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Coxiella Type IV Secretion and Cellular Microbiology

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Summary

Coxiella burnetii is a wide spread zoonotic bacterial pathogen that causes human Q fever. *In vivo*, *Coxiella* displays a tropism for mononuclear phagocytes where it participates in biogenesis of a lysosome-like replication compartment to conduct its obligate intracellular lifestyle. *Coxiella* actively regulates multiple events during infection, presumably via proteins with effector functions that are delivered to the host cytosol by a Dot/Icm type IV secretion system. Because the organism is currently refractory to genetic manipulation, *Coxiella* Dot/Icm substrates have been identified using bioinformatics and *Legionella pneumophila* as a surrogate type IV delivery system. Functional characterization of the biological activity of these effector proteins will dramatically aid our ability to model *Coxiella*-host cell interactions.

Introduction

Coxiella burnetii is a Gram-negative, obligate intracellular bacterial pathogen and the cause of Q fever in humans. The organism has a large and diverse zoonotic reservoir that includes birds, fish, and a variety of wild and domestic mammals [1]. In humans, *Coxiella* infection primarily occurs by inhalation of contaminated aerosols generated by domestic livestock operations. Symptomatic Q fever normally presents as an acute flu-like illness characterized by prolonged high fever, headache, and malaise [2]. However, approximately 50% of infections result in seroconversion without overt clinical signs/symptoms of disease. Rare chronic disease, usually manifested as endocarditis, can occur and is generally associated with patients that are immunocompromised and/or have heart valve defects [2]. Because of *Coxiella*'s aerosol route of transmission, pronounced environmental stability, and an infectious dose approaching one organism, the pathogen is classified as a United States Centers for Disease Control and Prevention category B select agent. To date, lipopolysaccharide is the only defined *Coxiella* virulence determinant, and this molecule is used to distinguish between virulent and avirulent organisms [3].

During natural infection, *Coxiella* appears to target mononuclear phagocytes. Intracellularly, the pathogen directs biogenesis of a unique lysosome-like replication compartment termed the parasitophorous vacuole (PV) [4]. Here, the organism replicates slowly (generation time ~ 11 h), taking approximately six days to reach the stationary phase of its growth cycle [5]. Similar to other Gram-negative bacterial pathogens, *Coxiella* possesses a type IV secretion system (T4SS) predicted to deliver proteins with effector functions into the host cytosol that mediate

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infection events. Unfortunately, genetic manipulation of *Coxiella* to directly identify T4SS substrates is currently not possible. Nonetheless, this problem has been circumvented by using bioinformatics to predict candidate *Coxiella* effector proteins and *Legionella pneumophila* as a surrogate host to screen these candidates for T4SS-dependent secretion. Here, we highlight host processes actively modulated by *Coxiella* during intracellular growth and discuss recent progress in identifying secreted effector proteins.

Coxiella cellular microbiology

Following uptake by a host cell, *Coxiella* does not subvert the canonical endolysosomal pathway but instead directs formation of a PV that is remarkably similar to a secondary lysosome [4]. The nascent *Coxiella*-containing phagosome is a tight-fitting compartment that engages the default endocytic cascade. The early PV (< 6 hours post-infection) recruits the small GTPases Rab5 and, to a lesser extent, Rab7, which are prototypic markers of early and late endosomes, respectively, that regulate membrane trafficking. The early *Coxiella* PV membrane also decorates with the autophagosome markers microtubule-associated protein light-chain 3 (LC3) and Rab24 [4]. At ~ 2 days post-infection, and coincident with entry of *Coxiella* into its exponential growth phase, the maturing PV dramatically expands to often occupy the majority of the host cell cytoplasm. At this point, the PV promiscuously fuses with endolysosomal vesicles and maintains interactions with the autophagic pathway. The vacuole contains the lysosomal enzymes acid phosphatase, 5′-nucleotidase, and cathepsin D and has a moderately acidic pH (~ pH 5) [4]. Moreover, the PV membrane loses Rab5 and decorates with the vacuolar H^+ ATPase, Rab7, lysosome-associated membrane proteins-1, -2, and -3, flotillin 1 and 2, LC3, and Rab24 [4]. The presence of the lipid raft proteins flotillin 1 and 2 correlates with a PV membrane rich in cholesterol, and inhibition of cholesterol biosynthesis or uptake dramatically antagonizes PV formation [6].

PV biogenesis and maintenance require *de novo Coxiella* protein synthesis. For example, in infected cells treated with chloramphenicol to inhibit bacterial protein synthesis, PV show diminished interactions with autophagosomes and lysosomes, in addition to losing their large and spacious character [•7,8]. *Coxiella* proteins also actively modulate eukaryotic pro-survival signaling pathways, presumably as a strategy to maintain the viability of host cells for the duration of the pathogen’s lengthy infectious cycle. Recently, we demonstrated that *Coxiella* potently inhibits death in macrophages exposed to inducers of the extrinsic and intrinsic pathways of apoptosis [•9]. Infected cells show decreased caspase activation and induction of a pro-survival transcriptional response [•9]. Similar anti-apoptotic responses, in addition to decreased release of cytochrome *c*, are observed in non-phagocytic HeLa and CHO cells [•10]. Moreover, *Coxiella*-mediated activation of the pro-survival host kinases Akt and Erk1/2 is required for full protection from apoptosis [•11]. Major events that occur during *Coxiella* infection of host cells are depicted in Figure 1, with an emphasis on processes requiring pathogen protein synthesis.

Temporal aspects of *Coxiella* type IV secretion

It is logical to assume that the *Coxiella* T4SS coordinately secretes sets of effector proteins that co-opt cellular functions associated with specific stages of infection. However, data is lacking on the temporal expression of individual T4SS substrates. *Coxiella* is metabolically quiescent in the extracellular environment with significant metabolic activity only observed at a pH approximating that of the PV lumen (~ pH 5) [12]. Thus, it is unclear how T4SS effector proteins might mediate very early infection events such as cellular uptake and interactions between the nascent phagosome and autophagosomes, an event that is observed as early as 5 min post-infection [•7]. One possibility is that the T4SS is “preloaded” and translocates effector proteins upon attachment to promote phagosome formation in the absence of significant

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pathogen metabolic activity. A similar scenario is proposed for *Chlamydia* whereby the organism's type III secretion system (T3SS) effector protein TARP mediates host cell entry by metabolically-inert elementary bodies [13]. Once internalized, data suggest that *Coxiella* Dot/Icm activity is constitutive. First, the PV appears promiscuously fusogenic with endolysosomal and autophagosomal vesicles throughout the course of infection [•7,8]. Second, continual disarmament of host apoptotic signaling likely occurs as minimal cytopathic effects are observed in cells infected for days to weeks [4]. Finally, *de novo* expression of *dotA* is detected as early as 8 h post-infection and expression is sustained into the stationary phase of *Coxiella*'s growth cycle (6-8 days post-infection) [5]. *Coxiella* Dot/Icm T4SS activity does not appear to function in pathogen host cell egress as there is no concerted lytic event associated with infection.

The Dot/Icm T4SS

Coxiella's T4SS appears strikingly similar to the Dot/Icm T4SS of *L. pneumophila*, which is a type IVB delivery apparatus as opposed to the type IVA machinery typified by the Vir system of *Agrobacterium tumefaciens* [14]. *Coxiella* encodes 23 of the 26 Dot/Icm proteins described for *L. pneumophila* but lacks homologs of the chaperone protein IcmR and the inner membrane proteins DotJ and DotV [14]. *Coxiella dotB*, *icmS*, *icmW*, and *icmT* complement the corresponding mutants in *L. pneumophila* indicating functional overlap between these two T4SSs [15,16]. Conversely, *Coxiella icmX*, *icmQ*, *dotM*, *dotL*, *dotN*, and *dotO* do not complement [15], suggesting pathogen-specific Dot/Icm interactions occur. While the lack of complementation by *icmQ* is attributed to the absence of its binding partner IcmR in *Coxiella* [16], a recent study showed that *Coxiella* produces a protein that is functionally similar to IcmR that binds IcmQ *in vitro* [17]. An important observation is that the *Coxiella* chaperones IcmS and IcmW are functional in *L. pneumophila*, suggesting conservation of a Dot/Icm substrate recognition system. Currently, over 70 *L. pneumophila* Dot/Icm effector proteins have been identified that subvert multiple host cell processes to generate a specialized replication vacuole [18]. However, with a few possible exceptions [19], *Coxiella* does not encode homologs of these proteins. This observation is consistent with the biologically distinct vacuolar compartments sheltering these pathogens [20].

Coxiella proteins with eukaryotic-like motifs/domains

Genome-wide genetic screens have revealed many *L. pneumophila* T4SS effector proteins [21,22]. Because *Coxiella* is currently genetically intractable, effector protein discovery in this pathogen requires indirect approaches. One approach is to bioinformatically screen the pathogen proteome for proteins with characteristics of known T4SS effector proteins. A common theme of these proteins is the presence of eukaryotic-like motifs/domains that functionally mimic or inhibit the activity of host cell proteins [23]. Indeed, de Felipe *et al*. recently demonstrated that *L. pneumophila* encodes numerous proteins with eukaryotic-like domains of which many are T4SS substrates [24]. *Coxiella* also encodes multiple proteins with eukaryotic-like features (Table 1). Examples include proteins with ankyrin repeat domains (Anks), tetratricopeptide repeats (TPR), coiled coil domains (CCD), leucine-rich repeats (LRR), GTPase domains, ubiquitination-related motifs, and multiple kinases and phosphatases. Eukaryotic-like genes in bacteria are thought to have arisen by interdomain horizontal gene transfer from a eukaryotic cell [24].

The predicted function of eukaryotic-like motifs/domains found in *Coxiella* proteins generally falls into one of two major categories. The Ank, TPR, CCD, and LRR domains represent the first category and routinely mediate protein-protein interactions in eukaryotic systems [25-28]. As such, these proteins are predicted to directly engage a host protein(s). A separate region is usually found in these proteins that performs a distinct function (*e. g.*, enzymatic

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activity). The second category consists of proteins with F-box, GTPase, kinase, and phosphatase homology that normally regulate signal transduction pathways. For example, F-box domains are well-characterized components of ubiquitination processes that either target eukaryotic proteins for proteasome-dependent degradation or direct re-targeting of proteins to specific subcellular sites [29]. Recently, the *L. pneumophila* Dot/Icm substrate LubX was shown to contain two U-box domains, which are similar to F-boxes and involved in ubiquitination processes [19]. LubX binds Cdc2-like kinase 1, a host protein that influences *L. pneumophila* growth in macrophages. As mentioned above, *Coxiella* actively regulates multiple host signaling cascades, making F-box domain-containing proteins good candidates for Dot/Icm substrates.

Coxiella Dot/Icm substrate identification using *L. pneumophila*

Phylogenetic relatedness to *Coxiella* [30] and production of a functionally similar T4SS [15, 16] suggested *L. pneumophila* could be applied as a surrogate host to define *Coxiella* T4SS substrates. Indeed, successful identification of *Coxiella* Dot/Icm effector proteins has recently been achieved using *L. pneumophila* in conjunction with the adenylate cyclase (CyaA) enzymatic reporter assay [••31]. Originally described in the characterization of *Yersinia pseudotuberculosis* Yop proteins [32], the assay involves secretion of candidate effector proteins fused to *Bordetella pertussis* calmodulin-dependent CyaA, which is only activated in the eukaryotic cytosol. *L. pneumophila* is transformed with constructs encoding candidate *Coxiella* T4SS substrates fused to CyaA and transformants used to infect mammalian cells. Following translocation of a *Coxiella* fusion protein into the host cytosol, CyaA binds calmodulin, thereby activating the enzyme, which converts ATP to cAMP. The supraphysiologic levels of cAMP that result from CyaA activation provide a sensitive readout for secretion. Using this approach, Pan *et al*. identified four *Coxiella* Anks (AnkA, B, F, and G) that are Dot/Icm substrates [••31]. Similar work from our laboratory has shown that additional Anks from disparate *Coxiella* isolates are translocated into the host cytosol in a Dot/Icm-dependent fashion. Moreover, we have demonstrated that the signal for Dot/Icm-mediated translocation likely resides in the C-terminus of effector proteins and that some, but not all, Anks require the chaperone IcmS for secretion (D. E. Voth and R. A. Heinzen, unpublished observations).

Genomic distribution of *Coxiella* T4SS genes

Genes encoding T3SSs and their associated effector molecules are often contained on pathogenicity islands (PIs) that have been transferred between pathogenic bacteria [33,34]. In addition, a *Helicobacter pylori* PI harbors a Vir-like T4SS and accompanying CagA effector protein [35]. An insertion sequence-flanked PI was proposed by Seshadri *et al.* [36] in their description of the *Coxiella* Nine Mile reference isolate genome that encompasses the region from CBU1186 to CBU1218. While the Dot/Icm-encoding region is located elsewhere (from CBU1622 to CBU1651), two candidate effector proteins with eukaryotic-like features are found in the putative PI: CBU1206, which encodes a sterol reductase, and CBU1213, which encodes an ankyrin repeat domain-containing protein (AnkI) (Table 1). However, this region is poorly conserved in other *Coxiella* isolates and has a G+C content (43.0%) nearly identical to the chromosome, suggesting this DNA was not acquired by horizontal gene transfer [37]. Moreover, of known [••31] and putative *Coxiella* effector-coding genes, only *ankL* resides close to the Dot/Icm locus with the remainder randomly dispersed throughout the chromosome [37].

Heterologous expression to obtain effector protein function clues

Lacking a system to knockout individual genes encoding *Coxiella* T4SS substrates, indirect approaches are needed to help define effector protein function. One approach employs ectopic

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expression in mammalian cells of Dot/Icm substrates fused to fluorescent proteins. The resultant trafficking behavior of a given fusion protein provides clues regarding potential effector activity, *i.e.*, proteins that localize to a particular host organelle may modulate host processes specific to this site [••31,••38]. Ectopically expressed *Coxiella* Dot/Icm substrates localize to a variety of subcellular locations including the PV membrane and host organelles. For example, AnkO (CBUD1108) and AnkJ (CBUD1338) fused to mCherry traffic to the PV membrane and mitochondria, respectively, in infected HeLa cells (D. E. Voth *et al.*, unpublished observations). PV localization of AnkO-mCherry suggests the protein may medicate vesicular fusion events required for vacuole biogenesis and maintenance. In support of this idea, recent studies show that *L. pneumophila* T4SS effector proteins traffic to the pathogen-containing compartment where they mediate acquisition of endoplasmic reticulum membrane [•39]. Localization of AnkJ-mCherry to host mitochondria suggests this protein may subvert apoptotic signaling. Direct mitochondrial trafficking has been demonstrated for EspF, a T3SS substrate of enteropathogenic *Escherichia coli* that exerts potent pro-apoptotic activity [40]. Moreover, the *L. pneumophila* T4SS substrate SidF interacts with mitochondrial-targeted BNIP3 and Bcl-rambo to neutralize their pro-apoptotic activities [41].

Dot/Icm-independent *Coxiella* secretion

Coxiella appears capable of type I secretion. The pathogen lacks a T3SS, autotransporter proteins (type V secretion), and a newly described Gram-negative type VI secretion system. While *Coxiella* also lacks prototypical proteins required for type II secretion, the organism does contain a number of Pil genes that are involved in type IV pilus biogenesis and evolutionarily related to type II secretion genes [37,42]. *Coxiella* encodes nine structural Pil proteins but lacks the critical ATPase PilT, which regulates pilus assembly [43]. However, a recent report indicates that *Francisella novicida* uses an incomplete set of type IV pilus proteins to secrete proteins beyond the periplasm that contain Sec-dependent signal peptides [•44]. These proteins, along with signal sequence-containing proteins delivered by the type II secretion system of *L. pneumophila*, are frequently enzymes with eukaryotic features [•44,•45]. *Coxiella* encodes abundant enzymes with predicted signal sequences (*e.g.*, phospholipase D, CBU0968; acid phosphatase, CBU0335; and D-alanine-D-alanine carboxypeptidase, CBU1261) that could presumably degrade macromolecules into simpler substrates, detoxify the PV environment, and/or modulate infection events. Indeed, a secreted signal sequence-containing *Coxiella* acid phosphatase is thought to detoxify the PV lumen by inhibiting superoxide production by professional phagocytes [46]. Modulation of infection events would likely require an additional mechanism for translocation beyond the PV lumen. This behavior would not be unprecedented as CPAF, a signal peptide-containing protease of *Chlamydia*, is delivered from the chlamydial replication vacuole to the cytosol where it degrades host cell proteins [47,48]. *Coxiella* also encodes a signal sequence-containing protein termed EnhC (**enh**anced entry protein **C)** with 21 tandemly-arranged eukaryotic-like TPR repeats of the Sel-1 variety [26]. Interestingly, the sole bacterial homolog of this protein is present in *L. pneumophila* where it acts in the early stages of pathogen uptake and/or replication vacuole biogenesis [49,50]. Eukaryotic TPR proteins are thought to function as adaptor proteins in assembling signaling complexes [26]; thus, surface-associated or secreted *Coxiella* EnhC might function in a similar manner.

Conclusions

Although *Coxiella* was defined as the etiologic agent of Q fever over six decades ago, our understanding of virulence factors used by the pathogen to cause disease remains rudimentary. Host processes such as apoptotic signaling have been identified that are modulated by *Coxiella* during infection. However, the specific *Coxiella* proteins involved in host cell manipulation are unknown. Nonetheless, recent reports identifying *Coxiella* Dot/Icm

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substrates have provided an intriguing list of potential effector proteins whose functional characterization will dramatically aid our ability to model *Coxiella*-host cell interactions and the pathophysiology of Q fever.

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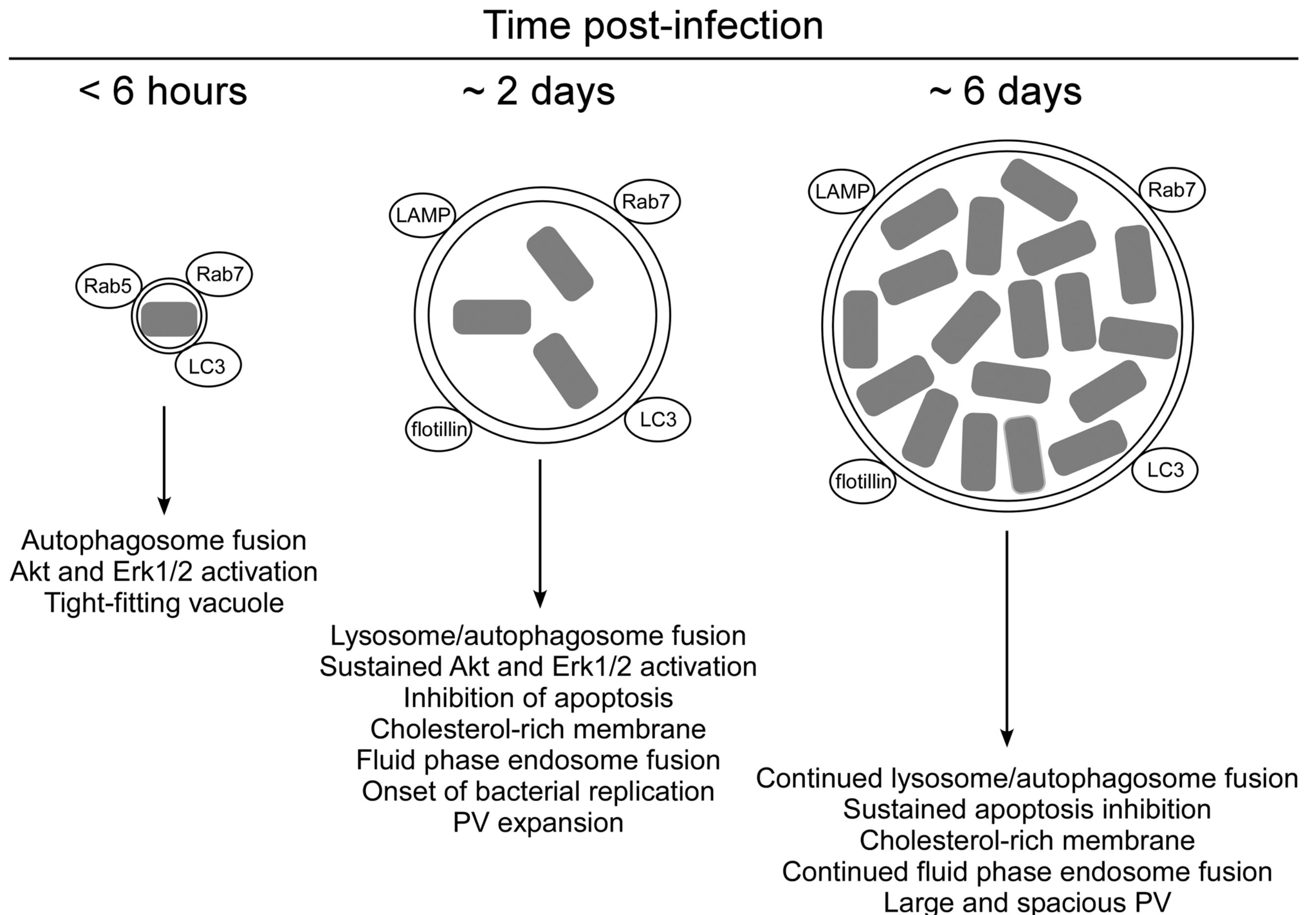

Figure 1.

Coxiella modulation of PV biogenesis and host cell signaling during intracellular growth. The nascent, tight-fitting *Coxiella* PV (< 6 hours post-infection) interacts with autophagosomes and early endosomes as evidenced by decoration with the markers LC3 and Rab5, respectively. Interaction with autophagic vesicles requires *Coxiella* protein synthesis, potentially in the form of Dot/Icm-secreted effectors. *Coxiella* proteins are also produced during this time that activate the pro-survival kinases Akt and Erk1/2. At ~ 2 days post-infection, and coincident with the onset of *Coxiella* replication, the maturing PV becomes large and spacious and usually harbors a low number of organisms. At this point, the PV is clearly acidic (~ pH 5), contains active acid hydrolases, retains LC3 and Rab7, and decorates with the lysosome-associated membrane proteins-1, -2, and -3 (LAMP). The PV membrane is also cholesterol-rich and contains lipid raft proteins (flotillin). The PV continually fuses with fluid phase endosomes as shown by trafficking of fluid phase markers to the vacuole lumen. At 2 days post-infection, *Coxiella*-infected cells are protected against inducers of apoptosis, a process that again requires pathogen protein synthesis and sustained phosphorylation of Akt and Erk1/2. The late PV (~ 6 days post-infection) sustains interactions with the autophagic and endolysosomal pathways and is filled with organisms entering the stationary phase of growth. Protein synthesis is needed throughout infection to maintain the spacious architecture of the PV and this requirement is predicted to involve Dot/Icm effector protein function.

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Table 1

Coxiella genes encoding proteins with eukaryotic-like motifs/domains[a]

Motif/domain	Predicted role	Gene aliases[b]
Ankyrin repeat	Protein-protein interactions	CBUK1330 (*ankN*)[c], CBUD0382 (*ankL*)[d], CBUD0829 (*ankG*)[d], CBUD1019 (*ankH*)[d], CBUD1028 (*ankM*)[d], CBUD1108 (*ankO*)[c], CBUD1298 (*ankI*)[d], CBUD1338 (*ankJ*)[d], CBUD1380 (*ankK*), CBUD1627 (*ankF*), CBUD1724 (*ankD*)[d], CBUD1894 (*ankC*), CBUD1960 (*ankB*)[d], CBUD2034 (*ankA*)[d], CBUD2035 (*ankP*)[d]
Tetratricopeptide repeat	Protein-protein interactions	CBU0295[d], CBU0530, CBU0547, CBU0870, CBU1457[d], CBUD0785[c], CBUD0795[c], CBUD1234[d], CBUD1257[d], CBUD1452[d], CBUDA0024[c]
Coiled coil	Protein-protein interactions	many (*e. g.*, CBU0547, CBU1366)
Leucine-rich repeat	Protein-protein interactions	CBUD0886[d]
F-box	Ubiquitin-related processes	CBU1217[d], CBUA0014[c], CBUK0684[d], CBUD1724[d], CBUD1107[e]
Regulator of chromatin condensation	Nuclear GTPase activity	CBU1217[d]
Protein kinase	Protein phosphorylation	CBU0175, CBUK1237[d], CBUD1261[d],
Phosphatase	Protein dephosphorylation	CBU0488, CBU1489, CBU1730, CBU1987, CBUA0032[c], CBUK0381[d], CBUK0919[d], CBUD1472[d], CBUD1744[d]
Sterol reductase	Cholesterol metabolism	CBU1158[d], CBU1206
Phospholipase	Membrane lipid interactions	CBUK0752[d], CBUK0919[d], CBUDA0012[c]
Stomatin/prohibitin homolog	Lipid raft-associated protein	CBU1482
Cardiolipin synthetase	Membrane stability	CBU0096
Thyroglobulin type-1 repeat	Hormone biosynthesis	CBU0898
SNARE-associated Golgi protein	Vesicular fusion	CBU0519

[a]this list was derived by bioinformatic analysis of the sequenced genomes of the *Coxiella* Nine Mile, G, K, and Dugway isolates with the GenBank accession numbers AE016828, CP001019, CP001020, and CP000733, respectively. Only one respective gene alias is listed if orthologous genes are present in more than one *Coxiella* isolate.

[b]*Coxiella* isolate gene abbreviations: CBU, Nine Mile; CBUK, K; CBUD, Dugway.

[c]gene not present in other isolates

[d]gene not full-length in one or more isolates

[e]frameshifted gene only present in the Dugway isolate