The *Coxiella burnetii* Cryptic Plasmid Is Enriched in Genes Encoding Type IV Secretion System Substrates^{∇†}

Daniel E. Voth,^{1*} Paul A. Beare,² Dale Howe,² Uma M. Sharma,¹ Georgios Samoilis,^{3,4}
Diane C. Cockrell,² Anders Omsland,² and Robert A. Heinzen²

Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205¹;
Coxiella Pathogenesis Section, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases,
National Institutes of Health, Hamilton, Montana 59840²; and Department of Chemistry³ and
Department of Clinical Bacteriology,⁴ University of Crete, Heraklion, Greece

Received 12 November 2010/Accepted 23 December 2010

The intracellular bacterial pathogen *Coxiella burnetii* directs biogenesis of a phagolysosome-like parasitophorous vacuole (PV), in which it replicates. The organism encodes a Dot/Icm type IV secretion system (T4SS) predicted to deliver to the host cytosol effector proteins that mediate PV formation and other cellular events. All *C. burnetii* isolates carry a large, autonomously replicating plasmid or have chromosomally integrated plasmid-like sequences (IPS), suggesting that plasmid and IPS genes are critical for infection. Bioinformatic analyses revealed two candidate Dot/Icm substrates with eukaryotic-like motifs uniquely encoded by the QpH1 plasmid from the Nine Mile reference isolate. CpeC, containing an F-box domain, and CpeD, possessing kinesin-related and coiled-coil regions, were secreted by the closely related *Legionella pneumophila* Dot/Icm T4SS. An additional QpH1-specific gene, *cpeE*, situated in a predicted operon with *cpeD*, also encoded a secreted effector. Further screening revealed that three hypothetical proteins (CpeA, CpeB, and CpeF) encoded by all *C. burnetii* plasmids and IPS are Dot/Icm substrates. By use of new genetic tools, secretion of plasmid effectors by *C. burnetii* during host cell infection was confirmed using β -lactamase and adenylate cyclase translocation assays, and a C-terminal secretion signal was identified. When ectopically expressed in HeLa cells, plasmid effectors trafficked to different subcellular sites, including autophagosomes (CpeB), ubiquitin-rich compartments (CpeC), and the endoplasmic reticulum (CpeD). Collectively, these results suggest that *C. burnetii* plasmid-encoded T4SS substrates play important roles in subversion of host cell functions, providing a plausible explanation for the absolute maintenance of plasmid genes by this pathogen.

Coxiella burnetii is a highly infectious intracellular bacterium that causes Q fever, a zoonotic disease that typically presents as an acute, influenza-like illness. Rare but serious chronic disease can also occur and normally manifests as endocarditis. *C. burnetii* displays an extensive array of animal reservoirs, with humans exposed to the pathogen primarily via contact with infected domestic livestock. Inhalation of contaminated aerosols is the main route of *C. burnetii* transmission to humans, with alveolar mononuclear phagocytes considered the pathogen's initial target cell (reviewed in reference 38).

Multiple *in vitro* studies indicate that *C. burnetii* replicates in a parasitophorous vacuole (PV) with lysosomal characteristics (34, 65). The early PV interacts with autophagosomes, which may provide nutrients to activate pathogen metabolism (51). Following an initial phagosome stall, the PV fuses with lysosomes and continually engages the endosomal pathway, as indicated by delivery of fluid-phase markers (28). During early stages of infection, *C. burnetii* differentiates from a nonreplicating small-cell-variant morphological form into a replicating large-cell-variant form (27). To accommodate pathogen

growth, the maturing PV expands by continual heterotypic fusion with endolysosomal compartments to ultimately occupy most of the host cell cytoplasm. PV expansion into a spacious structure visible by phase-contrast light microscopy occurs coincident with entry of *C. burnetii* into the log phase of its growth cycle (~1 to 3 days postinfection [dpi]) (17). The lysosomal character of the PV is shown by the presence of lysosomal membrane proteins, active acid hydrolases, and an acidic lumen (~pH 5) (34, 65).

C. burnetii's historical obligate intracellular nature has stymied attempts to identify pathogen factors required for successful infection. Lipopolysaccharide is the only confirmed virulence determinant of *C. burnetii* and is thought to shield the bacterial cell surface from innate immune recognition (42, 59). Predicted virulence factors include *C. burnetii* proteins that modulate host cell processes. For example, *C. burnetii* protein synthesis is required for PV maturation (33), autophagosome interactions (51), apoptosis subversion (68), and activation of the prosurvival kinases Akt and Erk1/2 (66). Maintenance of host cell viability by induction of prosurvival responses is considered a pathogenic strategy that accommodates *C. burnetii*'s slow growth rate.

C. burnetii protein effectors of host functions are likely delivered to the cytosol by a type IV secretion system (T4SS) with homology to the Dot/Icm machinery of *Legionella pneumophila* (57, 63). The refractory nature of *C. burnetii* to genetic manipulation has necessitated using *L. pneumophila* as a surrogate host to identify Dot/Icm substrates. These proteins often pos-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, 4301 W. Markham Street, Little Rock, AR 72205. Phone: (501) 686-8050. Fax: (501) 686-5359. E-mail: dvoth@uams.edu.

† Supplemental material for this article may be found at <http://jbb.asm.org/>.

[∇] Published ahead of print on 7 January 2011.

sess eukaryotic motifs/domains predicted to functionally mimic or antagonize the activity of host cell proteins (3, 14, 19, 46, 64). Indeed, multiple *C. burnetii* proteins with eukaryotic-like ankyrin repeat domains (Anks) are secreted by *L. pneumophila* in a Dot/Icm-dependent fashion (48, 67). An intriguing subset of secreted bacterial proteins contains eukaryotic F-box domains (2). F-box domain-containing proteins are components of the mammalian E3 ubiquitin ligase enzyme complex, which directs ubiquitination of target proteins, resulting in their proteasome-dependent degradation or functional alteration (2). *C. burnetii* isolates collectively contain three F-box-encoding open reading frames (ORFs): CBUA0014, CBU0355, and CBU0814 (2, 7).

CBUA0014 is present on the QpH1 plasmid, originally characterized from the *C. burnetii* Nine Mile reference isolate (55). All *C. burnetii* isolates examined to date maintain a related autonomously replicating plasmid or have chromosomally integrated plasmid-like sequences (IPS) (6, 29, 44, 55, 56, 62). Nucleotide sequences have been determined for QpH1, QpRS, QpDG, QpDV, and IPS of the G and S isolates (7). Early studies with a limited number of isolates demonstrated a correlation between plasmid content and disease presentation, i.e., isolates derived from ticks, infected animals, and patients with acute Q fever harbored QpH1, while isolates from patients with chronic disease carried QpRS or IPS (56). However, a recent genotyping study of 173 *C. burnetii* isolates revealed that QpH1 is also carried by some human chronic Q fever isolates, while showing correlations between QpDV and acute infection and QpRS and chronic infection (24). Whether plasmid type confers human disease potential is unresolved. However, genetically distinct *C. burnetii* isolates can clearly be grouped into pathotypes with different virulence in animal models of acute Q fever (52). Aside from genes involved in plasmid maintenance and segregation, plasmid genes primarily encode hypothetical proteins. Nonetheless, the absolute maintenance of plasmid sequences by all *C. burnetii* isolates suggests that they are critical for pathogen survival.

The presence of F-box-containing CBUA0014 on QpH1 prompted us to investigate whether this and other plasmid ORFs encode Dot/Icm substrates. Using *L. pneumophila* as a secretion model, we found that three QpH1-specific proteins (including the CBUA0014 protein) and three of five hypothetical proteins encoded by all *C. burnetii* plasmids and IPS are translocated into the host cell cytosol by the Dot/Icm T4SS. Importantly, plasmid effectors were also translocated into the cytosol by *C. burnetii*, demonstrating secretion in a native setting. Effector proteins contained a C-terminal region with similarity to the predicted *L. pneumophila* Dot/Icm translocation signal, and deletion analysis revealed that this region is necessary for secretion by *C. burnetii*. All plasmid effector genes were expressed during *in vitro* *C. burnetii* infection, and the encoded proteins trafficked to different subcellular sites when ectopically expressed. These results suggest that *C. burnetii* plasmids play an important role in host cell modifications.

MATERIALS AND METHODS

Bacteria and mammalian cell culture. Bacteria used in this study are described in Table 1. *C. burnetii* Nine Mile phase II, clone 4 (RSA439), was propagated in Vero (African green monkey kidney) cells (CCL-81; ATCC, Manassas, VA) and purified as previously described (16, 58). *L. pneumophila* strains were cultured on

charcoal yeast extract (CYE) agar plates. For plasmid selection, CYE plates contained 10 µg/ml chloramphenicol. For culture of DotA-deficient *L. pneumophila* LELA3118, plates also contained 25 µg/ml kanamycin. *L. pneumophila* transformations were conducted as previously described (67). THP-1 human monocytic cells (TIB-202; ATCC) and HeLa (human epithelioid carcinoma) cells (CCL-2; ATCC) were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (Invitrogen) at 37°C and 5% CO₂. THP-1 cells were differentiated into macrophage-like cells by use of phorbol 12-myristate 13-acetate (PMA; EMD Biosciences, San Diego, CA) as previously described (68). *Escherichia coli* TOP10 (Invitrogen) was used for recombinant DNA procedures. For host cell-free growth of *C. burnetii*, 6-well plates, T-75 flasks, or 0.2-µm-filter capped Erlenmeyer flasks containing ACCM (47) were inoculated with organisms and incubated at 37°C in a 2.5% O₂/5% CO₂ environment.

Effector gene expression. mRNA quantification was performed using the QuantiGene reagent system v.2.0 and custom-designed probes according to the manufacturer's directions (Panomics, Santa Clara, CA). THP-1 cells were incubated with *C. burnetii* at a multiplicity of infection (MOI) of 25 for 2 h, and then extracellular bacteria were washed from monolayers. Cells were lysed at various time points with QuantiGene lysis buffer supplemented with 150 ng/ml proteinase K (Invitrogen) and solubilized by incubation for 30 min at 55°C, followed by 3 freeze-thaw cycles. Lysates were diluted in QuantiGene lysis buffer and combined with blocking buffer and probes. Lysates were loaded into a 96-well capture plate and incubated for 16 to 20 h at 55°C. mRNA was detected by luminescence over 1,000 ms with a Safire² microplate reader (Tecan, Mannedorf, Switzerland). mRNA from uninfected THP-1 cells was used to determine the background signal, and this value was subtracted from each infected cell sample. Transcriptional signals were normalized to *C. burnetii* genome equivalents established as previously described (17).

Plasmid construction. The plasmid pJB2581 was used for expression of CyaA fusion proteins in *L. pneumophila* (4). *C. burnetii* genes were amplified from genomic DNA by PCR using Accuprime Taq polymerase (Invitrogen) and gene-specific primers (Integrated DNA Technologies, Coralville, IA) where the 5' primer incorporates a BamHI site and the 3' primer incorporates a SalI or PstI site (see Table S1 in the supplemental material). Products were cloned into pCR2.1-TOPO (Invitrogen), plasmids were digested with either BamHI/SalI or BamHI/PstI (New England BioLabs, Ipswich, MA), and gene-containing fragments were ligated to similarly digested pJB2581 by use of a Ligate-IT system (U.S. Biologicals, Cleveland, OH).

Modified versions of pJB2581, designated pJB-CAT-BlaM and pJB-CAT-CyaA, were constructed for expression of BlaM and CyaA fusion proteins, respectively, in *C. burnetii*. The chloramphenicol acetyltransferase (CAT) gene was amplified by PCR from pJB2581 using primers CAT-P1169F and CAT-pJB2581-HindIIIrecR. The CBU1169 promoter (P1169) was amplified from *C. burnetii* genomic DNA using primers P1169-pJB2581-Ab-HindIIIrecF and P1169-R. The CAT gene was placed downstream from P1169 to create P1169-CAT using overlapping PCR and the primers P1169-pJB2581-Ab-HindIIIrecF and CAT-pJB2581-HindIIIrecR. P1169-CAT was cloned into HindIII-digested pJB2581 by use of an In-fusion kit (BD Clontech, Mountain View, CA) to create pJB-CAT. P1169 was then amplified by PCR from *C. burnetii* genomic DNA using the primers P1169-pJB2581-F and P1169-R and fused by overlapping PCR to either *blaM*, amplified by PCR from pXDC61 using the primers BlaM-p1169-F and BlaM-pJB-CAT-R, or *cyaA*, amplified by PCR from pJB2581 using the primers Cya-P1169-F and Cya-pJB-CAT-R. P1169-*blaM* and P1169-*cyaA* fragments were cloned into EcoRI/PstI-digested pJB-CAT by use of the In-fusion kit to create pJB-CAT-BlaM and pJB-CAT-CyaA, respectively. pJB-CAT-BlaM and pJB-CAT-CyaA were then used to generate plasmids encoding BlaM or CyaA fused to the N terminus of *C. burnetii* plasmid effector genes. *C. burnetii* genes were amplified by PCR using gene-specific primers and cloned into a unique SalI site in pJB-CAT-BlaM or pJB-CAT-CyaA by use of the In-fusion kit.

For green fluorescent protein (GFP) fusion constructs, *C. burnetii* genes were amplified by PCR with gene-specific primers where the forward primer contains CACC at the 5' end for directional cloning and a 5' Kozac sequence (ATGGGC) for mammalian expression. Products were cloned into pENTR-D/TOPO (Invitrogen) and then subcloned into pcDNA6.2/N-GFP using LR Clonase II (Invitrogen). Plasmid constructions were confirmed by sequencing. All plasmids and primers used in the study are listed in Table 1 and Table S1 in the supplemental material.

***C. burnetii* transformation.** ACCM-cultured *C. burnetii* was collected, washed with 10% glycerol, and then resuspended in 10% glycerol. Ten micrograms of plasmid DNA was mixed with 50 µl of *C. burnetii* in a 0.1-cm cuvette, and organisms were electroporated as previously described (5). Following electroporation, bacteria were cultured in ACCM for 24 h, and then

TABLE 1. Bacteria and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
<i>C. burnetii</i> strains		
RSA439	Phase II, clone 4	6
<i>L. pneumophila</i> strains		
JR32	Salt-sensitive isolate of AM511	53
LELA3118	JR32 <i>dotA</i> ::Tn903dIII <i>lacZ</i> 3118, DotA ⁻ Km ^r	53
Paris	Serogroup 1, clinical isolate	X. Charpentier
Lens	Serogroup 1, clinical isolate	X. Charpentier
<i>E. coli</i> strain TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>dlacZ</i> ΔM15 Δ <i>lacX</i> 74 <i>recA1</i> <i>araD</i> 139 Δ(<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^r) <i>endA1</i> <i>nupG</i>	Invitrogen
Plasmids		
pJB2581	<i>cyaA</i> fusion vector, Cm ^r	4
pDHVAnkG	<i>ankG</i> in pJB2581	67
pDHVA0006	<i>cbua0006</i> in pJB2581	This study
pDHVA0007a	<i>cbua0007a</i> in pJB2581	This study
pDHVA0012	<i>cbua0012</i> in pJB2581	This study
pDHVA0013	<i>cbua0013</i> in pJB2581	This study
pDHVA0014	<i>cbua0014</i> in pJB2581	This study
pDHVA0015	<i>cbua0015</i> in pJB2581	This study
pDHVA0016	<i>cbua0016</i> in pJB2581	This study
pDHVA0023	<i>cbua0023</i> in pJB2581	This study
pDHVlpp1878	<i>lpp1878</i> in pJB2581	This study
pDHVlpl0189	<i>lpl0189</i> in pJB2581	This study
pCR2.1-TOPO	TA TOPO vector, Amp ^r	Invitrogen
pENTR-D/TOPO	Gateway entry vector, Km ^r	Invitrogen
pcDNA6.2/N-GFP	N-terminal GFP fusion vector, Amp ^r Cm ^r	Invitrogen
pGFPCpeA	GFP:: <i>cbua0006</i>	This study
pGFPCpeB	GFP:: <i>cbua0013</i>	This study
pGFPCpeC	GFP:: <i>cbua0014</i>	This study
pGFPCpeD	GFP:: <i>cbua0015</i>	This study
pGFPCpeE	GFP:: <i>cbua0016</i>	This study
pGFPCpeF	GFP:: <i>cbua0023</i>	This study
pJB-CAT	pJB2581 containing P1169 driving <i>cat</i>	This study
pJB-CAT-BlaM	<i>C. burnetii</i> <i>blaM</i> fusion vector, Cm ^r	This study
pJB-CAT-CyaA	<i>C. burnetii</i> <i>cyaA</i> fusion vector, Cm ^r	This study
pJB-CAT-BlaM-A6	<i>cbua0006</i> in pJB-CAT-BlaM	This study
pJB-CAT-CyaA-A6	<i>cbua0006</i> in pJB-CAT-CyaA	This study
pJB-CAT-BlaM-A7a	<i>cbua0007a</i> in pJB-CAT-BlaM	This study
pJB-CAT-BlaM-A12	<i>cbua00012</i> in pJB-CAT-BlaM	This study
pJB-CAT-BlaM-A13	<i>cbua0013</i> in pJB-CAT-BlaM	This study
pJB-CAT-CyaA-A13	<i>cbua0013</i> in pJB-CAT-CyaA	This study
pJB-CAT-BlaM-A14	<i>cbua0014</i> in pJB-CAT-BlaM	This study
pJB-CAT-CyaA-A14	<i>cbua0014</i> in pJB-CAT-CyaA	This study
pJB-CAT-BlaM-A15	<i>cbua0015</i> in pJB-CAT-BlaM	This study
pJB-CAT-CyaA-A15	<i>cbua0015</i> in pJB-CAT-CyaA	This study
pJB-CAT-BlaM-A16	<i>cbua0016</i> in pJB-CAT-BlaM	This study
pJB-CAT-CyaA-A16	<i>cbua0016</i> in pJB-CAT-CyaA	This study
pJB-CAT-BlaM-A23	<i>cbua0023</i> in pJB-CAT-BlaM	This study
pJB-CAT-CyaA-A23	<i>cbua0023</i> in pJB-CAT-CyaA	This study
pJB-CAT-CyaA-A15-2aa	<i>cbua0015</i> lacking 2 aa in pJB-CAT-BlaM	This study
pJB-CAT-CyaA-A15-5aa	<i>cbua0015</i> lacking 5 aa in pJB-CAT-BlaM	This study
pJB-CAT-CyaA-A15-7aa	<i>cbua0015</i> lacking 7 aa in pJB-CAT-BlaM	This study
pJB-CAT-CyaA-A15-10aa	<i>cbua0015</i> lacking 10 aa in pJB-CAT-BlaM	This study
pXDC61	pMMB207C containing <i>blaM</i>	18

chloramphenicol (3 µg/ml) was added to cultures for selection of transformants. The use of genes conferring resistance to chloramphenicol and ampicillin for *C. burnetii* genetic transformation studies at the Rocky Mountain Laboratories (RML) has been approved by the RML Institutional Biosafety Committee and the Centers for Disease Control and Prevention, Division of Select Agents and Toxins.

Immunoblotting. *L. pneumophila* transformant cultures were incubated with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (ICN Biomedicals, Costa Mesa, CA) for 2 h to induce protein expression. Cultures were analyzed by

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using a mouse monoclonal antibody directed against CyaA (clone 3D1; Santa Cruz Biotechnology, Santa Cruz, CA). Reacting proteins were detected using an anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL) and chemiluminescence using ECL Pico reagent (Pierce). To confirm fusion protein expression by *C. burnetii* transformants, THP-1 cells (1 × 10⁶ cells/well) were infected for 2 to 3 days and lysed with SDS-PAGE sample buffer, and lysates were immunoblotted. CyaA expression was detected as described above. BlaM expression was assessed using a mouse

FIG. 1. QpH1-specific genes encode Dot/Icm substrates. (A) Circular map of QpH1, showing the locations of QpH1-specific CBUA0014, CBUA0015, and CBUA0016 (red arrows) and five ORFs (CBUA0006, CBUA0007a, CBUA0012, CBUA0013, and CBUA0023) common to all *C. burnetii* plasmids and IPS (green arrows). (B) CyaA translocation assays showing Dot/Icm-dependent secretion of CBUA0014 (CpeC), CBUA0015 (CpeD), and CBUA0016 (CpeE). Intracellular cAMP levels were determined following infection of THP-1 cells with *L. pneumophila* expressing CyaA fused to individual *C. burnetii* proteins. Results are expressed as fold change over cAMP levels resulting from infection with *L. pneumophila* expressing CyaA alone (negative control). *L. pneumophila* expressing *C. burnetii* AnkG fused to CyaA was used as the positive control (67). Increased cAMP levels were observed when each fusion protein was expressed in wild-type *L. pneumophila*, and negative-control levels were observed following expression in DotA-deficient *L. pneumophila*, indicating that secretion requires a functional Dot/Icm T4SS. Experiments were performed in triplicate, and error bars indicate the standard deviations from the means.

monoclonal antibody directed against β -lactamase (QED; Bioscience, San Diego, CA).

***L. pneumophila* and *C. burnetii* CyaA translocation assays.** *L. pneumophila* CyaA assays were performed as previously described, using a cyclic AMP (cAMP) enzyme immunoassay (GE Healthcare, Piscataway, NJ) (67). For *C. burnetii* CyaA assays, THP-1 cells (1×10^6 cells/well) were infected with *C. burnetii* transformants (MOI of 100) for 3 days, cells were lysed, and then lysates were processed as described above for CyaA assays. Positive secretion of CyaA-effector fusion proteins was scored as ≥ 2.5 -fold more cytosolic cAMP than that for cells infected with organisms expressing CyaA alone. In *L. pneumophila* assays, CyaA fused to *C. burnetii* AnkG was used as a positive control (48, 67), and confirmation of Dot/Icm-dependent secretion was conducted by repeating the assay with the *L. pneumophila* DotA⁻ mutant LELA3118.

***C. burnetii* BlaM translocation assay.** For BlaM translocation assays, THP-1 cells were cultured in black, clear-bottomed, 96-well plates and infected with *C. burnetii* transformants (MOI of 100) for 2 days. Monolayers were loaded with the fluorescent substrate CCF4/AM (LiveBLAzer-FRET B/G loading kit; Invitrogen) in a solution containing 15 mM probenecid (Sigma). Cells were incubated in the dark for 1 h at room temperature and then fluorescence quantified on a Safire² microplate reader, with excitation detected at 405 nm and emission detected at 450 nm. Average fluorescence from 8 uninfected wells was subtracted from results from experimental wells. Results are presented as fold increase in fluorescence above that for cells infected with *C. burnetii* expressing BlaM alone (negative control). As with the CyaA assay, a fluorescence value ≥ 2.5 -fold above that for the negative control was considered positive for effector translocation. Infected cells processed for β -lactamase activity were also visualized by epifluorescence microscopy using an inverted Nikon TE2000 microscope. Representative images ($\times 20$ magnification) were captured with a β -lactamase ratiometric filter set (Chroma Technology, Rockingham, VT) and a Coolsnap HQ2 digital camera (Photometrics, Tucson, AZ) controlled by Metamorph software (Molecular Devices, Inc., Sunnyvale, CA). Images were processed using Adobe Photoshop (Adobe Systems, San Jose, CA).

HeLa cell transfections. Uninfected or infected HeLa cells cultured on 12-mm glass coverslips were transfected with individual GFP fusion constructs by use of Effectene reagent (Qiagen). At 18 h posttransfection, cells were processed for fluorescence microscopy. Cells were incubated with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) to stain host and bacterial DNA. A rabbit polyclonal anti-LC3B antibody (Cell Signaling Technology, Danvers, MA) was used to label autophagosomes, mouse monoclonal antiubiquitin (clone FK2; Sigma) was used to identify ubiquitinated proteins, and rabbit polyclonal anticalnexin (Stressgen,

Ann Arbor, MI) was used to detect the endoplasmic reticulum (ER). Antibodies were detected with anti-rabbit or anti-mouse secondary antibodies conjugated to Alexa Fluor 488 (Invitrogen). Fluorescence microscopy was performed using a Nikon Ti-U microscope, and images were acquired with a 60 $\times$ oil immersion objective and a DS-Qi1Mc camera (Nikon, Melville, NY). Images were processed using NIS-Elements software from Nikon.

RESULTS

Three QpH1-specific genes encode Dot/Icm substrates. CBUA0014 is specific to QpH1 (Fig. 1A and Table 2) and encodes a protein with a ubiquitination-related F-box domain (2, 7), which comprises 47 amino acids (aa) of this small, 77-aa protein. Immediately downstream of CBUA0014 is QpH1-specific CBUA0015, which encodes a protein with a predicted 55-aa coiled-coil domain (CCD). Common in eukaryotic proteins and increasingly found in secreted bacterial effectors, CCDs are α -helical conformations that mediate protein-protein interactions (8, 20). The CBUA0015 protein also contains a 25-aa region with similarity to kinesin-like protein 1 (KLP1) of *Giardia* spp. Kinesin is a microtubule-associated motor protein involved in intracellular transport of vesicular cargo (30). Because bacterial effector proteins often mimic host protein activities, the eukaryotic domains in the CBUA0014 and CBUA0015 proteins suggested that these proteins might be Dot/Icm substrates. To test this hypothesis, *Legionella pneumophila* was used as a surrogate model to assay secretion (48, 67). THP-1 cells were infected with *L. pneumophila* transformants harboring plasmids encoding the CBUA0014 or CBUA0015 protein N-terminally fused to the C terminus of *Bordetella pertussis* adenylate cyclase (CyaA) (60). The CyaA assay relies on effector-mediated delivery of the fusion protein to the cytosol, where the CyaA moiety is activated by binding cytosolic calmodulin, resulting in supraphysiological levels of

TABLE 2. *C. burnetii* plasmid effector family

ORF or protein designation in ^a :				Homology	Predicted molecular size (kDa)
Nine Mile (QpH1)	K (QpRS)	G (IPS)	Dugway (QpDG)		
CpeA (CBUA0006)	CBUKA0010	CBUG0089	CBUDA0032	Regions of <i>L. pneumophila</i> <i>lpp1878</i> and <i>lpl0189</i>	39
CpeB (CBUA0013)	CBUKA0019	CBUG0080	CBUDA0046	None	28
CpeC (CBUA0014)	Absent	Absent	Absent	F box	9
CpeD (CBUA0015)	Absent	Absent	Absent	KLP1 and CCD	26
CpeE (CBUA0016)	Absent	Absent	Absent	None	39
CpeF (CBUA0023)	CBUKA0021	CBUG0076	CBUDA0052	None	27

^a GenBank accession numbers are as follows: for Nine Mile, AE016828; for K, CP001020; for G, CP001019; and for Dugway, CP000733. The plasmid type is shown in parentheses beside the respective isolate.

cAMP (60). Elevated cAMP levels were observed in lysates from cells infected with wild-type *L. pneumophila*, but not a DotA[−] strain (53), expressing either CyaA fusion protein (Fig. 1B). Thus, the CBUA0014 and CBUA0015 proteins, now denoted CpeC (*Coxiella* plasmid effector protein C) and CpeD, respectively, are Dot/Icm substrates. CBUA0016 is QpH1 specific, encodes a hypothetical, hydrophilic protein, and resides in a predicted operon with *cpeD* (39). This genetic arrangement suggested that the CBUA0016 protein might also be a T4SS substrate. Indeed, the CBUA0016 protein, now designated CpeE, was also translocated into host cells in a Dot/Icm-dependent manner (Fig. 1B).

Conserved plasmid genes encode Dot/Icm substrates. Genus-specific hypothetical proteins lacking eukaryotic sequence similarity are also common T4SS substrates (23). This is not unexpected considering the broad repertoire of unique host-pathogen interactions. Therefore, we investigated whether five plasmid ORFs (CBUA0006, CBUA0007a, CBUA0012, CBUA0013, and CBUA0023) encoding hypothetical proteins

that are conserved among all *C. burnetii* plasmids (Fig. 1A) and IPS (6, 7) are Dot/Icm substrates. As shown in Fig. 2A, infection of THP-1 cells with wild-type, but not DotA[−], *L. pneumophila* expressing the CBUA0006, CBUA0013, and CBUA0023 proteins fused to CyaA resulted in significantly increased cAMP levels, indicating Dot/Icm-dependent translocation into the host cytosol (Fig. 2A). These proteins are now designated CpeA, CpeB, and CpeF, respectively (Fig. 2A and Table 2). Conversely, the CBUA0007a and CBUA0012 proteins were not translocated (Fig. 2A). By immunoblotting, all CyaA fusion proteins were equally expressed (data not shown); thus, negative results for CBUA0007a and CBUA0012 were not due to lack of synthesis.

Homologs of *C. burnetii* T4SS effectors in other bacterial species are unusual (67). While not a confirmed homolog, the N-terminal 250 aa of CpeA show ~50% similarity to the N termini of LPP1878 and LPL0189 from the *L. pneumophila* Paris and Lens strains, respectively. Therefore, we tested whether these proteins are secreted. As shown in Fig. 2B, both

FIG. 2. Conserved *C. burnetii* plasmid hypothetical proteins and *L. pneumophila* homologs are Dot/Icm substrates. Intracellular cAMP levels were determined following infection of THP-1 cells with *L. pneumophila* expressing the indicated CyaA fusion proteins. Results are expressed as fold change over cAMP levels resulting from infection with *L. pneumophila* expressing CyaA alone (negative control). *C. burnetii* AnkG fused to CyaA served as the positive control. (A) Elevated cAMP levels were observed after infection of THP-1 cells with *L. pneumophila* expressing CyaA fused to CBUA0006 (CpeA), CBUA0013 (CpeB), and CBUA0023 (CpeF), indicating secretion. Negative-control cAMP levels were observed when these fusion proteins were expressed in DotA-deficient *L. pneumophila*, indicating that translocation is mediated by the Dot/Icm T4SS. CBUA0007a and CBUA0012 were not secreted. (B) Elevated levels of cAMP were observed in THP-1 cells infected with *L. pneumophila* expressing the CpeA homologs LPP1878 and LPL0189 fused to CyaA, indicating secretion. Experiments were performed in triplicate, and error bars indicate the standard deviations from the means.

FIG. 3. Plasmid effector genes are expressed during *C. burnetii* intracellular growth. THP-1 cells were infected with *C. burnetii* for the indicated times, and then RNA was isolated. RNA was subjected to QuantiGene analysis to determine effector gene expression levels. Expression is shown as relative light units (RLUs). QpH1-specific effector genes were maximally expressed at 2 dpi, while conserved plasmid effector genes were maximally expressed at 3 dpi.

LPP1878 and LPL0189 caused increased levels of cAMP in host cells when expressed in wild-type *L. pneumophila*, suggesting a potential shared effector protein between these related pathogens.

Expression of Dot/Icm substrates is transcriptionally regulated. *L. pneumophila* Dot/Icm substrate expression is transcriptionally regulated to influence distinct infection events (36). To confirm that the six plasmid effectors are expressed by *C. burnetii* during infection and to resolve the kinetics of expression, we determined the transcriptional profile of each effector gene over a 7-day time course of infection. This time course represents the pathogen's growth cycle in human macrophages from initial infection through stationary phase (34). As shown in Fig. 3, maximal expression of QpH1-specific and conserved plasmid effector genes occurred at 2 and 3 days, respectively, after infection of THP-1 cells. Thus, expression of plasmid effector genes is temporally regulated, with highest expression observed concomitant with early-log-phase growth and rapid PV expansion.

Plasmid Dot/Icm substrates are translocated into the host cytosol by *C. burnetii*. *L. pneumophila* has been invaluable in identifying *C. burnetii* T4SS substrates (35, 48, 67). However, direct demonstration of effector secretion by *C. burnetii* would be ideal. Recent advances in *C. burnetii* genetic transformation (5) and the discovery that RSF1010 *ori*-containing plasmids autonomously replicate in the organism (15) allowed us to develop both CyaA and β -lactamase (BlaM) secretion assays for use in *C. burnetii*. Like the CyaA assay, the BlaM assay is an enzymatic reporter assay that relies on delivery of a BlaM-effector chimera to the cytosol. Here, the BlaM moiety cleaves the β -lactam ring of a cell-loaded fluorescent compound, resulting in blue cytosolic fluorescence (10, 18). The RSF1010 plasmid backbone used to create the *C. burnetii* expression vectors pJB-CAT-CyaA and pJB-CAT-BlaM (see Fig. S1 in the supplemental material) was derived from pJB2581 (4), the same plasmid used in our *L. pneumophila* CyaA assays. Fusion protein expression is driven by the *C. burnetii* CBU1169 promoter, which encodes Hsp20 and is constitutively expressed by *C. burnetii* (P. A. Beare, unpublished results).

Plasmid effector ORFs were cloned downstream and in frame with *cyaA* or *blaM*. THP-1 cells were infected with *C. burnetii* transformants cultured axenically under antibiotic selection, and then CyaA and BlaM assays were conducted at 3 and 2 dpi, respectively. By immunoblotting, CyaA and BlaM effector fusion proteins were all expressed in THP-1 cells (Fig. 4A and data not shown). THP-1 cells infected with *C. burnetii* expressing CpeA, CpeB, CpeD, CpeE, and CpeF fused to CyaA had ≥ 2.5 -fold-higher levels of cAMP than cells infected with *C. burnetii* expressing CyaA alone, indicating translocation into the cytosol (67) (Fig. 4A). CyaA-CpeC induced significantly more cAMP accumulation than CyaA alone; however, the level was slightly below the 2.5-fold cutoff. Based on the same elevation in blue fluorescence relative to the level for THP-1 cells infected with *C. burnetii* expressing BlaM alone, CpeA, CpeB, CpeC, CpeD, and CpeE were positive for translocation in the BlaM translocation assay (Fig. 4B). Levels of fluorescence with BlaM-CpeF were significantly higher than levels with BlaM alone but fell below the 2.5-fold cutoff. Furthermore, the CBU0007a and CBU0012 proteins were not secreted using the BlaM assay (data not shown), which corresponds with results from the *L. pneumophila* CyaA assay. Collectively, as assessed by one or both secretion assays, all plasmid effectors were secreted by *C. burnetii* during intracellular growth.

***C. burnetii* plasmid effectors contain a putative C-terminal translocation signal.** Deletion analysis indicates that an intact C terminus is required for translocation of effectors by *L. pneumophila*'s Dot/Icm T4SS (9, 43). Salient features of the putative C-terminal secretion signal include depletion of negative amino acids in positions -1 to -6 , enrichment of serine and threonine in positions -3 to -11 , and enrichment of hydrophobic amino acids in positions -1 to -3 (9, 43). While a lineup of the 20 C-terminal residues of each *C. burnetii* plasmid effector protein showed little sequence identity, C termini had the same general features of the putative *L. pneumophila* translocation signal, including the lack of negatively charged residues and enrichment of hydrophobic residues in positions -1 to -6 (Fig. 5A). To test the importance of the C

FIG. 4. Plasmid effectors are translocated by *C. burnetii*. (A) THP-1 cells were infected for 3 days with *C. burnetii* expressing individual effectors fused to CyaA, and then cell lysates were analyzed by immunoblotting and the cAMP assay. All CyaA-effector fusions were expressed by *C. burnetii* (top), and CpeA, CpeB, CpeD, CpeE, and CpeF were translocated into the host cytosol (bottom), as scored by ≥ 2.5 -fold more cytosolic cAMP than for cells infected with organisms expressing CyaA alone (negative control). Values at left in the top panel are molecular sizes in kilodaltons. (B) THP-1 cells were infected for 2 days with *C. burnetii* expressing individual effectors fused to BlaM and then processed for β -lactamase activity. (Top) Micrographs showing blue-fluorescence-associated THP-1 cells infected with *C. burnetii* expressing BlaM-CpeD but not BlaM alone. Bar, 30 μ M. (Bottom) BlaM assay results indicating that CpeA, CpeB, CpeC, CpeD, and CpeE are translocated into the host cytosol, as scored by ≥ 2.5 -fold more blue fluorescence than for cells infected with organisms expressing BlaM alone (negative control). Experiments were performed in triplicate, and error bars indicate the standard deviations from the means.

terminus for *C. burnetii* secretion, BlaM translocation assays were conducted with *C. burnetii* transformants expressing BlaM-CpeD fusion proteins with C-terminal deletions of 2, 5, 7, or 10 aa. All fusion proteins were equally expressed by *C. burnetii* following infection of THP-1 cells (data not shown). Deletion of only 2 aa impaired secretion, while deletion of 7 aa or more diminished secretion to near negative-control levels (Fig. 5B). Thus, *C. burnetii* Dot/Icm substrates contain a C-terminal region important for translocation.

Plasmid effectors traffic to distinct subcellular sites in mammalian cells. To probe effector function, we ectopically expressed plasmid T4SS substrates in HeLa cells as fusions to green fluorescent protein (GFP) to visualize the subcellular distribution. This commonly used approach provides clues about bacterial virulence factor function, as effector proteins typically modulate a host factor/process associated with the targeted subcellular site (11, 18, 26, 45, 67). Specific localization of GFP-CpeE and GFP-CpeF was not observed, while GFP-CpeA and GFP-CpeC localized to small cytoplasmic puncta, GFP-CpeB to vesicular structures, including the PV membrane, and GFP-CpeD to filamentous structures (Fig. 6 and data not shown). Further analysis demonstrated that CpeB trafficked to autophagosomes, as evidenced by colocalization with LC3B (Fig. 6). CpeD partially localized to the endoplasmic reticulum (ER), as indicated by colocalization with

calnexin, and caused accumulation and eventual breakdown of the ER network (Fig. 6). Finally, CpeC colocalized with ubiquitin-rich structures (Fig. 6), consistent with the presence of an F-box domain in this protein.

DISCUSSION

Here, we show that *C. burnetii* plasmids and IPS encode Dot/Icm T4SS substrates. The functional relevance of *C. burnetii* plasmids has been elusive, despite description of the molecules over 25 years ago and subsequent nucleotide sequencing (55, 56). An essential role in host cell modification provides the first plausible explanation for absolute maintenance of plasmid sequences by all *C. burnetii* isolates.

C. burnetii plasmid effectors were initially identified using *L. pneumophila* as a surrogate model (48, 67). This screen indicated that CpeA, CpeB, CpeC, CpeD, CpeE, and CpeF are secreted in a Dot/Icm-dependent manner. Previous confirmation of *C. burnetii* secretion of an effector originally identified using *L. pneumophila* was achieved for AnkF by immunoblotting the soluble cytosolic fraction of infected cells with specific antibody (48). A major accomplishment of the current work is direct demonstration of secretion by *C. burnetii* using genetic methods. This screen was made possible using an RSF1010 *ori*-containing plasmid that autonomously replicates in *C. bur-*

FIG. 5. C termini of plasmid effectors have features required for efficient translocation. (A) The C-terminal 20 aa of the plasmid Dot/Icm substrates were aligned, and residues having similar properties are denoted. Each plasmid effector contained at least 2 hydrophobic residues (L, V, I, and F) in the -1 to -6 positions relative to the C terminus. Negatively charged residues (E and D) were absent in these positions but present in the -10 to -20 region. Amino acids with hydroxyl side chains (Ser and Thr) were randomly distributed across each C-terminal segment. (B) A C-terminal deletion series of CpeD fused to BlaM was tested for secretion using the BlaM assay conducted as described in the legend for Fig. 4. Removal of only 2 aa impaired secretion, while removal of 7 aa or more resulted in fluorescence near negative-control levels. Experiments were performed in triplicate, and error bars indicate the standard deviations from the means.

netii, thereby providing a system for heterologous gene expression (15). When expressed in *C. burnetii*, five plasmid effectors were positive in the CyaA assay and five were positive in the BlaM assay. We are unsure why CpeC and CpeF were negative in the CyaA and BlaM assays, respectively. Fusion proteins are expressed at similar levels in *C. burnetii*; thus, negative results are not associated with translational levels. However, native CpeC is ~ 9 kDa in size, and attachment of CyaA (43 kDa) to this small protein may alter folding and subsequent secretion. In agreement with this hypothesis, CyaA-CpeC was also translocated at low levels in *L. pneumophila* compared to the other plasmid effectors. The RSF1010 *ori* plasmid used in this study has a copy number of 3 to 6 in *C. burnetii* (P. A. Beare and R. A. Heinzen, unpublished data), and effectors are constitutively expressed. Thus, overexpression of effector fusions might

inhibit the secretion apparatus, resulting in a negative readout. However, all *C. burnetii* effector transformants productively infect THP-1 cells to form a typical PV, suggesting that translocation of the effector repertoire required for intracellular growth is unaltered. In the *L. pneumophila* CyaA assay, isopropyl- β -D-thiogalactopyranoside (IPTG) is added to cultures before infection to induce fusion protein expression. A similar system of temporally induced expression may be optimal for *C. burnetii* translocation assays. Finally, fusion partners may affect chaperone recognition of some effectors. Little is known about chaperone function in *C. burnetii* type IV secretion, although some, but not all, *C. burnetii* CyaA-Ank fusions require the chaperone IcmS for translocation by *L. pneumophila*. Nonetheless, combined results from both assays indicate that all six plasmid effectors are secreted by *C. burnetii*.

The C termini of *C. burnetii* plasmid effectors show amino acid enrichments similar to the proposed C-terminal secretion signal of *L. pneumophila* Dot/Icm T4SS substrates (9, 43). We previously demonstrated that deletion of 10 amino acids from the C terminus of *C. burnetii* AnkI eliminates Dot/Icm-mediated secretion by *L. pneumophila* (67). Here, we show that deletion of only 2 residues from the C terminus of CpeD adversely affects translocation by *C. burnetii*, with deletion of 7 or 10 aa lowering secretion to near negative-control levels. Collectively, these data demonstrate a critical C-terminal secretion signal in *C. burnetii* T4SS substrates.

Because Dot/Icm-translocated CpeC, CpeD, and CpeE are specific to QpH1, their functions are strictly associated with isolates that maintain this plasmid (24). Functional analysis of specific and conserved plasmid effectors would be aided by phenotyping respective gene knockouts. Unfortunately, methods of allelic exchange in *C. burnetii* are currently unavailable. Therefore, to provide clues about effector protein activity, we ectopically expressed plasmid effectors as GFP fusion proteins in mammalian cells and monitored their subcellular localization.

Consistent with the presence of an F-box domain (31), GFP-CpeC traffics to ubiquitin-positive structures throughout the cytoplasm. Ubiquitination mediated by bacterial F-box-containing proteins can modify protein function or target proteins for degradation by the proteasome (2). This process modulates diverse host functions, including innate immune signaling, inflammation, and apoptosis (2). *L. pneumophila* produces five F-box-containing proteins that are Dot/Icm substrates (22), the most thoroughly characterized being AnkB (LegAU13). AnkB forms a functional Skp-Cullin-F-box E3 ubiquitin ligase complex (37) and is targeted to the cytosolic face of the *L. pneumophila*-containing vacuole by a process requiring host cell farnesylation (50). The substrate(s) (bacterial or host) targeted by AnkB for ubiquitination is unknown, as is its role in *L. pneumophila* parasitism, with some (1, 37, 49), but not all (22), strains with inactivated AnkB showing strong infection defects. Further characterization of CpeC-interacting proteins will provide insight into the specific function of this protein.

Ectopically expressed GFP-CpeD partially colocalizes with the ER and disrupts the organelle's dispersed architecture. CpeD contains a region of homology to a kinesin-related *Giardia* protein. Kinesins are well-characterized motor proteins that direct plus-end vesicular cargo trafficking along microtubules (30) and mediate processes such as Golgi complex-

FIG. 6. CpeB, CpeC, and CpeD traffic to distinct subcellular compartments. GFP-CpeB, GFP-CpeC, and GFP-CpeD were ectopically expressed in HeLa cells, and cells were processed for fluorescence microscopy. GFP fusion proteins, antibody-labeled proteins, and DNA are shown as green, red, and blue, respectively, in the merged images. Labeled proteins are indicated in the panels. CpeB trafficked to autophagosomes, as evidenced by colocalization with LC3B. CpeC colocalized with ubiquitinated proteins. CpeD partially localized to the ER, as demonstrated by calnexin staining, and caused disruption of the ER network. Bar, 10 μ m.

directed secretory transport (69). CpeD also contains a CCD that may mediate protein-protein interactions. When ectopically expressed, CCD-containing Dot/Icm effectors of *L. pneumophila* can alter *Saccharomyces cerevisiae* secretory transport and/or cause accumulation of ER-derived structures in CHO cells (9, 18). Recent evidence suggests that *C. burnetii* manipulates host secretory processes, with Rab1 recruitment to the PV involved in proper vacuole formation (13). Additionally, the ER protein Bip associates with a fractionated PV (32), and the PV membrane decorates with calnexin (D. E. Voth, unpublished results). Thus, CpeD and potentially other Dot/Icm substrates may influence ER function. It should be noted that CpeD was also identified in a proteomic screen of *C. burnetii* secreted proteins (54).

The third QpH1-specific Dot/Icm substrate, CpeE, showed no specific localization when ectopically expressed. CpeE is a hypothetical protein lacking any obvious homology to known eukaryotic or prokaryotic proteins. CpeE was originally identified as a hydrophilic protein specific to acute Q fever isolates and was termed CbhE' in reference to unique restriction mapping on QpH1 (40, 41). *cpeE* and *cpeD* reside in a putative

operon (39), a prediction supported by similar expression profiles. Like that for *cpeC*, maximal expression of both genes occurs at 2 days postinfection, coincident with the onset of rapid PV enlargement. Thus, CpeE and CpeD may act together to modulate PV formation or other host cell functions.

Of the five hypothetical proteins encoded by all *C. burnetii* plasmids and IPS, three (CpeA, CpeB, and CpeF) are Dot/Icm substrates. This effector group is maximally expressed at 3 days postinfection, 1 day later than QpH1-specific effectors. CpeA is similar to two hypothetical *L. pneumophila* proteins, LPL0189 and LPP1878, that we demonstrate are also Dot/Icm substrates. The relatedness of these three secreted proteins may correlate with a common biological function during intracellular growth. Only GFP-CpeB showed specific subcellular trafficking in localizing to LC3B-positive autophagosome-derived vesicles, including the PV membrane. Autophagosomes normally remove unwanted material, such as damaged organelles, from the host cell cytoplasm and mature into autophagolysosomes, where this material is degraded (61). Numerous intracellular pathogens subvert autophagic signaling to provide a suitable growth environment (12). For example, *Mycobacte-*

rium tuberculosis inhibits autophagosome/lysosome fusion to avoid lysosomal destruction (21). Conversely, exogenous induction of autophagy benefits *C. burnetii* replication (25, 51), and the organism actively engages autophagosomes a few minutes after infection (25, 51). It is intriguing to speculate that CpeB benefits *C. burnetii* by acting alone, or in concert with other effectors, to induce autophagy.

In summary, *C. burnetii* plasmids and IPS are enriched in genes encoding Dot/Icm substrates. Functional characterization of these proteins will provide needed insight into *C. burnetii* host cell parasitism. Host cell-free cultivation of *C. burnetii* (47) may provide a means to cure the pathogen of its plasmid. Generation of an isogenic strain lacking plasmid sequences will help define the role of these molecules in host cell infection and pathotype-specific virulence.

ACKNOWLEDGMENTS

We thank Anita Mora for graphic illustrations, Katja Mertens and James Samuel for unpublished information regarding the autonomous replication of RSF1010 *ori*-containing plasmids in *C. burnetii*, and Seth Winfree for BlaM microscopy. We thank Raphael Valdivia and Sergey Konstantinov for helpful discussions and Xavier Charpentier for the generous gift of *L. pneumophila* Paris and Lens genomic DNA.

This work was supported by funding from NIH NIAID grants K22AI081753 (D.E.V.) and R01AI087669 (D.E.V.), the Arkansas Biosciences Institute (D.E.V.), and the Intramural Research Program of the National Institutes of Health, National Institute of Allergy and Infectious Diseases (R.A.H.).

REFERENCES

- Al-Khodori, S., C. T. Price, F. Habyarimana, A. Kalia, and Y. Abu Kwaik. 2008. A Dot/Icm-translocated ankyrin protein of *Legionella pneumophila* is required for intracellular proliferation within human macrophages and protozoa. *Mol. Microbiol.* **70**:908–923.
- Angot, A., A. Vergunst, S. Genin, and N. Peeters. 2007. Exploitation of eukaryotic ubiquitin signaling pathways by effectors translocated by bacterial type III and type IV secretion systems. *PLoS Pathog.* **3**:e3.
- Backert, S., and T. F. Meyer. 2006. Type IV secretion systems and their effectors in bacterial pathogenesis. *Curr. Opin. Microbiol.* **9**:207–217.
- Bardill, J. P., J. L. Miller, and J. P. Vogel. 2005. IcmS-dependent translocation of SdeA into macrophages by the *Legionella pneumophila* type IV secretion system. *Mol. Microbiol.* **56**:90–103.
- Beare, P. A., et al. 2009. Characterization of a *Coxiella burnetii* *ftsZ* mutant generated by *HimarI* transposon mutagenesis. *J. Bacteriol.* **191**:1369–1381.
- Beare, P. A., et al. 2006. Genetic diversity of the Q fever agent, *Coxiella burnetii*, assessed by microarray-based whole-genome comparisons. *J. Bacteriol.* **188**:2309–2324.
- Beare, P. A., et al. 2009. Comparative genomics reveal extensive transposon-mediated genomic plasticity and diversity among potential effector proteins within the genus *Coxiella*. *Infect. Immun.* **77**:642–656.
- Burkhard, P., J. Stetefeld, and S. V. Strelkov. 2001. Coiled coils: a highly versatile protein folding motif. *Trends Cell Biol.* **11**:82–88.
- Burstein, D., et al. 2009. Genome-scale identification of *Legionella pneumophila* effectors using a machine learning approach. *PLoS Pathog.* **5**:e1000508.
- Campbell, R. E. 2004. Realization of beta-lactamase as a versatile fluorogenic reporter. *Trends Biotechnol.* **22**:208–211.
- Campodónico, E. M., L. Chesnel, and C. R. Roy. 2005. A yeast genetic system for the identification and characterization of substrate proteins transferred into host cells by the *Legionella pneumophila* Dot/Icm system. *Mol. Microbiol.* **56**:918–933.
- Campoy, E., and M. I. Colombo. 2009. Autophagy in intracellular bacterial infection. *Biochim. Biophys. Acta* **1793**:1465–1477.
- Campoy, E. M., F. C. M. Zoppino, and M. I. Colombo. 2010. The early secretory pathway contributes to the growth of the *Coxiella*-replicative niche. *Infect. Immun.* **79**:402–413.
- Cazalet, C., et al. 2004. Evidence in the *Legionella pneumophila* genome for exploitation of host cell functions and high genome plasticity. *Nat. Genet.* **36**:1165–1173.
- Chen, C., et al. Large-scale identification and translocation of type IV secretion substrates by *Coxiella burnetii*. *Proc. Natl. Acad. Sci. U. S. A.*, in press.
- Cockrell, D. C., P. A. Beare, E. R. Fischer, D. Howe, and R. A. Heinzen. 2008. A method for purifying obligate intracellular *Coxiella burnetii* that employs digitonin lysis of host cells. *J. Microbiol. Methods* **72**:321–325.
- Coleman, S. A., E. R. Fischer, D. Howe, D. J. Mead, and R. A. Heinzen. 2004. Temporal analysis of *Coxiella burnetii* morphological differentiation. *J. Bacteriol.* **186**:7344–7352.
- de Felipe, K. S., et al. 2008. *Legionella* eukaryotic-like type IV substrates interfere with organelle trafficking. *PLoS Pathog.* **4**:e1000117.
- de Felipe, K. S., et al. 2005. Evidence for acquisition of *Legionella* type IV secretion substrates via interdomain horizontal gene transfer. *J. Bacteriol.* **187**:7716–7726.
- Delahay, R. M., and G. Frankel. 2002. Coiled-coil proteins associated with type III secretion systems: a versatile domain revisited. *Mol. Microbiol.* **45**:905–916.
- Dereic, V., et al. 2006. *Mycobacterium tuberculosis* inhibition of phagolysosome biogenesis and autophagy as a host defence mechanism. *Cell. Microbiol.* **8**:719–727.
- Ensminger, A. W., and R. R. Isberg. 2010. E3 ubiquitin ligase activity and targeting of BAT3 by multiple *Legionella pneumophila* translocated substrates. *Infect. Immun.* **78**:3905–3919.
- Ensminger, A. W., and R. R. Isberg. 2009. *Legionella pneumophila* Dot/Icm translocated substrates: a sum of parts. *Curr. Opin. Microbiol.* **12**:67–73.
- Glazunova, O., et al. 2005. *Coxiella burnetii* genotyping. *Emerg. Infect. Dis.* **11**:1211–1217.
- Gutierrez, M. G., et al. 2005. Autophagy induction favours the generation and maturation of the *Coxiella*-replicative vacuoles. *Cell. Microbiol.* **7**:981–993.
- Heidtman, M., E. J. Chen, M. Moy, and R. R. Isberg. 2009. Large scale identification of *Legionella pneumophila* Dot/Icm substrates that modulate host cell vesicle trafficking pathways. *Cell. Microbiol.* **11**:230–248.
- Heinzen, R. A., T. Hackstadt, and J. E. Samuel. 1999. Developmental biology of *Coxiella burnetii*. *Trends Microbiol.* **7**:149–154.
- Heinzen, R. A., M. A. Scidmore, D. D. Rockey, and T. Hackstadt. 1996. Differential interaction with endocytic and exocytic pathways distinguish parasitophorous vacuoles of *Coxiella burnetii* and *Chlamydia trachomatis*. *Infect. Immun.* **64**:796–809.
- Hendrix, L. R., J. E. Samuel, and L. P. Mallavia. 1991. Differentiation of *Coxiella burnetii* isolates by analysis of restriction-endonuclease-digested DNA separated by SDS-PAGE. *J. Gen. Microbiol.* **137**:269–276.
- Hirokawa, N., and Y. Noda. 2008. Intracellular transport and kinesin superfamily proteins, KIFs: structure, function, and dynamics. *Physiol. Rev.* **88**:1089–1118.
- Ho, M. S., P. I. Tsai, and C. T. Chien. 2006. F-box proteins: the key to protein degradation. *J. Biomed. Sci.* **13**:181–191.
- Howe, D., and R. A. Heinzen. 2008. Fractionation of the *Coxiella burnetii* parasitophorous vacuole. *Methods Mol. Biol.* **445**:389–406.
- Howe, D., J. Melnicakova, I. Barak, and R. A. Heinzen. 2003. Maturation of the *Coxiella burnetii* parasitophorous vacuole requires bacterial protein synthesis but not replication. *Cell. Microbiol.* **5**:469–480.
- Howe, D., J. G. Shannon, S. Winfree, D. W. Dorward, and R. A. Heinzen. 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.
- Huang, B., et al. 2010. *Anaplasma phagocytophilum* APH_0032 is expressed late during infection and localizes to the pathogen-occupied vacuolar membrane. *Microb. Pathog.* **49**:273–284.
- Isberg, R. R., T. J. O'Connor, and M. Heidtman. 2009. The *Legionella pneumophila* replication vacuole: making a cosy niche inside host cells. *Nat. Rev. Microbiol.* **7**:13–24.
- Lomma, M., et al. 2010. The *Legionella pneumophila* F-box protein Lpp2082 (AnkB) modulates ubiquitination of the host protein parvin B and promotes intracellular replication. *Cell. Microbiol.* **12**:1272–1291.
- Madariaga, M. G., K. Rezaei, G. M. Trenholme, and R. A. Weinstein. 2003. Q fever: a biological weapon in your backyard. *Lancet Infect. Dis.* **3**:709–721.
- Mao, F., P. Dam, J. Chou, V. Olman, and Y. Xu. 2009. DOOR: a database for prokaryotic operons. *Nucleic Acids Res.* **37**:D459–D463.
- Minnick, M. F., C. L. Small, M. E. Frazier, and L. P. Mallavia. 1991. Analysis of strain-specific plasmid sequences from *Coxiella burnetii*. *Acta Virol.* **35**:497–502.
- Minnick, M. F., C. L. Small, M. E. Frazier, and L. P. Mallavia. 1991. Analysis of the *cbhE'* plasmid gene from acute disease-causing isolates of *Coxiella burnetii*. *Gene* **103**:113–118.
- Moos, A., and T. Hackstadt. 1987. Comparative virulence of intra- and interstrain lipopolysaccharide variants of *Coxiella burnetii* in the guinea pig model. *Infect. Immun.* **55**:1144–1150.
- Nagai, H., et al. 2005. A C-terminal translocation signal required for Dot/Icm-dependent delivery of the *Legionella* RalF protein to host cells. *Proc. Natl. Acad. Sci. U. S. A.* **102**:826–831.
- Ning, Z., S. R. Yu, Y. G. Quan, and Z. Xue. 1992. Molecular characterization of cloned variants of *Coxiella burnetii* isolated in China. *Acta Virol.* **36**:173–183.
- Ninio, S., J. Celli, and C. R. Roy. 2009. A *Legionella pneumophila* effector protein encoded in a region of genomic plasticity binds to Dot/Icm-modified vacuoles. *PLoS Pathog.* **5**:e1000278.

46. Ninio, S., and C. R. Roy. 2007. Effector proteins translocated by *Legionella pneumophila*: strength in numbers. *Trends Microbiol.* **15**:372–380.
47. Omsland, A., et al. 2009. Host cell-free growth of the Q fever bacterium *Coxiella burnetii*. *Proc. Natl. Acad. Sci. U. S. A.* **106**:4430–4434.
48. Pan, X., A. Luhrmann, A. Satoh, M. A. Laskowski-Arce, and C. R. Roy. 2008. Ankyrin repeat proteins comprise a diverse family of bacterial type IV effectors. *Science* **320**:1651–1654.
49. Price, C. T., et al. 2009. Molecular mimicry by an F-box effector of *Legionella pneumophila* hijacks a conserved polyubiquitination machinery within macrophages and protozoa. *PLoS Pathog.* **5**:e1000704.
50. Price, C. T., T. Al-Quadan, M. Santic, S. C. Jones, and Y. Abu Kwaik. 2010. Exploitation of conserved eukaryotic host cell farnesylation machinery by an F-box effector of *Legionella pneumophila*. *J. Exp. Med.* **207**:1713–1726.
51. Romano, P. S., M. G. Gutierrez, W. Beron, M. Rabinovitch, and M. I. Colombo. 2007. The autophagic pathway is actively modulated by phase II *Coxiella burnetii* to efficiently replicate in the host cell. *Cell. Microbiol.* **9**:891–909.
52. Russell-Lodrigue, K. E., et al. 2009. *Coxiella burnetii* isolates cause group-specific virulence in mouse and guinea pig models of acute Q fever. *Infect. Immun.* **77**:5640–5650.
53. Sadosky, A. B., L. A. Wiater, and H. A. Shuman. 1993. Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. *Infect. Immun.* **61**:5361–5373.
54. Samoilis, G., et al. 2010. Proteomic screening for possible effector molecules secreted by the obligate intracellular pathogen *Coxiella burnetii*. *J. Proteome Res.* **9**:1619–1626.
55. Samuel, J. E., M. E. Frazier, M. L. Kahn, L. S. Thomashow, and L. P. Mallavia. 1983. Isolation and characterization of a plasmid from phase I *Coxiella burnetii*. *Infect. Immun.* **41**:488–493.
56. Samuel, J. E., M. E. Frazier, and L. P. Mallavia. 1985. Correlation of plasmid type and disease caused by *Coxiella burnetii*. *Infect. Immun.* **49**:775–779.
57. Seshadri, R., et al. 2003. Complete genome sequence of the Q-fever pathogen *Coxiella burnetii*. *Proc. Natl. Acad. Sci. U. S. A.* **100**:5455–5460.
58. Shannon, J. G., and R. A. Heinzen. 2008. Infection of human monocyte-derived macrophages with *Coxiella burnetii*. *Methods Mol. Biol.* **431**:189–200.
59. Shannon, J. G., D. Howe, and R. A. Heinzen. 2005. Virulent *Coxiella burnetii* does not activate human dendritic cells: role of lipopolysaccharide as a shielding molecule. *Proc. Natl. Acad. Sci. U. S. A.* **102**:8722–8727.
60. Sory, M. P., and G. R. Cornelis. 1994. Translocation of a hybrid YopE-adenylate cyclase from *Yersinia enterocolitica* into HeLa cells. *Mol. Microbiol.* **14**:583–594.
61. Swanson, M. S. 2006. Autophagy: eating for good health. *J. Immunol.* **177**:4945–4951.
62. Valkova, D., and J. Kazar. 1995. A new plasmid (QpDV) common to *Coxiella burnetii* isolates associated with acute and chronic Q fever. *FEMS Microbiol. Lett.* **125**:275–280.
63. Vogel, J. P. 2004. Turning a tiger into a house cat: using *Legionella pneumophila* to study *Coxiella burnetii*. *Trends Microbiol.* **12**:103–105.
64. Voth, D. E., and R. A. Heinzen. 2009. *Coxiella* type IV secretion and cellular microbiology. *Curr. Opin. Microbiol.* **12**:74–80.
65. Voth, D. E., and R. A. Heinzen. 2007. Lounging in a lysosome: the intracellular lifestyle of *Coxiella burnetii*. *Cell. Microbiol.* **9**:829–840.
66. Voth, D. E., and R. A. Heinzen. 2009. Sustained activation of Akt and Erk1/2 is required for *Coxiella burnetii* antiapoptotic activity. *Infect. Immun.* **77**:205–213.
67. Voth, D. E., et al. 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.
68. Voth, D. E., D. Howe, and R. A. Heinzen. 2007. *Coxiella burnetii* inhibits apoptosis in human THP-1 cells and monkey primary alveolar macrophages. *Infect. Immun.* **75**:4263–4271.
69. Yildiz, A., M. Tomishige, R. D. Vale, and P. R. Selvin. 2004. Kinesin walks hand-over-hand. *Science* **303**:676–678.