C. burnetii* suspended in RPMI plus 10% FBS. Plates were centrifuged at 500 × *g* for 20 min. Infected cells were washed once with PBS and then cultured in RPMI plus 10% FBS for the remainder of the experiment. For each of three independent experiments, samples were collected in duplicate on the day of infection (day 0) and 6 days postinfection. *C. burnetii* growth was assessed by quantifying genomic equivalents (GE) as previously described (14).

To measure PV size, Vero cells ($2 \times 10^4$) on 12-mm coverslips in a 24-well plate were infected at an MOI of 5 with *C. burnetii* suspended in RPMI plus 2% FBS. Plates were centrifuged at 500 × *g* for 20 min. Infected Vero cells were incubated 6 days and then fixed and stained with antibodies against LAMP1 and *Coxiella* as described above. Fluorescence micrographs were acquired, and 25 PV were measured for each *C. burnetii* strain in three independent experiments. For mutant complementation by coinfection, Vero cells ($2 \times 10^4$) on 12-mm coverslips in a 24-well plate were infected at MOIs of 5 and 2, respectively, with the Δ*cvp* mutant and wild-type *C. burnetii* expressing mCherry red fluorescent protein (16). The Δ*cvp* mutants were visualized in PVs cohabited with wild-type *C. burnetii* at 6 days postinfection by immunostaining with antibodies against *C. burnetii* and LAMP1. Assessment of mutant PV fusion was conducted with Vero cells ($2 \times 10^4$) infected at an MOI of 100 with *C. burnetii* in RPMI plus 2% FBS. Cells were immunostained for LAMP1 and *C. burnetii* at 6 days postinfection, and the number of PVs in each cell ($n = 100$) enumerated using fluorescence microscopy.

**PV acidification.** PV acidification was examined using LysoTracker Green DND-26 (Invitrogen) according to the manufacturer's protocol. Vero cells ($2 \times 10^4$) were infected at an MOI of 100 with *C. burnetii* in RPMI plus 2% FBS. Cells were incubated 6 days, treated with LysoTracker, and then immunostained for LAMP1 and *C. burnetii*.

**Data analysis.** GraphPad Prism 6.0 software (San Diego, CA) was used to perform one-way ANOVA or two-way ANOVA statistical tests. All image processing and measurements were conducted using ImageJ software (W. S. Rasband, National Institutes of Health, Bethesda, MD).

## RESULTS

**Identification of *C. burnetii* T4BSS effector proteins.** Bioinformatic analysis of the *C. burnetii* Nine Mile (RSA493), Dugway (5J108-111), and K (Q154) genomes (33) revealed 14 genes encoding predicted proteins with characteristics of T4BSS substrates (Table 1). The identified candidate proteins contained eukaryote-like features, including coiled-coil and leucine-rich repeat domains associated with ligand recognition, and transmembrane domains predicted to mediate membrane attachment. CBU0885 contains a haloacid dehalogenase (HAD)-like domain associated with phosphatase activity (34), while CBU1457 harbors multiple Sel1 repeats known to promote protein-protein interactions (35). In addition, several genes encoding candidate proteins contained PmrA regulatory elements (28). Genes encoding each of the candidate effectors were inserted into pJB-CAT-CyaA, allowing *C. burnetii* expression of proteins N-terminally fused to the adenylate cyclase reporter protein CyaA (14, 18). PMA-differentiated THP-1 human macrophages were infected with wild-type *C. burnetii* or Δ*dotA* mutant strains expressing each of the CyaA constructs. THP-1 macrophages were also infected with wild-type *C. burnetii* transformed with the empty CyaA vector as a negative control. At 48 h postinfection, THP-1 cells were lysed, and the concentration of cytosolic cAMP measured (Fig. 1). Expression of 13 CyaA fusion proteins by *C. burnetii* resulted in a significant fold increase in cAMP concentration relative to *C. burnetii* expressing CyaA alone. No increase in cAMP concentration was detected after infection with *C. burnetii* Δ*dotA* expressing CyaA fusion proteins, indicating that secretion is Dot/Icm dependent. Collectively, these data indicated that CBU0021, CBU0534, CBU0885, CBUD0886, CBUK0790, CBU1493, CBUD0487, CBU1543,

**TABLE 1** Features of candidate Dot/Icm effectors

| Candidate effector | Size (kDa) | Features of gene or protein | Source or reference(s) |
|---|---|---|---|
| CBU0021 | 93.1 | Coiled-coil domain | 22, 30, 63 |
| CBUD0487 | 90.6 | Coiled-coil domain | This study |
| CBU0534 | 46.1 | Coiled-coil domain:, transmembrane domain | This study |
| CBUK0790 | 84.7 | PmrA regulatory element | This study |
| CBU0885 | 43.3 | Haloacid dehalogenase (HAD)-like domain | 22, 25 |
| CBUD0886 | 50.8 | Leucine-rich repeats | This study |
| CBU1457 | 78.3 | Sel1-like repeats | 21, 25 |
| CBU1493 | 60.9 | PmrA regulatory element | This study |
| CBU1543 | 22.3 | Coiled-coil domain, transmembrane domain | 21, 25 |
| CBU1556 | 64.6 | Coiled-coil domain, transmembrane domains | 21, 25 |
| CBU1676 | 41.8 | Paralog of CBU0885 | 62 |
| CBU1818 | 53.9 | Coiled-coil domain, transmembrane domains | 62 |
| CBU1819 | 42.3 | Coiled-coil domain | This study |
| CBU1863 | 33.2 | PmrA regulatory element, transmembrane domains | This study |

CBU1556, CBU1676, CBU1818, CBU1819, and CBU1863 are Dot/Icm substrates delivered to the host cell cytosol by the *C. burnetii* T4BSS. Negative translocation of CyaA-CBU1457 was not due to lack of expression since all CyaA fusion proteins were expressed as assessed by immunoblotting (data not shown).

***C. burnetii* effectors traffic to the PV.** A subset of effector proteins within the repertoire of *C. burnetii* Dot/Icm substrates is predicted to localize to the PV membrane where they modify vesicle budding, transport, and fusion events to promote vacuole expansion. Indeed, CvpA was previously shown to localize to the PV membrane and contribute to PV biogenesis by a mechanism involving subversion of clathrin-mediated vesicular trafficking (14). To identify additional PV-localized effector proteins, the 13 validated *C. burnetii* Dot/Icm substrates were ectopically expressed as N-terminal fusions to GFP or mCherry fluorescent protein in HeLa cells infected with *C. burnetii*. At 3 days postinfection, cells were fixed and stained with antibodies against LAMP1 and *C. burnetii*. The ectopically expressed Dot/Icm substrates displayed subcellular localizations ranging from diffuse cytoplasmic to punctate perinuclear (Fig. 2A; see also Fig. S1 in the supplemental material). Of the 13 proteins examined, CBU0021, CBU1556, CBU1818, and CBU1863 localized to the LAMP1-positive PV membrane (Fig. 2A; see also Fig. S1 in the supplemental material), suggesting they confer effector functions associated with PV biogenesis. Consequently, these proteins were termed CvpB, CvpC, CvpD, and CvpE, respectively. To examine whether Cvp effectors also interact with the PV early after infection, HeLa cells ectopically expressing the Cvp proteins were examined at 6 h postinfection. More than 90% of *C. burnetii* PV labeled with ectopically expressed CvpB, CvpC, CvpD, or CvpE, suggesting the effectors target vesicular components during the initial stages of PV maturation (see Fig. S2 in the supplemental material).

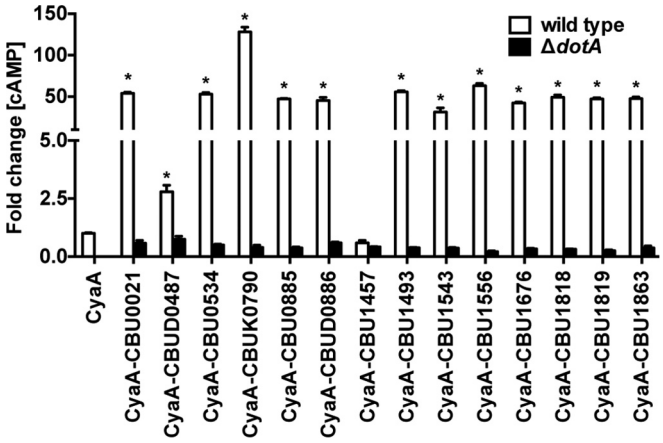


**FIG 1** *C. burnetii* translocates 13 proteins via its Dot/Icm T4BSS. Cytosolic concentrations of cAMP were determined after infection of THP-1 macrophages for 48 h with wild-type *C. burnetii* or a Δ*dotA* mutant expressing CyaA fusions to candidate Dot/Icm substrates. The results are expressed as the fold change relative to wild-type *C. burnetii* expressing CyaA alone. Increased cytosolic cAMP concentrations indicative of protein translocation were detected for CBU0021, CBUD0487, CBU0534, CBUK0790, CBU0886, CBUD0886, CBU1493, CBU1543, CBU1556, CBU1676, CBU1818, CBU1819, and CBU1863. The results are from one experiment conducted in duplicate and are representative of three independent experiments. Error bars indicate the standard deviations from the means. Asterisks indicate a significantly greater difference ($P < 0.05$) compared to values for the CyaA only control as determined by one-way ANOVA.

Although Cvp effectors traffic to the LAMP1-positive PV membrane, it remained possible that they also interact with membrane components outside the late endosomal compartment, as is the case with CvpA (14). To further examine their subcellular itineraries, each Cvp protein was ectopically expressed N-terminally fused to GFP or mCherry red fluorescent protein in uninfected HeLa cells. Transfected cells were then stained with antibodies against protein markers of early, recycling, or late endosomes, the endoplasmic reticulum (ER), the ER-Golgi intermediate compartment (ERGIC), or the *cis*-Golgi or *trans*-Golgi. As expected, all of the Cvp effectors localized with late endosomal marker LAMP1 in uninfected cells. In addition, CvpC exhibited partial localization with transferrin receptor, a marker of recycling endosomes (Fig. 2B). We have previously shown that ectopically expressed CvpA localizes to recycling endosomes and inhibits the uptake of transferrin (14). However, ectopic expression of CvpC did not alter transferrin uptake (data not shown). Moreover, none of the Cvp proteins localized with structures that labeled with antibodies against EEA1 (early endosomes), calreticulin (ER), ERGIC53 (ERGIC), giantin (*cis*-Golgi), or p230/golgin-245 (*trans*-Golgi) (data not shown). Collectively, these results suggest CvpB, CvpC, CvpD, and CvpE are *C. burnetii* effectors that target components of the late endosomal system, and that CvpC also targets recycling endosomes.

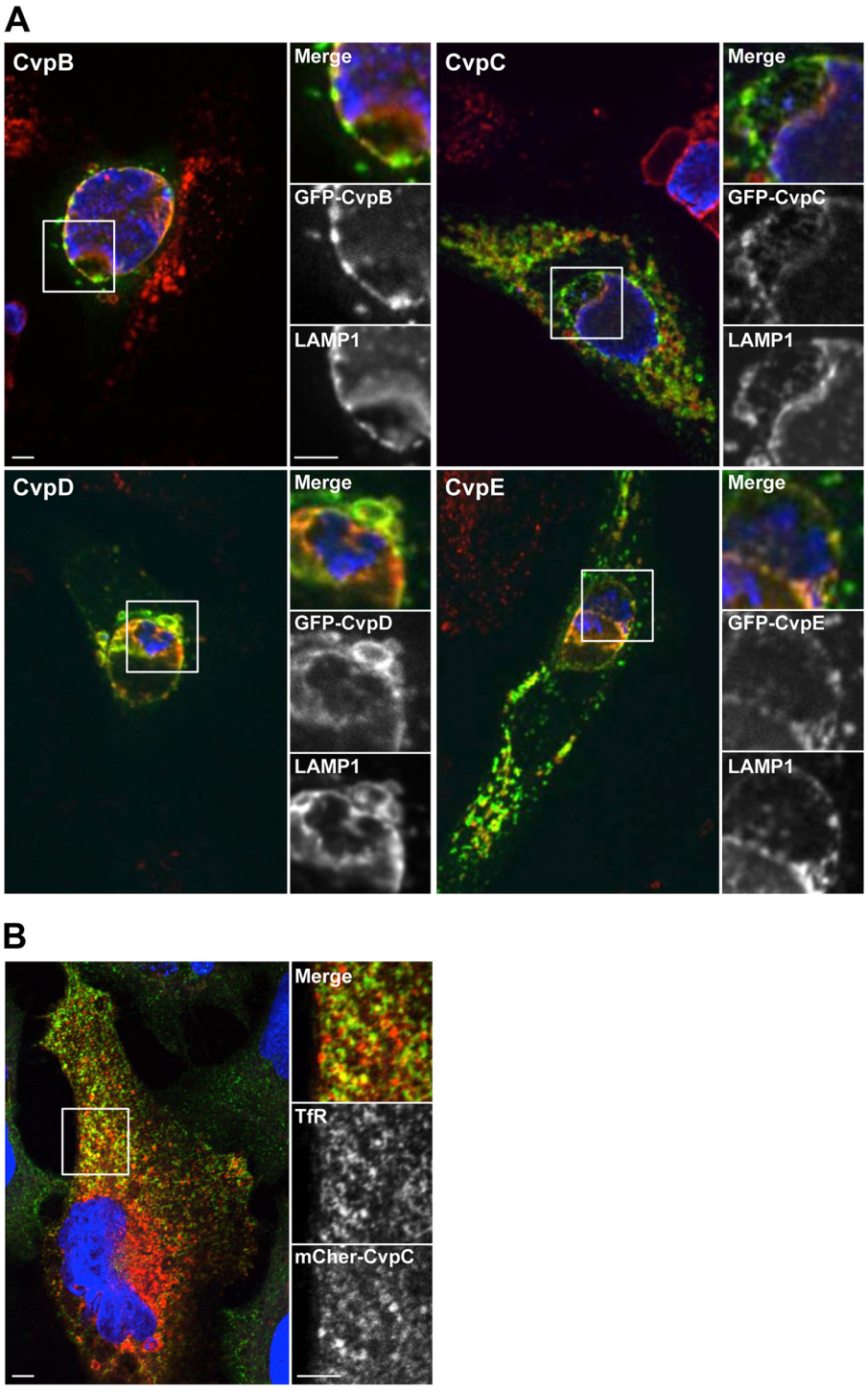


**FIG 2** Four *C. burnetii* Dot/Icm T4BSS substrates localize to the PV membrane. (A) Representative confocal fluorescence micrographs of *C. burnetii*-infected HeLa cells ectopically expressing CvpB, CvpC, CvpD, or CvpE N-terminally fused to GFP. At 72 h postinfection, cells were fixed and stained with antibodies against the lysosomal membrane protein LAMP1 (red) and *C. burnetii* (blue). (B) CvpC-mCherry colocalizes with transferrin receptor. HeLa cells were transfected with a plasmid encoding CvpC N-terminally fused to mCherry red fluorescent protein. Cells were immunostained for transferrin receptor (green) with the DNA stained by DAPI (blue). Scale bar, 5 μm.

**Cvp effectors promote intracellular replication and PV expansion.** Localization to the PV membrane suggested CvpB, CvpC, CvpD, and CvpE promote PV biogenesis and *C. burnetii* intracellular replication. To assess this possibility, *C. burnetii cvp* deletion mutants were generated and growth was assessed in THP-1 macrophages by measuring the increase in GE over 6 days. Replication of the *C. burnetii* Δ*cvpB*, Δ*cvpC*, Δ*cvpD*, and Δ*cvpE* mutants was significantly impaired with 49-, 276-, 160-, and 110-

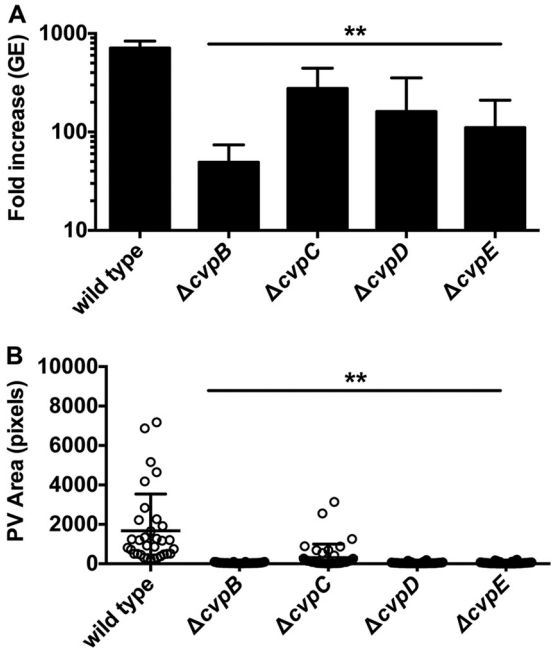


**FIG 3** *C. burnetii* Δ*cvp* mutants exhibit defects in intracellular growth and PV biogenesis. (A) Replication of wild-type *C. burnetii* and Δ*cvp* mutants. Fold increases in bacterial GE at 6 days postinfection of THP-1 macrophages relative to the 0 day time point are depicted from three independent experiments, each performed in duplicate. (B) Sizes of PVs generated by wild-type *C. burnetii* and the Δ*cvp* mutants 6 days postinfection in Vero cells. PV size was measured using ImageJ ($n = 25$), and the data are representative of three independent experiments. Error bars indicate the standard deviations from the means. Asterisks indicate a statistically significant difference ($P < 0.01$) compared to values for wild-type bacteria as determined by one-way ANOVA.

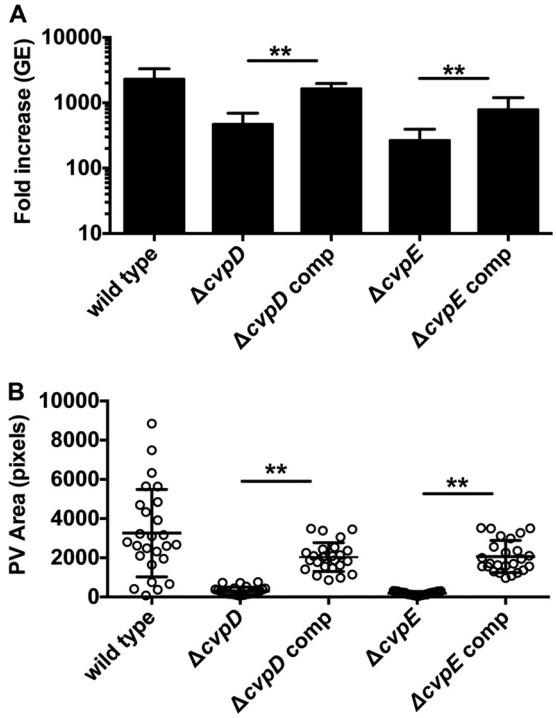


**FIG 4** Genetic complementation of the Δ*cvpD* and Δ*cvpE* mutants rescues intracellular growth and PV biogenesis. (A) Replication of wild-type *C. burnetii* and complemented Δ*cvp* mutants. Fold increases in bacterial GE at 6 days postinfection of THP-1 macrophages relative to the 0 day time point are depicted from three independent experiments, each performed in duplicate. (B) Sizes of PVs generated by the wild-type *C. burnetii* and complemented Δ*cvp* mutants at 6 days postinfection of Vero cells. PV size was measured using ImageJ ($n = 25$), and the data are representative of three independent experiments. Error bars indicate the standard deviations from the means. Asterisks indicate a statistically significant difference ($P < 0.01$) compared to values for wild-type bacteria as determined by one-way ANOVA.

fold increases in GE, respectively, relative to the 713-fold increase in GE observed for wild-type *C. burnetii* (Fig. 3A). Defects in *C. burnetii* replication correlated with impaired PV biogenesis. All mutants occupied aberrantly small, tight-fitting vacuoles in Vero cells compared to the large and spacious PVs generated by wild-type *C. burnetii* (Fig. 3B). PVs harboring *C. burnetii* Δ*cvpB*, Δ*cvpD*, and Δ*cvpE* strains in Vero cells also exhibited impaired homotypic fusion with 57.5, 35.2, and 32.6%, respectively, containing two or more PVs compared to 16.2% of cells infected with wild-type *C. burnetii* (see Fig. S3A in the supplemental material). Consistent with the presence of LAMP1, PVs containing Δ*cvp* mutants were acidified, as evidenced by the accumulation of the acidotropic fluorescent dye LysoTracker DND-26 (see Fig. S3B in the supplemental material). These acidified vacuoles supported metabolic activation of Δ*cvp* mutants as each was capable of secreting a CyaA-CvpA fusion protein (14) (see Fig. S4 in the supplemental material). Thus, Δ*cvp* mutant growth defects likely result from inactivation of the targeted effector.

To confirm that the specific gene deletion was responsible for the intracellular growth defect of a Δ*cvp* mutant, complementation was attempted using Tn*7* to introduce a single gene copy into the chromosome under the control of a native promoter. Tn*7* complementation rescued intracellular growth of the Δ*cvpD* and Δ*cvpE* mutants, as evidenced by significant increases in replication within THP-1 cells (Fig. 4A) and PV size in Vero cells (Fig. 4B). This strategy of genetic complementation failed to rescue impaired growth of *C. burnetii* Δ*cvpB* and Δ*cvpC*. Transformation with a multicopy plasmid encoding constitutively expressed CvpB and CvpC also failed to complement mutant growth defects (data not shown). Therefore, we used a coinfection strategy that has been previously used to show that the growth deficiencies of *C. burnetii* Dot/Icm mutants can be complemented by functions provided in *trans* by wild-type *C. burnetii* (16, 25). Vero cells were coinfected with wild-type *C. burnetii* and the Δ*cvpB* or Δ*cvpC* strain. When sequestered within a PV co-occupied by wild-type *C. burnetii*, the Δ*cvpB* and Δ*cvpC* mutants exhibited robust replication (Fig. 5).

## DISCUSSION

The pronounced fusogenicity of the *C. burnetii* PV is predicted to be mediated by *C. burnetii* Dot/Icm effector proteins that modulate vesicle budding, transport, and fusion events on the PV membrane (6, 9, 14, 36). In support of this model is CvpA, a PV-interacting Dot/Icm effector protein that promotes PV formation (14). CvpA targets clathrin-dependent vesicular trafficking, presumably allowing *C. burnetii* to acquire membrane components for PV biogenesis (14). In the present study, we used genetic manipulation of *C. burnetii* to directly identify Dot/Icm T4BSS substrates secreted by the pathogen and to validate their importance in intracellular replication. Based on interactions with the PV membrane, four new Cvp proteins were identified among the 13

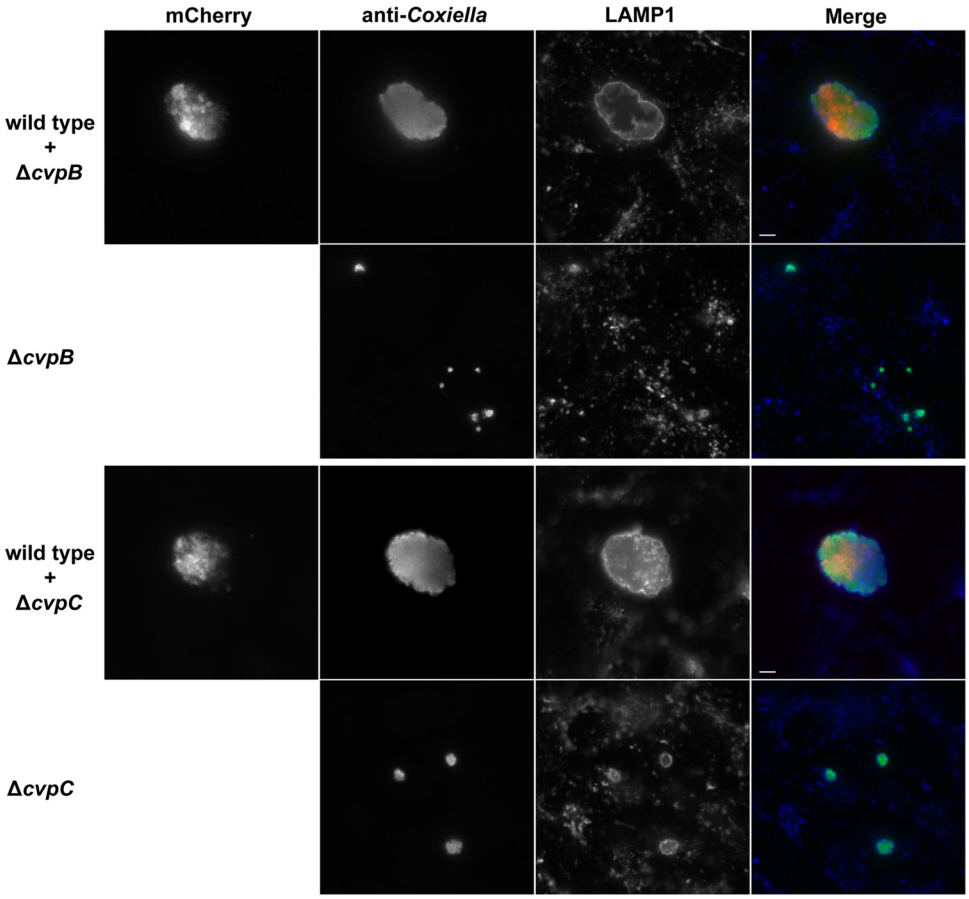


**FIG 5** Coinfection with wild-type *C. burnetii* rescues growth of Δ*cvpB* and Δ*cvpC* mutants. THP-1 macrophages were infected with the Δ*cvpB* or Δ*cvpC* mutant alone or coinfected with the Δ*cvpB* or Δ*cvpC* mutant and wild-type *C. burnetii* expressing mCherry red fluorescent protein. At 6 days postinfection, *C. burnetii* (wild type and mutant) and LAMP1 were immunostained green and blue, respectively. Robust growth of Δ*cvpB* and Δ*cvpC* mutants was only observed in PVs cohabited with wild-type *C. burnetii*. Scale bar, 5 μm.

identified Dot/Icm substrates. Defects in intracellular replication and PV expansion observed for *C. burnetii* Δ*cvp* mutants support the hypothesis that *C. burnetii* encodes a family of Dot/Icm effectors that localize to the PV membrane and modulate membrane trafficking events necessary for PV development and pathogen replication.

The Δ*cvp* mutants occupy a LAMP1-positive, acidified PV and can secrete proteins via the T4BSS. Thus, their growth deficiencies are not related to overall failings in PV maturation and Dot/Icm function. The Δ*cvpB*, Δ*cvpD*, and Δ*cvpE* mutants, but not the Δ*cvpC* mutant, generated multi-PV in Vero cells that displayed impaired homotypic fusion. These phenotypes correlated with a severe replication defect. Rescue of mutant growth defects by Tn*7* *cis*-complementation verified mutation of CvpD and CvpE impairs *C. burnetii* intracellular growth, but this strategy failed to complement growth of the Δ*cvpB* and Δ*cvpC* mutants. The reasons for lack of genetic complementation remain unclear, but may include temporal problems with gene expression, insufficient chaperone engagement, and/or altered protein levels. To bypass these issues, we and others have used coinfection with wild-type *C. burnetii* to rescue impaired growth of strains with mutations in the T4BSS apparatus (16, 30) and Dot/Icm effectors (25). Although coinfection rescued the growth defects of Δ*cvpB* and Δ*cvpC* mutants, we cannot rule out the possibility that the phenotypes are due to a secondary mutation that can also be complemented in *trans*.

The observation that *cvpB*, *cvpC*, *cvpD*, and *cvpE* are maintained as full-length genes among sequenced *C. burnetii* strains suggests that they modulate core host cell functions required for successful intracellular parasitism by the genus *Coxiella*. Polymorphisms among several previously identified *C. burnetii* Dot/Icm substrates (17, 24) are hypothesized to contribute to pathogenic potential (36). Of the substrates identified in the present study, *cbud0487* is full length only in the Dugway (5J108-111) strain, *cbud0886* is full length only in the Dugway (5J108-111) and K (Q154) strains, and *cubk0790* is only full length only in the K (Q154) strain. The Nine Mile strain used here is representative of acute disease isolates, the K strain is a human chronic endocarditis isolate, and the Dugway strain is an attenuated rodent isolate (33).

Localization of bacterial effector proteins to pathogen-occupied vacuoles is commonly observed. For example, *L. pneumophila* translocates multiple effector proteins via its Dot/Icm T4BSS that interact with the membrane of the *Legionella* vacuole (LV). Dot/Icm effectors LidA, SidC, and DrrA/SidM bind specific phosphoinositides (37, 38), while LepA, LepB, LegC3, LegC2/YlfB, and LegC7/YlfA contain transmembrane domains that facilitate inter-

action with the LV membrane (39, 40). Similarly, *Chlamydia* spp. deploy a type 3 secretion system (T3SS) to deliver multiple Inc family proteins that localize to the chlamydial inclusion via a conserved bilobed hydrophobic motif (41). CvpC, CvpD, and CvpE all contain predicted transmembrane domains that may facilitate their interaction with the LAMP1-positive PV membrane. In contrast, CvpB lacks obvious transmembrane domains and therefore might target the PV membrane by binding phosphoinositides. When examined in uninfected cells, CvpB, CvpD, and CvpE consistently localize only to the late endolysosomal compartment. CvpC, in addition to LAMP1-positive structures, also localizes with transferrin receptor, an endocytic carrier protein that undergoes clathrin-dependent endocytosis and traffics through the endocytic recycling system. Thus, CvpC may traffic between late and recycling endosomal compartments, as is the case with CvpA (14).

The activities of vesicular effectors produced by other pathogens may provide clues to Cvp biochemical activities. Modulation of Rab GTPases is a prominent strategy used by intracellular pathogens to remodel their phagosome into a replication-permissive compartment (42, 43). Rab GTPases play critical roles in regulating transport of lipids and proteins that ultimately determine organelle identity (44, 45). Guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) regulate the rate of cycling between inactive GDP-bound and active GTP-bound forms of Rab GTPases (45). Pathogens secrete effectors that subvert these regulatory mechanisms to control spatial and temporal aspects of membrane transport. Of the LV-localized Dot/Icm effectors produced by *L. pneumophila*, multiple proteins modulate Rab1 activity to refurbish the LV with ER components and to prevent LV fusion with lysosomes. For example, LidA binds Rab1, which stabilizes its activation (46) and promotes the acquisition of ER-derived vesicles (47). Furthermore, DrrA/SidM is a GEF that posttranslationally modifies Rab1 with AMP (48–51), thereby preventing inactivation by GAP proteins (52–54). Rab1 (12), as well as the regulators of endocytic traffic Rab5 and Rab7, have been implicated in *C. burnetii* PV biogenesis (15, 55, 56). Thus, it is plausible that *C. burnetii* Cvp effectors disregulate Rab GTPases to promote maturation of its strikingly large phagolysosome-like replication compartment.

The Cvp effectors lack homology to proteins of known function, but CvpB, CvpC, and CvpD contain predicted eukaryote-like coiled-coil domains similar to bacterial effectors known to functionally mimic the activity of eukaryotic SNARE proteins (57). SNAREs regulate fusion between transport vesicles and target compartment membranes, and pathogens subvert SNARE function to promote fusion events beneficial to pathogen growth (57). The chlamydial T3SS substrate IncA harbors two SNARE-like coiled-coil motifs (58) and inhibits membrane fusion mediated by endocytic, but not exocytic, SNARE complexes (59). Expression of the *L. pneumophila* effector LegC3 produces a vacuolar protein sorting defect in yeast (40) linked to the disruption of trans-SNARE complexes by coiled-coil domains within the effector (60). In contrast to *L. pneumophila* and *Chlamydia* spp. that block fusion of their vacuoles with lysosomes, phagosome acidification and lysosomal fusion promotes *Coxiella* replication (6). Moreover, *C. burnetii* also requires membrane components from autophagic and secretory compartments for PV expansion (10–12, 15, 61). Several SNARE proteins, including syntaxin-8, syntaxin-17, and vesicle-associated membrane protein 7 (VAMP7), localize to the PV membrane, with syntaxin-17 and VAMP7 having demonstrated roles in vacuole expansion (11, 15). *Coxiella* researchers are just beginning to investigate the molecular mechanisms that control PV fusogenicity, a process that likely involves the activities of multiple Rab GTPases, SNARE proteins, and Dot/Icm effectors (11, 14–16).

When this manuscript was originally submitted, 4 of the 13 proteins we report here as substrates of the *C. burnetii* Dot/Icm system had also been described as *L. pneumophila* Dot/Icm substrates (21, 22, 25). Using a hidden semi-Markov model, Lifshitz et al. (22) identified a group of *C. burnetii* proteins, termed C-terminal signal for effector translocation of *C. burnetii* (CetCb) proteins, that have C-terminal amino acid sequences resembling those of known Dot/Icm substrates. In agreement with our findings using *C. burnetii*, CvpB/CetCb1 (CBU0021) and CetCb4 (CBU0885) fusions with CyaA are translocated by *L. pneumophila* in a Dot/Icm-dependent manner (22). Chen et al. (21) expressed multiple *C. burnetii* proteins in *L. pneumophila* fused to the β-lactamase (BlaM) reporter. These researchers concluded that CBU1457, CBU1543, and CvpC (CBU1556) are Dot/Icm substrates based on 2, 25, and 50% translocation efficiencies of the respective BlaM fusion protein (21, 25). We found that CBU1543 and CvpC fusions with CyaA are translocated by *C. burnetii* in a Dot/Icm-dependent fashion. In contrast, we did not detect translocation of CyaA-CBU1457 by *C. burnetii* at the 48-h postinfection time point. Translocation was also not detected at 72 or 96 h postinfection, nor with *L. pneumophila* using standard assay conditions (data not shown) (18). The reason for this disparate result is unclear but emphasizes the need to validate Dot/Icm effectors directly in *C. burnetii*.

Since submission of the manuscript, a study by Lifshitz and coworkers (62) demonstrated *L. pneumophila* Dot/Icm-dependent secretion of CBU1676 and CBU1818 (CvpD). Expression of CBU1676, or its paralog CBU0885, inhibits mitogen-activated protein kinase signaling in *Saccharomyces cerevisiae*. Newton et al. (63) reported that *C. burnetii cig2* (*cvpB*) Tn insertion mutants exhibit a multi-PV growth phenotype in HeLa cells without showing an overall growth defect. We found that *C. burnetii* Δ*cvpB* replicates poorly in THP-1 human macrophages, a behavior that correlates with generation of multiple, small PVs in Vero cells. Martinez et al. (30) also found that *C. burnetii* strains harboring a Tn insertion in *cbu0021* (*cvpB*) display impaired growth in Vero cells. Thus, accumulating evidence from independent laboratories indicates CvpB is a critical Dot/Icm effector that subverts cellular functions involved in PV biogenesis. Indeed, because PVs containing the *cvpB* mutant do not accumulate the autophagosome protein LC3, and autophagy inhibition of cells infected with wild-type bacteria produce multiple PVs similar to the *C. burnetii cvpB* mutant, Newton et al. (63) conclude that CvpB promotes PV interactions with autophagosomes that enhance PV maturation. Biochemical characterization of the functional activities CvpB and other Cvp effectors will provide important insight into mechanisms governing PV development.

## ACKNOWLEDGMENTS

This study was supported by funding from NIH NIAID grants AI072485 (R.H.V.) and R01AI087669 (D.E.V.) and by the Intramural Research Program of the National Institutes of Health, National Institute of Allergy and Infectious Diseases (R.A.H.).

## REFERENCES


1. **Maurin M, Raoult D.** 1999. Q fever Clin Microbiol Rev **12:**518–553.
2. **Coleman SA, Fischer ER, Howe D, Mead DJ, Heinzen RA.** 2004. Temporal analysis of *Coxiella burnetii* morphological differentiation. J Bacteriol **186:**7344–7352. http://dx.doi.org/10.1128/JB.186.21.7344-7352.2004.
3. **Heinzen RA, Hackstadt T, Samuel JE.** 1999. Developmental biology of *Coxiella burnetii*. Trends Microbiol **7:**149–154. http://dx.doi.org/10.1016/S0966-842X(99)01475-4.
4. **McCaul TF, Williams JC.** 1981. Developmental cycle of *Coxiella burnetii*: structure and morphogenesis of vegetative and sporogenic differentiations. J Bacteriol **147:**1063–1076.
5. **McCaul TF, Hackstadt T, Williams JC.** 1981. Ultrastructural and biological aspects of *Coxiella burnetii* under physical disruptions, p 267–280. *In* Burgdorfer W, Anacker RL (ed), Rickettsiae and rickettsial diseases. Academic Press, Inc, New York, NY.
6. **Voth DE, Heinzen RA.** 2007. Lounging in a lysosome: the intracellular lifestyle of *Coxiella burnetii*. Cell Microbiol **9:**829–840. http://dx.doi.org/10.1111/j.1462-5822.2007.00901.x.
7. **Howe D, Shannon JG, Winfree S, Dorward DW, Heinzen RA.** 2010. *Coxiella burnetii* phase I and II variants replicate with similar kinetics in degradative phagolysosome-like compartments of human macrophages. Infect Immun **78:**3465–3474. http://dx.doi.org/10.1128/IAI.00406-10.
8. **Hackstadt T, Williams JC.** 1981. Biochemical stratagem for obligate parasitism of eukaryotic cells by *Coxiella burnetii*. Proc Natl Acad Sci U S A **78:**3240–3244. http://dx.doi.org/10.1073/pnas.78.5.3240.
9. **Howe D, Melnicakova J, Barak I, Heinzen RA.** 2003. Maturation of the *Coxiella burnetii* parasitophorous vacuole requires bacterial protein synthesis but not replication. Cell Microbiol **5:**469–480. http://dx.doi.org/10.1046/j.1462-5822.2003.00293.x.
10. **Beron W, Gutierrez MG, Rabinovitch M, Colombo MI.** 2002. *Coxiella burnetii* localizes in a Rab7-labeled compartment with autophagic characteristics. Infect Immun **70:**5816–5821. http://dx.doi.org/10.1128/IAI.70.10.5816-5821.2002.
11. **Campoy EM, Mansilla ME, Colombo MI.** 2013. Endocytic SNAREs are involved in optimal *Coxiella burnetii* vacuole development. Cell Microbiol **15:**922–941. http://dx.doi.org/10.1111/cmi.12087.
12. **Campoy EM, Zoppino FC, Colombo MI.** 2011. The early secretory pathway contributes to the growth of the *Coxiella*-replicative niche. Infect Immun **79:**402–413. http://dx.doi.org/10.1128/IAI.00688-10.
13. **Howe D, Melnicakova J, Barak I, Heinzen RA.** 2003. Fusogenicity of the *Coxiella burnetii* parasitophorous vacuole. Ann N Y Acad Sci **990:**556–562. http://dx.doi.org/10.1111/j.1749-6632.2003.tb07426.x.
14. **Larson CL, Beare PA, Howe D, Heinzen RA.** 2013. *Coxiella burnetii* effector protein subverts clathrin-mediated vesicular trafficking for pathogen vacuole biogenesis. Proc Natl Acad Sci U S A **110:**E4770–E4779. http://dx.doi.org/10.1073/pnas.1309195110.
15. **McDonough JA, Newton HJ, Klum S, Swiss R, Agaisse H, Roy CR.** 2013. Host pathways important for *Coxiella burnetii* infection revealed by genome-wide RNA interference screening. mBio **4:**e00606–12. http://dx.doi.org/10.1128/mBio.00606-12.
16. **Beare PA, Gilk SD, Larson CL, Hill J, Stead CM, Omsland A, Cockrell DC, Howe D, Voth DE, Heinzen RA.** 2011. Dot/Icm type IVB secretion system requirements for *Coxiella burnetii* growth in human macrophages. mBio **2:**e00175–11. http://dx.doi.org/10.1128/mBio.00175-11.
17. **Carey KL, Newton HJ, Lührmann A, Roy CR.** 2011. The *Coxiella burnetii* Dot/Icm system delivers a unique repertoire of type IV effectors into host cells and is required for intracellular replication. PLoS Pathog **7:**e1002056. http://dx.doi.org/10.1371/journal.ppat.1002056.
18. **Voth DE, Beare PA, Howe D, Sharma UM, Samoilis G, Cockrell DC, Omsland A, Heinzen RA.** 2011. The *Coxiella burnetii* cryptic plasmid is enriched in genes encoding type IV secretion system substrates. J Bacteriol **193:**1493–1503. http://dx.doi.org/10.1128/JB.01359-10.
19. **Vogel JP.** 2004. Turning a tiger into a house cat: using *Legionella pneumophila* to study *Coxiella burnetii*. Trends Microbiol **12:**103–105. http://dx.doi.org/10.1016/j.tim.2004.01.008.
20. **Beare PA, Larson CL, Gilk SD, Heinzen RA.** 2012. Two systems for targeted gene deletion in *Coxiella burnetii*. Appl Environ Microbiol **78:**4580–4589. http://dx.doi.org/10.1128/AEM.00881-12.
21. **Chen C, Banga S, Mertens K, Weber MM, Gorbaslieva I, Tan Y, Luo ZQ, Samuel JE.** 2010. Large-scale identification and translocation of type IV secretion substrates by *Coxiella burnetii*. Proc Natl Acad Sci U S A **107:**21755–21760. http://dx.doi.org/10.1073/pnas.1010485107.
22. **Lifshitz Z, Burstein D, Peeri M, Zusman T, Schwartz K, Shuman HA, Pupko T, Segal G.** 2013. Computational modeling and experimental validation of the *Legionella* and *Coxiella* virulence-related type IVB secretion signal. Proc Natl Acad Sci U S A **110:**E707–E715. http://dx.doi.org/10.1073/pnas.1215278110.
23. **Pan X, Luhrmann A, Satoh A, Laskowski-Arce MA, Roy CR.** 2008. Ankyrin repeat proteins comprise a diverse family of bacterial type IV effectors. Science **320:**1651–1654. http://dx.doi.org/10.1126/science.1158160.
24. **Voth DE, Howe D, Beare PA, Vogel JP, Unsworth N, Samuel JE, Heinzen RA.** 2009. The *Coxiella burnetii* ankyrin repeat domain-containing protein family is heterogeneous, with C-terminal truncations that influence Dot/Icm-mediated secretion. J Bacteriol **191:**4232–4242. http://dx.doi.org/10.1128/JB.01656-08.
25. **Weber MM, Chen C, Rowin K, Mertens K, Galvan G, Zhi H, Dealing CM, Roman VA, Banga S, Tan Y, Luo ZQ, Samuel JE.** 2013. Identification of *Coxiella burnetii* type IV secretion substrates required for intracellular replication and *Coxiella*-containing vacuole formation. J Bacteriol **195:**3914–3924. http://dx.doi.org/10.1128/JB.00071-13.
26. **Burstein D, Zusman T, Degtyar E, Viner R, Segal G, Pupko T.** 2009. Genome-scale identification of *Legionella pneumophila* effectors using a machine learning approach. PLoS Pathog **5:**e1000508. http://dx.doi.org/10.1371/journal.ppat.1000508.
27. **Huang L, Boyd D, Amyot WM, Hempstead AD, Luo ZQ, O'Connor TJ, Chen C, Machner M, Montminy T, Isberg RR.** 2011. The E block motif is associated with *Legionella pneumophila* translocated substrates. Cell Microbiol **13:**227–245. http://dx.doi.org/10.1111/j.1462-5822.2010.01531.x.
28. **Zusman T, Aloni G, Halperin E, Kotzer H, Degtyar E, Feldman M, Segal G.** 2007. The response regulator PmrA is a major regulator of the *icm/dot* type IV secretion system in *Legionella pneumophila* and *Coxiella burnetii*. Mol Microbiol **63:**1508–1523. http://dx.doi.org/10.1111/j.1365-2958.2007.05604.x.
29. **Maturana P, Graham JG, Sharma UM, Voth DE.** 2013. Refining the plasmid-encoded type IV secretion system substrate repertoire of *Coxiella burnetii*. J Bacteriol **195:**3269–3276. http://dx.doi.org/10.1128/JB.00180-13.
30. **Martinez E, Cantet F, Fava L, Norville I, Bonazzi M.** 2014. Identification of OmpA, a *Coxiella burnetii* protein involved in host cell invasion, by multi-phenotypic high-content screening. PLoS Pathog **10:**e1004013. http://dx.doi.org/10.1371/journal.ppat.1004013.
31. **Bonifacino JS, Traub LM.** 2003. Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem **72:**395–447. http://dx.doi.org/10.1146/annurev.biochem.72.121801.161800.
32. **Omsland A, Beare PA, Hill J, Cockrell DC, Howe D, Hansen B, Samuel JE, Heinzen RA.** 2011. Isolation from animal tissue and genetic transformation of *Coxiella burnetii* are facilitated by an improved axenic growth medium. Appl Environ Microbiol **77:**3720–3725. http://dx.doi.org/10.1128/AEM.02826-10.
33. **Beare PA, Unsworth N, Andoh M, Voth DE, Omsland A, Gilk SD, Williams KP, Sobral BW, Kupko JJ, III, Porcella SF, Samuel JE, Heinzen RA.** 2009. Comparative genomics reveal extensive transposon-mediated genomic plasticity and diversity among potential effector proteins within the genus *Coxiella*. Infect Immun **77:**642–656. http://dx.doi.org/10.1128/IAI.01141-08.
34. **Takeuchi H, Hirano T, Whitmore SE, Morisaki I, Amano A, Lamont RJ.** 2013. The serine phosphatase SerB of *Porphyromonas gingivalis* suppresses IL-8 production by dephosphorylation of NF-κB RelA/p65. PLoS Pathog **9:**e1003326. http://dx.doi.org/10.1371/journal.ppat.1003326.
35. **Mittl PR, Schneider-Brachert W.** 2007. Sel1-like repeat proteins in signal transduction. Cell Signal **19:**20–31. http://dx.doi.org/10.1016/j.cellsig.2006.05.034.
36. **van Schaik EJ, Chen C, Mertens K, Weber MM, Samuel JE.** 2013. Molecular pathogenesis of the obligate intracellular bacterium *Coxiella burnetii*. Nat Rev Microbiol **11:**561–573. http://dx.doi.org/10.1038/nrmicro3049.
37. **Brombacher E, Urwyler S, Ragaz C, Weber SS, Kami K, Overduin M, Hilbi H.** 2009. Rab1 guanine nucleotide exchange factor SidM is a major phosphatidylinositol 4-phosphate-binding effector protein of *Legionella pneumophila*. J Biol Chem **284:**4846–4856. http://dx.doi.org/10.1074/jbc.M807505200.
38. **Ragaz C, Pietsch H, Urwyler S, Tiaden A, Weber SS, Hilbi H.** 2008. The *Legionella pneumophila* phosphatidylinositol-4 phosphate-binding type IV substrate SidC recruits endoplasmic reticulum vesicles to a replication-

permissive vacuole. Cell Microbiol **10:**2416–2433. http://dx.doi.org/10.1111/j.1462-5822.2008.01219.x.
39. **Chen J, Reyes M, Clarke M, Shuman HA.** 2007. Host cell-dependent secretion and translocation of the LepA and LepB effectors of *Legionella pneumophila*. Cell Microbiol **9:**1660–1671. http://dx.doi.org/10.1111/j.1462-5822.2007.00899.x.
40. **de Felipe KS, Glover RT, Charpentier X, Anderson OR, Reyes M, Pericone CD, Shuman HA.** 2008. *Legionella* eukaryotic-like type IV substrates interfere with organelle trafficking. PLoS Pathog **4:**e1000117. http://dx.doi.org/10.1371/journal.ppat.1000117.
41. **Betts HJ, Wolf K, Fields KA.** 2009. Effector protein modulation of host cells: examples in the *Chlamydia* spp. arsenal Curr Opin Microbiol **12:**81–87. http://dx.doi.org/10.1016/j.mib.2008.11.009.
42. **Alix E, Mukherjee S, Roy CR.** 2011. Subversion of membrane transport pathways by vacuolar pathogens. J Cell Biol **195:**943–952. http://dx.doi.org/10.1083/jcb.201105019.
43. **Brumell JH, Scidmore MA.** 2007. Manipulation of rab GTPase function by intracellular bacterial pathogens. Microbiol Mol Biol Rev **71:**636–652. http://dx.doi.org/10.1128/MMBR.00023-07.
44. **Behnia R, Munro S.** 2005. Organelle identity and the signposts for membrane traffic. Nature **438:**597–604. http://dx.doi.org/10.1038/nature04397.
45. **Zerial M, McBride H.** 2001. Rab proteins as membrane organizers. Nat Rev Mol Cell Biol **2:**107–117. http://dx.doi.org/10.1038/35052055.
46. **Cheng W, Yin K, Lu D, Li B, Zhu D, Chen Y, Zhang H, Xu S, Chai J, Gu L.** 2012. Structural insights into a unique *Legionella pneumophila* effector LidA recognizing both GDP and GTP bound Rab1 in their active state. PLoS Pathog **8:**e1002528. http://dx.doi.org/10.1371/journal.ppat.1002528.
47. **Machner MP, Isberg RR.** 2006. Targeting of host Rab GTPase function by the intravacuolar pathogen *Legionella pneumophila*. Dev Cell **11:**47–56. http://dx.doi.org/10.1016/j.devcel.2006.05.013.
48. **Hardiman CA, Roy CR.** 2014. AMPylation is critical for Rab1 localization to vacuoles containing *Legionella pneumophila*. mBio **5:**e01035–13. http://dx.doi.org/10.1128/mBio.01035-13.
49. **Ingmundson A, Delprato A, Lambright DG, Roy CR.** 2007. *Legionella pneumophila* proteins that regulate Rab1 membrane cycling. Nature **450:**365–369. http://dx.doi.org/10.1038/nature06336.
50. **Muller MP, Peters H, Blumer J, Blankenfeldt W, Goody RS, Itzen A.** 2010. The *Legionella* effector protein DrrA AMPylates the membrane traffic regulator Rab1b. Science **329:**946–949. http://dx.doi.org/10.1126/science.1192276.
51. **Murata T, Delprato A, Ingmundson A, Toomre DK, Lambright DG, Roy CR.** 2006. The *Legionella pneumophila* effector protein DrrA is a Rab1 guanine nucleotide-exchange factor. Nat Cell Biol **8:**971–977. http://dx.doi.org/10.1038/ncb1463.
52. **Gazdag EM, Schobel S, Shkumatov AV, Goody RS, Itzen A.** 2014. The structure of the N-terminal domain of the *Legionella* protein SidC. J Struct Biol **186:**188–194. http://dx.doi.org/10.1016/j.jsb.2014.02.003.
53. **Mishra AK, Del Campo CM, Collins RE, Roy CR, Lambright DG.** 2013. The *Legionella pneumophila* GTPase activating protein LepB accelerates Rab1 deactivation by a non-canonical hydrolytic mechanism. J Biol Chem **288:**24000–24011. http://dx.doi.org/10.1074/jbc.M113.470625.
54. **Yu Q, Hu L, Yao Q, Zhu Y, Dong N, Wang DC, Shao F.** 2013. Structural analyses of *Legionella* LepB reveal a new GAP fold that catalytically mimics eukaryotic RasGAP. Cell Res **23:**775–787. http://dx.doi.org/10.1038/cr.2013.54.
55. **Newton HJ, McDonough JA, Roy CR.** 2013. Effector protein translocation by the *Coxiella burnetii* Dot/Icm type IV secretion system requires endocytic maturation of the pathogen-occupied vacuole. PLoS One **8:**e54566. http://dx.doi.org/10.1371/journal.pone.0054566.
56. **Romano PS, Gutierrez MG, Beron W, Rabinovitch M, Colombo MI.** 2007. The autophagic pathway is actively modulated by phase II *Coxiella burnetii* to efficiently replicate in the host cell. Cell Microbiol **9:**891–909. http://dx.doi.org/10.1111/j.1462-5822.2006.00838.x.
57. **Wesolowski J, Paumet F.** 2010. SNARE motif: a common motif used by pathogens to manipulate membrane fusion. Virulence **1:**319–324. http://dx.doi.org/10.4161/viru.1.4.12195.
58. **Delevoye C, Nilges M, Dautry-Varsat A, Subtil A.** 2004. Conservation of the biochemical properties of IncA from *Chlamydia trachomatis* and *Chlamydia caviae*: oligomerization of IncA mediates interaction between facing membranes. J Biol Chem **279:**46896–46906. http://dx.doi.org/10.1074/jbc.M407227200.
59. **Paumet F, Wesolowski J, Garcia-Diaz A, Delevoye C, Aulner N, Shuman HA, Subtil A, Rothman JE.** 2009. Intracellular bacteria encode inhibitory SNARE-like proteins. PLoS One **4:**e7375. http://dx.doi.org/10.1371/journal.pone.0007375.
60. **Bennett TL, Kraft SM, Reaves BJ, Mima J, O'Brien KM, Starai VJ.** 2013. LegC3, an effector protein from *Legionella pneumophila*, inhibits homotypic yeast vacuole fusion in *vivo* and in *vitro*. PLoS One **8:**e56798. http://dx.doi.org/10.1371/journal.pone.0056798.
61. **Gutierrez MG, Vazquez CL, Munafo DB, Zoppino FC, Beron W, Rabinovitch M, Colombo MI.** 2005. Autophagy induction favours the generation and maturation of the *Coxiella*-replicative vacuoles. Cell Microbiol **7:**981–993. http://dx.doi.org/10.1111/j.1462-5822.2005.00527.x.
62. **Lifshitz Z, Burstein D, Schwartz K, Shuman HA, Pupko T, Segal G.** 2014. Identification of novel *Coxiella burnetii* Icm/Dot effectors and genetic analysis of their involvement in modulating a mitogen-activated protein kinase pathway. Infect Immun **82:**3740–3752. http://dx.doi.org/10.1128/IAI.01729-14.
63. **Newton HJ, Kohler LJ, McDonough JA, Temoche-Diaz M, Crabill E, Hartland EL, Roy CR.** 2014. A screen of *Coxiella burnetii* mutants reveals important roles for Dot/Icm effectors and host autophagy in vacuole biogenesis. PLoS Pathog **10:**e1004286. http://dx.doi.org/10.1371/journal.ppat.1004286.