REVIEW

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Future MICROBIOLOGY

Right on Q: genetics begin to unravel *Coxiella burnetii* host cell interactions

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Invasion of macrophages and replication within an acidic and degradative phagolysosome-like vacuole are essential for disease pathogenesis by *Coxiella burnetii*, the bacterial agent of human Q fever. Previous experimental constraints imposed by the obligate intracellular nature of *Coxiella* limited knowledge of pathogen strategies that promote infection. Fortunately, new genetic tools facilitated by axenic culture now allow allelic exchange and transposon mutagenesis approaches for virulence gene discovery. Phenotypic screens have illuminated the critical importance of *Coxiella*'s type 4B secretion system in host cell subversion and discovered genes encoding translocated effector proteins that manipulate critical infection events. Here, we highlight the cellular microbiology and genetics of *Coxiella* and how recent technical advances now make *Coxiella* a model organism to study macrophage parasitism.

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• apoptosis • autophagy • *Coxiella burnetii* • effector protein • host cell invasion • macrophages • mutagenesis • phagolysosome • type 4B secretion • vacuole remodeling

Coxiella: a wide-ranging zoonotic pathogen

The expansive geographical distribution and host range of *Coxiella burnetii*, a Gram-negative intracellular bacterium, was noted soon after its discovery as the causative agent of human Q fever. Acute Q fever typically presents as an incapacitating but self-limited flu-like illness with other common clinical presentations being atypical pneumonia and/or hepatitis. Rare chronic infections also occur that normally manifest as endocarditis or vascular disease, with increased incidence associated with valvular heart disease, vascular pathology and/or a compromised immune system [1,2]. Evidence for infection exists in a multitude of vertebrate and invertebrate hosts [3]. Infected domestic livestock, especially goats, sheep and dairy cattle are responsible for the vast majority of human Q fever cases. These zoonotic reservoirs shed *Coxiella* into the environment that can fuel human outbreaks [4]. Especially relevant to pathogen transmission are the vast numbers of bacteria present in birth products. Desiccation of placental material promotes aerosol transmission of highly infectious, environmentally stable *Coxiella* that can infect individuals miles away from a single point source [5]. Exacerbating pathogen dissemination are abortion waves in goats and sheep caused by *Coxiella* infection [6]. Detection of the *Coxiella* using PCR amplification of chromosomal *IS1111* repetitive elements has revealed the extent to which zoonotic reservoirs have dispersed *Coxiella* into the USA, with 23.8% of over 1600 random environmental samples containing *Coxiella* DNA [7]. Two caveats of this study are that *IS1111*-like sequences are widespread in *Coxiella*-like endosymbionts [8],

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and that PCR does not distinguish between live and dead bacteria. However, mice were infected with material from several field samples, indicating environmental samples can contain viable *C. burnetii* [7]. Q fever outbreaks can have substantial financial impact as illustrated by the 2007–2011 Netherlands outbreak (>4000 cases) where human disease burden and infection control measures were estimated to cost 307 million Euro [9].

Although *Coxiella* is frequently referenced as a close relative of the γ-proteobacterium *Legionella pneumophila* [10], the two bacteria actually have a distant relationship [11]. A new study shows the evolutionary progenitor of *C. burnetii* is in fact an ancient monophyletic group of *Coxiella*-like endosymbionts of ticks [12]. Genetic diversity among *C. burnetii* strains suggests it recently emerged from this group as a parasite of vertebrate cells, possibly through acquisition of virulence genes from a coinfecting pathogen [12]. A rooted phylogeny of sequenced *C. burnetii* strains using SNPs implies the ancestor of *Coxiella* was more likely to cause persistent or chronic infection [11]. Further pathoadaptive evolution within the genus, driven by expansion of insertion sequence elements, accumulation of pseudogenes and extensive genomic rearrangements, led to more acutely virulent strains [10,11,13]. The observation that the naturally attenuated Dugway 5J108-111 strain has the largest genome with the fewest pseudogenes suggests this strain lineage is at a more primitive stage of pathoadaptation [10,11]. It is interesting to speculate that novel gene content of the Dugway strain includes antivirulence genes enabling persistent, subclinical infection of animal hosts [11,14].

Animal models of acute Q fever clearly demonstrate a relationship between genome content and pathotype [15,16]. SNP-based typing has revealed 35 unique genotypes of *Coxiella* [11,17,18]. Some genotypes have defined associations with animal reservoirs and disease in humans. For example, genotype 8 has a goat reservoir and is associated with human chronic disease [18]. Researchers hypothesize that this genotype is more likely to cause a subclinical infection that goes untreated, thereby persisting and causing chronic disease such as endocarditis [18]. Genotype 17 is associated with severe, acute Q fever pneumonia in French Guiana [19]. In the USA, millions of dairy cows appear exclusively infected with genotype 20 strains. However, despite tremendous shedding of these strains into the environment, they have not been isolated from human Q fever cases and thus may be attenuated in causing human disease [20].

Pressure exerted by serial laboratory passage also drives *Coxiella* strain variation. *Coxiella* isolated from natural sources and infections produces full-length lipopolysaccharide (LPS) that is required for virulence. During serial passage in immunoincompetent hosts, such as embryonated hen's eggs and tissue culture cells, or synthetic media [21], variants emerge within the population that produce truncated LPS. Extensive passage (~90 passages) culminates in avirulent organisms with a severely truncated, deep-rough LPS consisting of lipid A and core sugars, but no *O*-antigen [22–24]. This change in LPS structure corresponds with a change in reactivity to postvaccination sera referred to as phase variation. Organisms displaying full-length and severely truncated LPS are serologically in Phase I and Phase II, respectively. *O*-antigen is the primary surface antigen recognized by Phase I antiserum [25]. Some, but not all, Phase II strains contain a chromosomal deletion eliminating roughly 20 genes associated with sugar metabolism [26–28]. Moreover, deep-rough LPS of Phase II strains can be antigenically distinct [29]. Collectively, these observations suggest multiple mutational pathways can lead to phase transition. Nonetheless, a plaque-cloned Phase II variant (NMII) of the Nine Mile Phase I (NMI) strain containing a large chromosomal deletion is widely used by *Coxiella* researchers, as the strain is considered nonrevertable to full virulence and thus suitable for use at biosafety level-2 [23,30]. All other strains are considered biosafety level-3 organisms. Importantly, the growth kinetics and intracellular trafficking of NMI and NMII in human macrophages appear indistinguishable, making infection by NMII a relevant model for *ex vivo* pathogen–host interaction studies [31,32]. NMII also has the advantage of being roughly 500-fold more infectious on a per particle basis for nonprofessional phagocytic cells, such as Vero epithelial cells, aiding cell biology studies [33]. Increased surface hydrophobicity and protein exposure due to missing *O*-antigen are thought to facilitate NMII–host plasma membrane interactions [34,35]. Greater exposure of lipoprotein, a potent TLR-2 ligand, may also explain the unusual growth restriction of NMII, but not NMI, by primary mouse macrophages [36,37]. Ligation of TLR-2 triggers synthesis of the

proinflammatory cytokine tumor necrosis factor, which in turn elicits production of the antibacterial effector nitric oxide that inhibits NMII replication [37–39]. Although potentiated innate immune signaling also occurs during NMII interactions *ex vivo* with primary human macrophages and dendritic cells [32,40], no growth defects relative to NMI are observed. However, the resulting robust immune response elicited by NMII during primary infection of mammals is thought to render the strain avirulent [37,40].

Making a home in host cells

As an intracellular bacterial pathogen, *Coxiella* must be internalized by host cells to cause disease. The primary target of *Coxiella* during natural infection is alveolar macrophages [32,41–43]. The organism can subsequently disseminate to colonize and replicate in resident macrophages of different tissues and organs [44]. Trophoblasts of infected females can also become heavily infected with adipoctyes potentially serving as a reservoir of persistent *Coxiella* [45,46]. *Ex vivo,* multiple cell lines have been used to investigate *Coxiella* infections including primary macrophages, macrophage-like cells, epithelial cells and fibroblasts [47]. Macrophages and fibroblasts internalize live or dead *Coxiella* with equal efficiency, suggesting *Coxiella* plays a passive role in cellular uptake by both phagocytic and nonphagocytic cells [48,49]. Because protease treatment of *Coxiella* inhibits uptake, surface proteins likely serve as invasins [48]. Indeed, as described below, OmpA has recently been identified as the first *Coxiella* invasin inducing cellular uptake, specifically by nonprofessional phagocytes [50]. Two monocyte surface receptors are known for *Coxiella*: $\alpha_V\beta_3$ integrin, engaged by NMI and NMII, and complement receptor 3, co-engaged by NMII [51]. Membrane protrusions induced during attachment by NMI are thought to restrict interactions with CR3, suggesting LPS plays a role in *Coxiella* infection of human phagocytes [51,52]. Consistent with the induction of membrane projections, *Coxiella* internalization requires F (filamentous)-actin rearrangements [48,51–53]. The signaling cascade associated with *Coxiella* uptake by phagocytic and nonphagocytic cells involves Rho family GTPases. RhoA appears particularly important considering the activity of the downstream effectors ROCK, a protein kinase and mDia1, an actin nucleating factor, are required for optimal internalization [53]. Src-family kinases [54] and the F-actin binding protein cortactin [55] also play roles in internalization of *Coxiella* by phagocytic and nonphagocytic cells, respectively.

Following uptake, *Coxiella* is confined to a nascent vacuole that transits through the default endocytic maturation pathway from early endosome to phagolysosome [31,56,57]. Full maturation of the *Coxiella*-containing vacuole (CCV) to fusion with lysosomes is indicated by the presence of active cathepsins [31,56–60]. *Coxiella* is uniquely adapted among intravacuolar bacterial pathogens to replicate in an acidic phagolysosome-like compartment that retains significant hydrolytic activity [31,57]. Consequently, the pathogen does not deploy the sophisticated mechanisms used by other pathogens to avoid death by lysosome [61,62]. In fact, *Coxiella* requires the acidic conditions of the vacuole for metabolic activation and production of proteins that manipulate infection events, such as vacuole expansion and inhibition of apoptosis [63–65]. Roughly coincident with the onset of *Coxiella* replication (~1–2 days postinfection), the CCV becomes highly fusogenic, a property that generally produces a single vacuole that occupies the majority of the host cell cytoplasm [65]. Studies have recently highlighted CCV engagement with several cellular vesicular trafficking pathways predicted to provide membrane and/or nutrients to the *Coxiella* replicative niche.

Much attention has been devoted to defining the role of autophagy in *Coxiella* infection as the pathway can exert both pro- and antimicrobial effects [66]. Several lines of evidence indicate the CCV fuses with autophagosomes, and that this interaction benefits *Coxiella*. The CCV clearly decorates with the autophagy markers LC3 [56,57,59,60,67–69], Beclin 1 [59,70], Rab24 [70] and p62 [67]. Autophagosome interactions with the CCV occur within a few hours of infection and require *Coxiella* protein synthesis and an active type 4B secretion system (T4BSS) [56,67]. Although levels of lipidated LC3 (LC3-II) increase during *Coxiella* infection [56,60,67], the overall rate of autophagic flux does not increase as evidenced by the absence of p62 turnover [60,67]. As the CCV expands, endogenous LC3 continues to accumulate in the vacuole lumen, but not the limiting membrane, suggesting constitutive fusion between the CCV and autophagosomes delivering LC3-labeled cargo [60,71]. CCV fusion with autophagosomes is presumed to assist *Coxiella* by providing membrane for vacuole expansion and nutrients for

pathogen growth [67]. However, there are conflicting data on the overall benefit of autophagy to *Coxiella* infection. Inhibition of autophagy with 3-methyladenine reduces vacuole expansion and pathogen growth whereas the opposite occurs with induction of autophagy by host cell starvation or treatment with the mTOR inhibitor, rapamycin [68,70]. Others have shown siRNA knockdown of essential autophagy proteins results in multiple CCV without negatively impacting *Coxiella* replication, and that a *Coxiella* mutant in the effector gene *cbu0021* (*cvpB, cig2*) (discussed in the 'The hard part: effector function' section) replicates normally in an LC3-negative CCV [60,71].

Interactions with secretory and endocytic pathways also contribute to CCV development. The vacuole decorates with Rab1b, a small GTPase that regulates transport between the ER and Golgi apparatus [69]. Expression of a dominant-negative version of Rab1 or siRNA knockdown inhibits CCV biogenesis. Similar effects are observed if the secretory pathway is disrupted by brefeldin A treatment or expression of a dominant-negative version of Sar1. Separate studies have shown disruption of Rab5 or Rab7, GTPases that direct endocytic maturation, negatively impacts CCV formation [56,68,70,72]. Clathrin-mediated vesicular trafficking is an important component of CCV endosome recruitment as depletion of clathrin or the clathrin-adaptor protein AP2, inhibits vacuole expansion and *Coxiella* replication [73]. Through an unknown mechanism, the major lysosmal membrane proteins LAMP-1 and LAMP-2 also participate in CCV formation by increasing the rate of endosome fusion [57].

A genome-wide siRNA screen using a HeLa cell infection model identified several components of the retromer, a complex that retrieves receptors from the endolysosomal system, that contribute to CCV biogenesis and *Coxiella* replication [72]. The same study identified soluble NSF attachment receptor (SNARE) syntaxin-17 as a key host factor mediating homotypic fusion of CCVs. Multiple nascent CCVs that occur in cells infected with more than one bacterium typically fuse to form a single large and spacious vacuole [65]. The multivacuolar phenotypes observed after depletion of syntaxin-17 or ATG5/12 are similar, suggesting constitutive fusion between the CCV and autophagosomes maintains the vacuole in fusogenic, autophagolysosomal state [60,72]. Indeed, syntaxin-17 has recently been described as an autophagosome-associated SNARE required for proper autophagosome–lysosome fusion [74]. The endocytic SNAREs VAMP3, 7 and 8 also localize to the CCV [75]. Optimal recruitment of VAMP7 requires *Coxiella* protein synthesis and disruption of VAMP7 activity disrupts CCV hetero- and homo-typic fusion events and *Coxiella* replication [75].

The CCV is surrounded by F-actin puncta that potentially provide structural integrity and/or tracks for vesicular fusion events [58]. Chemical disruption of F-actin results in aberrantly small CCVs that fully mature through the endocytic pathway but cannot fuse with latex bead-containing phagosomes. The actin-associated small GTPases Cdc42 and RhoA traffic to the CCV, with peak recruitment requiring *Coxiella* protein synthesis Expression of a dominant-negative RhoA results in a small, multivacuole phenotype reminiscent of that associated with disrupted autophagy or syntaxin 17 activity [58,60].

The *Coxiella* infection cycle occurs over several days with minimal cytopathic effect and no concerted egress event [76]. Thus, the organism is adept at maintaining host cell viability by disrupting both intrinsic and extrinsic apoptotic cell death signaling [63,77]. Apoptosis inhibition involves pathogen activation of the prosurvival kinases Akt, Erk1/2 and PKA [32,78,79]. Host cell maintenance requires active *Coxiella* protein synthesis and a functional T4BSS [63,77,80]. Indeed, recent data show PKA is recruited to the CCV membrane in a T4BSS-dependent fashion. PKA inactivates the proapoptotic protein Bad by phosphorylation, a modification that also stimulates localization of Bad to the CCV [79]. PKA activity in general is also required for optimal CCV development and bacterial replication [81]. A similar dual functionality is observed with CCV-localized Beclin 1, which benefits *Coxiella* by promoting autophagy and by binding the antiapoptotic protein Bcl-2 and modulating its activity [59].

Type 4 home remodeling

As discussed above, successful infection by *Coxiella* requires pathogen-directed manipulation of several host cell processes including apoptosis, autophagy, secretion and endolysosomal trafficking. It is now recognized that translocation of proteins with effector functions by a Dot/Icm T4BSS is critical for co-opting host cell

functions. The *Coxiella* NMI genome encodes 23 homologs of the 26 *L. pneumophila dot/icm* genes [82,83]. Predicted protein identities range from 20.8 (IcmD) to 63.4% (DotB) [84]. Four of ten *dot/icm* genes tested complemented the corresponding mutant in *L. pneumophila*, suggesting functional similarity between the two systems [85,86]. Consequently, and owing to the lack of genetic tools for *Coxiella, L. pneumophila* was extensively used as a surrogate host to identify *Coxiella* T4BSS substrates using both CyaA and BlaM cytosolic translocation reporter assays [87–96]. Indeed, of the 143 currently recognized *Coxiella* Dot/Icm effectors, 125 were initially identified in *L. pneumophila.* Gain-of-function studies using *L. pneumophila* also helped define *Coxiella* effector function [97,98].

Although *L. pneumophila* has proven utility as a heterologous system to examine *Coxiella* Dot/Icm functions, traditional gene mutation/complementation studies in *Coxiella* are necessary to validate surrogate results and to fulfill molecular Koch's postulates [99]. However, *Coxiella*'s obligate intracellular lifestyle made development of genetic methods technically challenging. Reliance on a eukaryotic host cell for growth dramatically complicates selection, scoring, cloning and expansion of genetic transformants [100]. Moreover, mutants with lowered intracellular growth fitness may not be recovered. Despite these obstacles, a mutagenesis protocol for *Coxiella* was developed using the mariner-based *Himar1* transposon (Tn) and Vero host cell system [101]. Promptly thereafter, *Coxiella* was liberated from its host cell by growth in the synthetic medium, acidified citrate cysteine medium (ACCM) [102,103]. Under microaerobic conditions, second-generation ACCM-2 now supports robust axenic growth in liquid media and development of small (~0.5 mm in diameter) colonies on solid media [103]. Axenic growth dramatically accelerated several advances in *Coxiella* genetic manipulation. Multiple methods for gene inactivation are now available including random mutagenesis using *Himar1* [80] and targeted mutagenesis using a 'loop in/loop out' strategy coupled with *sacB* counterselection or a Cre-*lox* approach that allows deletion of large chromosomal regions [104]. For single and multiple copy complementation, a Tn7-based system and RSF1010 *ori*-based shuttle vectors, respectively, are available [80,88,91,100]. Additional tools include inducible gene expression using the anhydrotetracycline-inducible promoter *tetA* [80] and a luciferase-based gene reporter system [105]. A method of nonantibiotic positive selection of *Coxiella* genetic transformants has recently been reported that uses nutritionally defined ACCM (ACCM-D) and complementation of *Coxiella's* natural arginine auxotrophy by expression of heterologous arginine biosynthesis genes capable of converting citrulline into arginine [106].

Confirmation that *Coxiella* translocates T4BSS substrates originally identified using *L. pneumophila* was first accomplished using shuttle vector technology [87,88,91]. The essential nature of the T4BSS for productive infection was then demonstrated by showing strains harboring *Himar1* insertions in *icmD* or *icmL* or targeted deletions in *dotA* or *dotB*, had severe intracellular growth defects associated with failure to secrete effector proteins in a *dot/icm*-dependent fashion [80,87,104]. Direct screening of potential Dot/Icm substrates in *Coxiella* is now feasible [60,73,87,94,107,108]. Moreover, targeted mutagenesis [73,98,107] and large-scale Tn screens [50,60] have revealed the importance of individual effectors in host cell colonization and helped resolve their function.

Mining for effectors

The bulk of *Coxiella* Dot/Icm substrates have been identified using bioinformatic criteria. Eukaryotic-like features (ELF), a C-terminal Dot/Icm translocation signal and similarity to known *Coxiella* effectors are strong predictors of effector proteins. Indicators of effector genes are upstream PmrA regulatory motifs and genomic location [73,87–92,94–96,107]. When assembling candidate effector gene lists, the predictive power of these features is amplified when multiple criteria are combined in machine learning [95] and manual approaches [88,94]. Ultimately, Dot/Icm substrates must be empirically validated using translocation assays. In an unbiased screen using translocation as a positive readout, Carey *et al.* [87] generated a library of random *Coxiella* genomic fragments cloned downstream from the *cyaA* reporter gene. Screening of *L. pneumophila* transformants in pools of 50 identified 7 Dot/Icm substrates. This list was expanded to 18 upon screening predicted paralogs of identified effectors and proximal genes.

Studies of *L. pneumophila* Dot/Icm substrates revealed a C-terminal translocation signal that varies substantially in primary sequence, but exhibits an overall regular distribution of charged, polar and hydrophobic amino

acids [109–111]. Computational modeling of the complex physiochemical parameters of amino acids comprising translocation signals demonstrated enrichment of hydrophobic amino acids within the three carboxyl terminal residues, serine and threonine at positions -4 to -11 and negatively charged amino acids between residues -8 and -18 [111]. A subsequent analysis revealed a motif within some Dot/Icm substrates comprised of 3–6 glutamate residues at position -11 to -18 termed the E-block [112]. This information was applied in a hidden semi-Markov model that predicted 44 *Coxiella* effector genes, 28 of which encoded *bona fide* Dot/Icm substrates [89].

Translocated effector proteins of pathogenic bacteria operate at the pathogen–host interface. Effectors have consequently evolved to contain ELFs in the form of domains or motifs that functionally mimic eukaryotic host cell proteins [113]. The higher frequency of ELFs in bacterial effectors versus noneffectors provides a powerful predictive tool for assembling candidate effector lists [73,88,90–92,94–96,107,113]. Indeed, recognition of a cohort of *Coxiella* genes encoding 33 amino acid ankyrin repeat domains (ARDs) resulted in identification of the first class of *Coxiella* Dot/Icm effectors [90,92]. ARDs, along with tetratricopeptide and leucine-rich repeats, are structural recognition modules typically found in eukaryotic proteins. Coiled-coil and helix–loop–helix domains, as well as transmembrane helices, are not specifically ELFs, but because they frequently engage proteins, lipids and/or nucleotides, they have been included in *Coxiella* effector searches. Although the presence of these structural modules is useful in assembling candidate effector gene lists, they provide little information on effector function. ELFs associated with biochemical functions include regulator of chromosome condensation 1-like domains linked with guanine nucleotide exchange,HAD-like domains found in phosphotransferases, filamentation induced by cAMP domains involved in AMPylation, serine/threonine protein kinase domains associated with phosphorylation and HECT-like E3 ligase and F-Box domains involved in ubiquitination. Another type of ELF found in effectors, termed short linear motifs (SLMs), consist of short amino acid sequences (3–10 residues long). The protein binding activities of SLMs can be used to unravel effector function. For example, endocytic sorting SLMs within the effector CvpA were critical for establishing its interaction with clathrin-coated vesicles [73].

At the nucleic acid level, a strong predictor of effector genes is the presence of an upstream PmrA regulatory element [88,95,114]. PmrA is a response regulator that couples with the sensory histidine kinase PmrB to form a two-component system. Work in the *Legionella* system demonstrated PmrA regulates expression of approximately 15% of known effector genes by binding the tandem repeat sequence cTTAATatT-N_2-cTTAATatT [114,115]. Functional expression of *Coxiella* PmrA in *Legionella* was also achieved [114]. As detailed below, *Coxiella* PmrAB has subsequently been shown by multiple groups to be essential for productive infection by *Coxiella* [50,60,105].

A final effector screen relies on binding to DotF in a bacterial-two hybrid system [88]. DotF, which comprises the core transmembrane complex of the T4BSS [116], was previously shown to bind a subset of *Legionella* effectors [117]. Screening of a genomic library revealed six *Coxiella* DotF-binding proteins that were validated as Dot/Icm substrates using the CyaA translocation assay [88]. The small number of effectors identified in both *Legionella* and *Coxiella* screens may reflect new data indicating DotF is not a major receptor of cytoplasmically expressed Dot/Icm substrates [116].

As effector lists were assembled, it was quickly recognized that considerable heterogeneity exists among effector gene repertoires of *Coxiella* genotypes associated with distinct virulence potential in animal models of Q fever [87,92,94]. The vast majority (>96%) of Dot/Icm substrates have been identified using the genome of the NMI RSA493 strain (multispacer sequencing typing genotype 18, 26), which is highly virulent [16,18]. Cross-genome comparisons with Henzerling RSA331 (high virulence; genotype 18, 25), G Q212 (moderate virulence; genotype 21), K Q154 (low virulence; genotype 8) and Dugway 5J108-111 (low virulence; genotype 111) [16,18,118], showed that many effector genes are pseudogeneized, due to insertion/deletions resulting in frameshifts, or completely missing **(Figure 1)**. Indeed, only 44 out of 143 identified effector genes are intact among all 5 strains. Several annotated effector genes encode proteins with small N-terminal truncations that may still be functional if synthesized and critical domains are not disrupted. Effectors with altered C-terminal sequences are likely nonfunctional due to failed translocation, although a *Legionella* Dot/Icm effector with an internal translocation signal has recently been

described [119]. An interesting subset of *Coxiella* effectors exhibiting strain variation is plasmid encoded [91]. All *Coxiella* strains examined to date harbor a moderately sized (~37–54 kb) plasmid or have approximately 17.5 kb of plasmid DNA integrated into their chromosome [10]. Plasmids share 22 conserved genes and have eight to 13 unique genes each. Thirteen of 15 genes within the integrated plasmid sequences are also conserved [10]. Screening of all plasmid-encoded hypothetical proteins uncovered 12 effector genes. Three effector genes are conserved between all plasmids and the integrated plasmid sequences [93], while three and four are uniquely encoded by QpH1 (carried by Nine Mile and Henzerling strains) and QpDG (carried by the Dugway strain), respectively [93]. The strict maintenance of plasmid sequences suggests they serve critical functions in *Coxiella* biology, with conserved effector genes predicted to play important roles in host cell manipulation [93]. By analyzing genome sequences from 10 *Coxiella* strains, Pearson *et al.* [11] recently defined the *Coxiella* pan genome as consisting of 2148 genes, with 341 genes not conserved among all strains. The attenuated Dugway strain contains the largest number of unique genes with 98. Some undoubtedly encode novel effector proteins that possibly contribute to infection outcome. Testing of effector mutants in animal models will provide needed insight into the contributions of effector pools to pathotype-specific virulence.

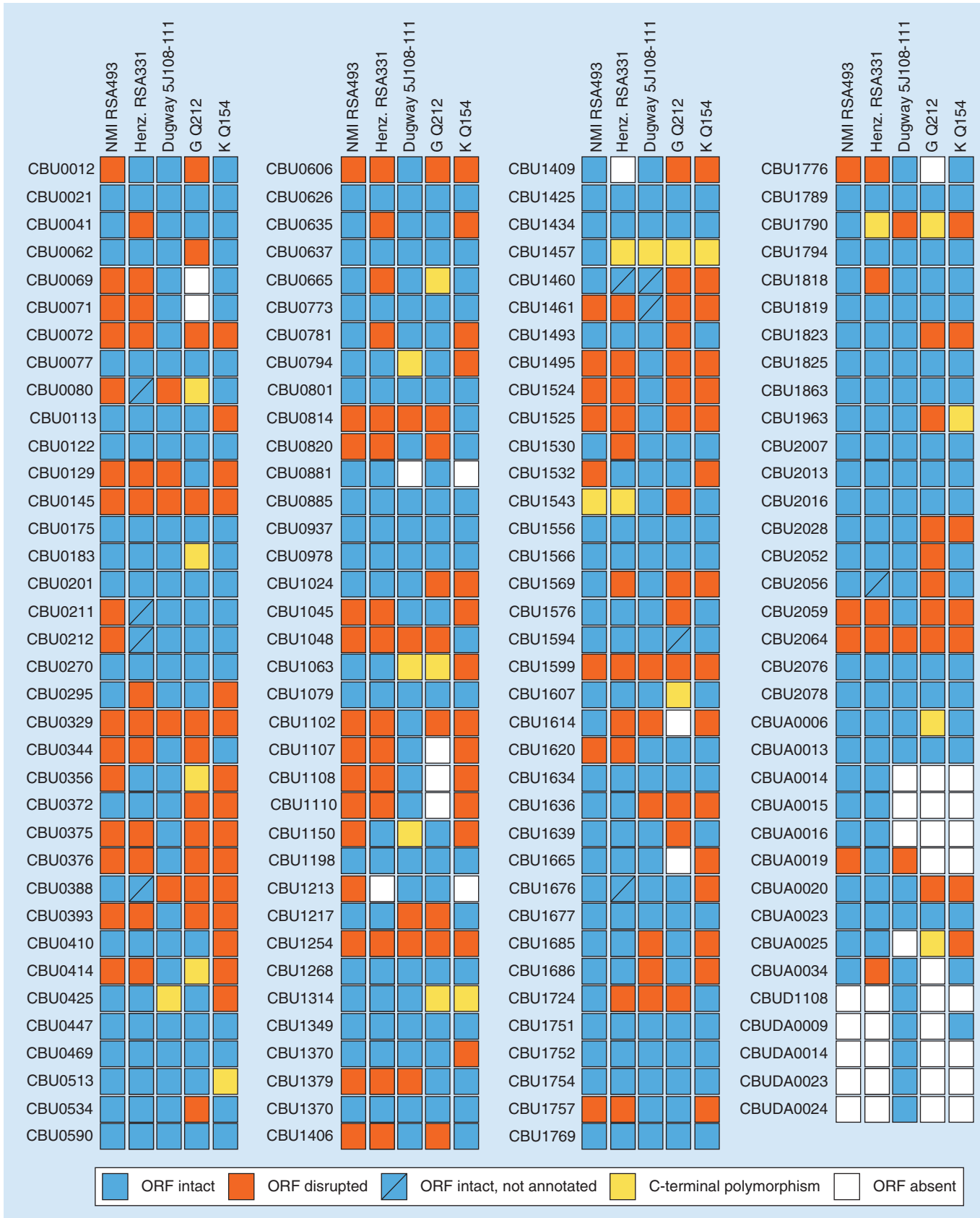
Finally, it is worth noting that, despite similar Dot/Icm apparatuses, effectors of *Coxiella* and *Legionella* are unique to each bacterium with virtually no homologs in other proteobacteria. Their distinct effector cohorts reflect disparate intracellular lifestyles, host range and acquisition of new genetic material by unknown horizontal gene transfer mechanisms that benefit lifestyle changes.

The hard part: effector function

With the greater part of the *Coxiella* Dot/Icm effector cohort known, focus has now shifted toward functional analysis. Effectors target host systems with defined roles in *Coxiella* cellular parasitism, and identifying their subcellular destinations can help design laboratory inquiries into function. However, detection of native effectors is exceedingly difficult; thus, ectopic expression of effector–fluorescent protein chimeras has been extensively used for trafficking studies, despite known artifacts of this procedure [120]. Effectors traffic to mitochondria [87,92,94,121], endoplasmic reticulum [91,94,96,122], Golgi apparatus [87], lysosomes [87,92,107,122], autophagosomes [91,93], endocytic vesicles [73,107], nucleus [87,94,121–123], microtubules [92,122] and ubiquitinated proteins [91,93]. An interesting subset of five effectors designated Cvp (*Coxiella* vacuolar protein) also traffic to the CCV [73,107]. The vast majority of effectors display a nonspecific, diffuse or punctate cytoplasmic appearance. Also relying on ectopic expression is the SEAP assay used to identify effectors that potentially disturb mammalian secretory trafficking. Several effectors inhibit release of SEAP in a HEK293T reporter cell system including CBU0635 [87], CBU1566 (CvpC), CBU1825, CBUA0019 [94] and CBUD1884 (ElpA) [96].

For a handful of effectors, functional insight has benefited from ectopic expression that confers gain-of-function and phenotype changes. Biochemical assays are then conducted to define pathway involvement. Because effectors often modulate the functions of eukaryotic pathways conserved between higher and lower eukaryotes, the yeast *Saccharomyces cerevisiae* is commonly used as a surrogate system to provide clues concerning effector function. Specific synthetic inhibition phenotypes observed in yeast ectopically expressing bacterial proteins can provide insight into effector identity and/or the cell processes they target [124]. Four studies have used yeast toxicity as a screening tool for *Coxiella* effectors [87,94,95,122]. Lifshitz *et al.* [95] specifically examined growth phenotypes associated with medium containing caffeine, a compound that activates the yeast cell wall integrity MAPK pathway. Interestingly, expression of CBU0885 (CetCb4) or CBU1676 (Cem9) potentiates yeast growth inhibition in the presence of caffeine, whereas CBU0388 (CetCb2) suppresses growth inhibition. Enhanced caffeine toxicity induced by Cem9 is rescued by coexpression of CetCb4 [95], suggesting antagonizing roles in modulating the cell wall integrity MAPK pathway. CetCb4 and Cem9 both contain an HAD domain required for activity. Whether these effectors promote *Coxiella* infection by coordinately regulating MAPKs, such as Erk1/2, through phosphorylation events remains to be determined.

Pathogen-associated molecular patterns (PAMPs) of invading microbes are sensed by pathogen recognition receptors of macrophages.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CBU1769
CBU1776
CBU1789
CBU1790
CBU1794
CBU1818
CBU1819
CBU1823
CBU1825
CBU1863
CBU1963
CBU2007
CBU2013
CBU2016
CBU2028
CBU2052
CBU2056
CBU2059
CBU2064
CBU2076
CBU2078
CBUA0006
CBUA0013
CBUA0014
CBUA0015
CBUA0016
CBUA0019
CBUA0020
CBUA0023
CBUA0025
CBUA0034
CBUD1108
CBUDA0009
CBUDA0014
CBUDA0023
CBUDA0024
ORF intact
ORF disrupted
ORF intact, not annotated
C-terminal polymorphism
ORF absent

Figure 1. Polymorphisms of *Coxiella* effector genes (see facing page). ORFs encoding validated Dot/Icm substrates were used to BLAST the genomes of the Nine Mile Phase I (NMI) RSA493, Henzerling RSA331, Dugway 5J108-111, G Q212 and K Q154 strains for homologous sequences. Effector ORFs were classified as intact (blue) if the length of the translated amino acid sequence was at least 90% of the longest representative amino acid sequence. Homologous ORFs with polymorphisms leading to protein sequence lengths less than 90% of the longest representative amino acid sequence were considered disrupted (red). In some cases, an intact effector gene was present but not annotated in GenBank (blue with slash), or completely missing (white). Amino acid sequences encoded by intact ORFs were further inspected for C-terminal polymorphisms, resulting largely from frame shift mutations, predicted to disrupt the translocation signal (yellow). Only 44 effector ORFs are conserved among the *Coxiella* strains in this analysis. The ORF identifiers CBU and CBUA correspond to chromosomal and plasmid genes, respectively, of the NMI RSA493 strain. The ORF identifiers CBUD and CBUDA correspond to chromosomal and plasmid genes, respectively, of the Dugway 5J108-111 strain.
ORF: Open reading frame.

Among the innate immune responses elicited by these interactions are apoptotic and pyroptotic programmed cell death [125]. *Coxiella* has a lengthy intracellular replication cycle lasting over a week [31], and thus deploys mechanisms to prevent both extrinsic (death receptor-mediated) and intrinsic (mitochondria-mediated) apoptosis [63,77]. The potent antiapoptotic activity of *Coxiella* requires a functional Dot/Icm apparatus [80]. A phenotypic screen of mammalian cells ectopically expressing *Coxiella* ARD effectors revealed that CBU0781 (AnkG) protects cells against staurosporine-induced intrinsic apoptosis [90,97]. In gain-of-function experiments, *Legionella* expressing AnkG replicated in mouse dendritic cells that normally die apoptotically following infection [97]. Subcellular localization of AnkG to the mitochondria coincides with its binding to p32, an interaction that is required for AnkG translocation to the nucleus and antiapoptotic activity [97,121]. A similar screen of known *Coxiella* effectors identified CBU1524 (CaeA) and CBU1532 (CaeB) as antiapoptotic effectors [87,126]. CaeA localizes to the nucleus and reduces both intrinsic and extrinsic apoptotic cascades between the initiator caspase-9 and the executioner caspase-7 by a mechanism requiring a CaeA glutamate/lysine repeat region [127]. More potent inhibition of intrinsic apoptosis is observed with CaeB that localizes to mitochondria and blocks mitochondrial outer membrane permeabilization in response to staurosporine treatment by a mechanism independent of p32 [126]. Prolonged activation of the prosurvival kinases Erk1/2 and Akt by *Coxiella* is also associated with antiapoptotic activity [78].

Genetic dissection of the importance of effectors implicated in *Coxiella* apoptosis inhibition and MAPK signaling has not been conducted. Advances in *Coxiella* genetic manipulation now make mutant generation and phenotyping an increasingly important requirement for publication of effector studies. Allelic exchange was described in a recent report by Cunha *et al.* [98] on a *Coxiella* effector involved in inflammasome inhibition. As opposed to noninflammatory apoptosis, pyroptosis is an inflammatory process mediated by inflammasome activation of caspase-1. Essential for inflammasome activity is PAMP recognition by cytosolic Nod-like receptors. *Coxiella* infection of primary mouse macrophages fails to induce caspase-1 and capase-11 activation and the subsequent release of IL-1 cytokines [37,98]. Furthermore, *Coxiella* inhibits induction of caspase-11-mediated, noncanonical activation of the inflammasome by *L. pneumophila* in macrophage coinfection experiments [98]. By expressing a panel of *Coxiella* effectors in *L. pneumophila*, CBU1823 (IcaA) was found to inhibit caspase-11 activation [98]. Importantly, a *Coxiella icaA* mutant failed to suppress caspase-11-mediated inflammasome activation induced by *L. pneumophila* in coinfection experiments. Cytosolic delivery of a *Coxiella* extract enriched in LPS induces caspase-11 dependent pore formation, indicating the bacterium produces molecules that induce pyroptosis. However, infection with the *Coxiella icaA* mutant alone fails to activate the inflammasome, suggesting that, like antiapoptotic effectors, *Coxiella* deploys functionally redundant effectors that contribute to protection [98]. A potential caveat is related to the observation that human primary alveolar macrophages infected with NMII, but not virulent strains of *Coxiella,* release mature IL-1β [32]. Thus, inflammasome activation may be *Coxiella* strain and/or host cell specific.

Functional insight has been gained for CvpA (CBU0665) [73,107]. A Δ*cvpA* mutant shows poor intracellular growth in unusually small CCVs. CvpA contains several dileucine ([DERQ] XXXL[L,I]) and tyrosine (YXXφ, φ-bulky hydrophobic residue) SLMs that interact in pull-down assays with clathrin and the heterotetrameric clathrin adaptor complex AP2,

suggesting CvpA targets clathrin-mediated vesicular trafficking originating from the plasma membrane [128]. Consistent with this hypothesis, ectopically expressed mCherry-CvpA localizes with clathrin on the cell periphery and endocytic recycling vesicles in the pericentrosomal region in uninfected cells, known trafficking itineraries of endogenous endocytic cargo

Table 1. Intracellular growth phenotypes of *Coxiella burnetii* effector gene mutants.

ORF	Aliases	Intracellular growth phenotype	Ref.
CBU0012		Growth defect (50, 94)	[50,94]
CBU0021	*cvpB/cig2*	Growth defect (50, 107); CCV fusion defect (107, 60, 131)	[50,60,107,131]
CBU0041	*cirA*	Growth defect (94)	[94]
CBU0069-CBU0071	*ankP*	No growth defect (60); growth defect (50)	[50,60]
CBU0072	*ankA*	No growth defect (60); growth defect (50)	[50,60]
CBU0077		No growth defect (50)	[50]
CBU0145	*ankB*	No growth defect (60)	[60]
CBU0295		No growth defect (60)	[60]
CBU0388		Growth defect (94)	[94]
CBU0425	*cirB*	Growth defect (94); no growth defect (50)	[50,94]
CBU0447	*ankF*	No growth defect (50)	[50]
CBU0626		Growth defect (50)	[50]
CBU0635		No growth defect (50)	[50]
CBU0665	*cvpA*	Growth and CCV fusion defects (73)	[73]
CBU0937	*cirC*	Growth defect (50, 94)	[50,94]
CBU1079		No growth defect (60)	[60]
CBU1198		Growth defect (94)	[94]
CBU1217		No growth defect (50)	[50]
CBU1251		No growth defect (60)	[60]
CBU1376	*coxK2*	No growth defect (60)	[60]
CBU1457		Growth defect (94)	[94]
CBU1460		Growth defect (50)	[50]
CBU1461	*coxCC8*	Growth defect (60)	[60]
CBU1525		Growth defect (50)	[50]
CBU1556	*cvpC*	Growth defect (107)	[107]
CBU1569	*coxCC12*	No growth defect (60)	[60]
CBU1639		No growth defect (50)	[50]
CBU1665		Growth defect (50)	[50,60]
CBU1751	*cig57*	Growth defect (60); no growth defect (50)	[50]
CBU1754		Growth defect (60)	[60]
CBU1780		Growth defect (60)	[60]
CBU1789		No growth defect (60)	[60]
CBU1818	*cvpD*	Growth and CCV fusion defects (107)	[107]
CBU1823	*icaA*	No growth defect reported (98)	[98]
CBU1863	*cvpE*	Growth and CCV fusion defects (107)	[107]
CBU2013		Growth defect (94)	[94]
CBU2016		Growth defect (50)	[50]
CBU2028		No growth defect (60)	[60]
CBU2052	*cirD*	Growth defect (94); no growth defect (60)	[60,94]
CBU2056		No growth defect (60)	[60]
CBU2059	*cirE*	Growth defect (94)	[94]
CBU2076		No growth defect (60)	[60]
CBU2078		No growth defect (50, 60)	[50,60]
CBU2082		No growth defect (60)	[60]
CBUA0023	*cpeF*	Growth defect (50)	[50]

CCV: *Coxiella*-containing vacuole; ORF: Open reading frame.

proteins. mCherry-CvpA expression also inhibits endocytosis of transferrin [73], a cargo molecule internalized at the plasma membrane by clathrin-mediated endocytosis [129,130]. *Coxiella* replication and CCV biogenesis are dramatically impaired in cells depleted of AP2 or clathrin. Collectively, these data suggest CvpA targets clathrin-mediated vesicular trafficking that contributes to CCV biogenesis.

Three groups have demonstrated that mutation of the effector gene *cbu0021* (*cvpB, cig2*) results in aberrant CCV formation and/or *Coxiella* growth [50,60,107,131]. Vacuoles harboring *cbu0021* mutants do not homotypically fuse, resulting in a multivacuole phenotype [60,107,131]. In two investigations, *cbu0021* mutants also display deficient replication [50,107]. Autophagosome interactions with the CCV require an active T4BSS [67] and CCV recruitment of the autophagy protein LC3 requires functional CBU0021 [60]. Depletion of the autophagy regulators syntaxin 7, ATG5 or ATG7 during infection with wild-type bacteria results in a multiple vacuole phenotype similar to that associated the *cbu0021* mutant, suggesting a functional link between the *Coxiella* effector, autophagy and CCV fusogenic properties [60]. Indeed, CBU0021 binds phosphoinositides on host cell membranes and perturbs the activity of the phosphatidylinositol 3-kinase PIKfyve to enrich phosphatidylinositol 3-phosphate (PI[3]P) on CCVs [131]. This favors association of autophagosomal machinery with CCVs and their homotypic fusion [131].

Allelic exchange demonstrated the importance of the remaining *cvp* genes (*cvpC, -D* and *-E*) in host cell parasitism [107]. Seven *cir* (*Coxiella* effector for intracellular replication) genes with Tn insertions that result in intracellular growth defects by *Coxiella* were also recovered from a library following bioinformatics-based effector discovery [94]. With the exception of *cbu0021, cbu1751* (described below) and *cvpA, -D* and *-E*, effector mutants have not been genetically complemented to eliminate potential contributions of polar effects and/or secondary mutations on phenotypes. Nonetheless, thus far a surprisingly high percentage of *Coxiella* mutants in effector genes show discernable intracellular growth defects when compared with *L. pneumophila* effector gene mutants. There is extensive functional redundancy in the effector pool of *L. pneumophila,* maintenance of which is attributed to selective pressure to survive in a multiple species of amoebae [132]. **Supplementary Table 1** summarizes characteristics of known *Coxiella* Dot/Icm substrates and the strategies used for identification.

Blasting the chromosome with *Himar1*

The past few years have witnessed remarkable advances in microscopy-based high-content screening applied to the study of infectious diseases [133,134]. Multiphenotypic screens can be developed that simultaneously extrapolate a large number of features from bacteria and host cells [133]. The creation of an exceptionally large, single vacuole filled with bacteria makes the *Coxiella* infectious process an ideal system for this type of analysis. When combined with robust *Himar1*-mediated insertional mutagenesis, large and ordered libraries of *Coxiella* mutants can be generated and screened in a high-throughput manner for mutations that confer infection deficiencies. Mapping Tn insertions on the *Coxiella* genome identifies the repertoire of coding and noncoding regions that promote infection. Moreover, genes with no insertions potentially represent those universally required for growth.

The first application of this technology was reported by Martinez *et al.* [50] who evaluated 1082 *Coxiella* Tn mutants for the capacity to invade, replicate within and/or protect host cells from apoptosis. Mutagenesis was conducted using a *Himar1*-derived Tn designed to express green fluorescent protein [101]. Automated fluorescence microscopy and image analysis were employed to score nuclei features (apoptosis), *Coxiella* features (intracellular growth) and the number of CCV per host nuclei (internalization) from an average of 15,000 infected Vero cells per mutant. Intracellular growth was scored by measuring both CCV number and *Coxiella* colony size. The Tn insertion point for each isolated mutant, regardless of phenotype, was mapped using single primer PCR coupled to DNA sequencing to provide a global view of putative virulence determinants and *Coxiella* genome architecture. Mutation of 483 of the 1831 NMII coding sequences (26.1%) was reported, with a remarkable 83.2% of mutated genes conferring a mild or strong phenotype in one or more scored feature. As predicted, 40 Tn insertions in 16 *dot/icm* genes resulted in severe intracellular growth defects. Furthermore, mutation of 12 of 20 genes encoding confirmed Dot/Icm substrates produced severe intracellular replication phenotypes, results that allow prioritization of effectors for further study. Critical transcription factors were identified, including PmrA (CBU1227) and OmpR (CBU2006), which are response regulators

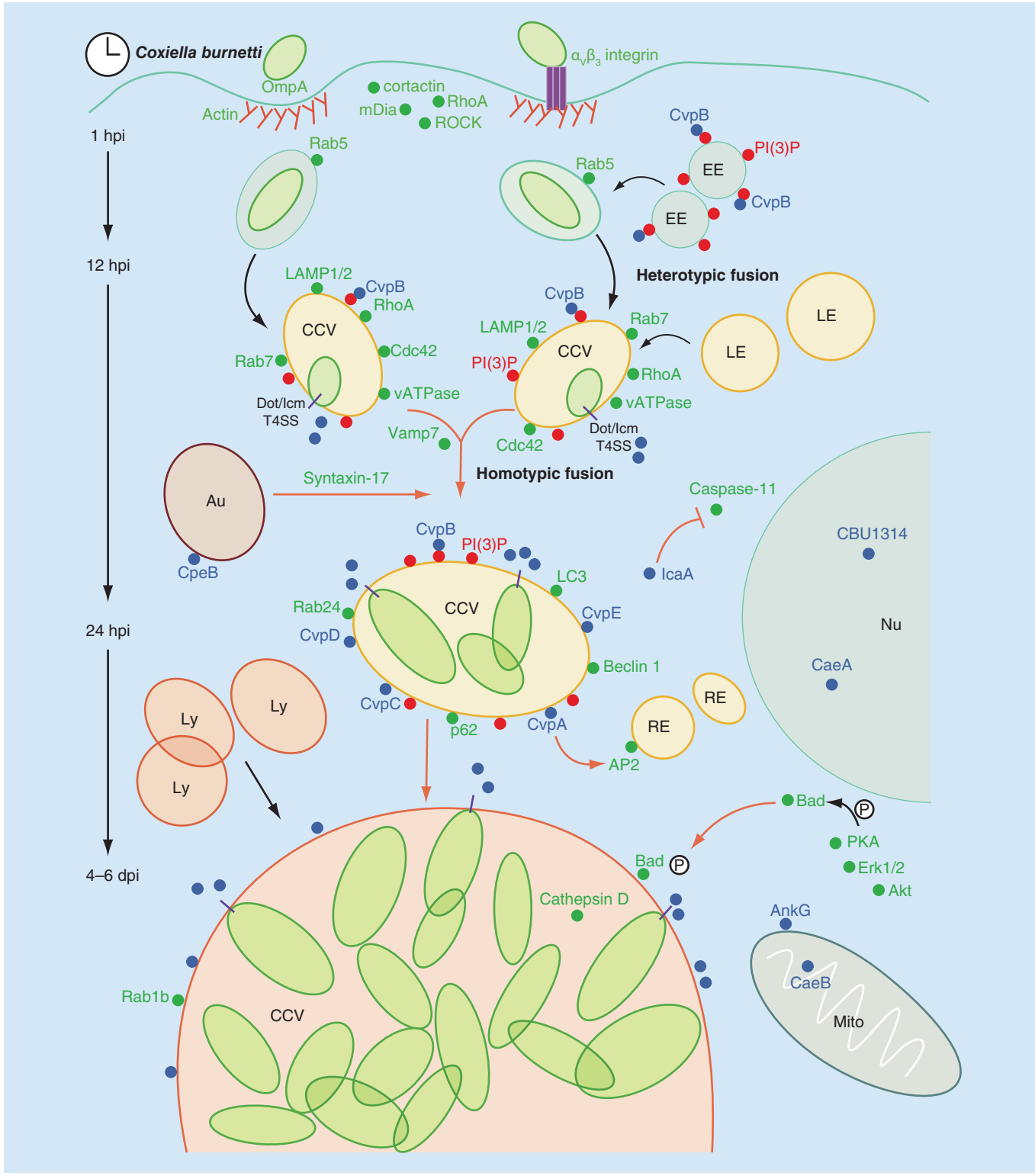

Figure 2. Steps in *Coxiella* infection and *Coxiella*-containing vacuole formation and host cell processes potentially modulated by *Coxiella* effector proteins. *Coxiella* internalization is facilitated by the rearrangement of the host actin cytoskeleton, mediated by the actin-binding proteins cortactin and mDia and requiring the activation of the small GTP-ases RhoA and ROCK. The *Coxiella* invasin OmpA mediates bacterial internalization by nonprofessional phagocytes, whereas internalization by macrophages requires interaction with cell surface $\alpha_v\beta_3$ integrins.

Figure 2. Steps in *Coxiella* infection and *Coxiella*-containing vacuole formation and host cell processes potentially modulated by *Coxiella* effector proteins (cont.). Inside cells bacteria reside within CCVs that progressively acquire endosomal markers by heterotypic fusion with early and late endosomes and lysosomes (EE, LE and Ly, respectively). CCV acidification activates bacterial metabolism, which likely coincides with translocation of effectors (blue dots) by the Dot/Icm T4BSS (purple rods). This is required for vacuole expansion and for the recruitment of the autophagosomal and endocytic SNARE proteins syntaxin-17 and Vamp7, mediating the homotypic fusion of multiple CCVs into a single vacuole. CCV maturation requires *Coxiella* effectors to reroute several vesicle trafficking pathways that contribute membrane to the expanding CCV. The *Coxiella* effector CvpA interacts with AP2 on recycling endosomes (RE). The effector CvpB localizes at EE and CCVs, where it binds PI(3)P (red dots) and manipulates its metabolism to favor vacuole biogenesis and autophagy (Au)-mediated homotypic fusion of CCVs. Vacuole decoration by the small GTPase Rab1b suggests that other *Coxiella* effectors manipulate ER-to-Golgi traffic. Additional *Coxiella* effectors inhibit the intrinsic and extrinsic apoptotic pathways of infected cells. Dot/Icm-mediated translocation of yet unidentified effectors is required to activate the prosurvival kinases Akt, Erk1/2 and PKA. In turn, PKA phosphorylates and inactivates the proapoptotic protein Bad, triggering its recruitment by the CCV. The effectors AnkG and CaeA localize at Mito where they prevent membrane permeabilization induced by staurosporin. CaeA localizes at the Nu of infected cells where it prevents apoptosis by preventing caspase-9 and -7 cleavage whereas the effector IcaA has been recently associated with the inhibition of caspase-11 cleavage in macrophages to prevent pyroptosis. Finally, the *Coxiella* effector CBU1314 localizes at the nucleus of infected cells where it may act as nucleomodulin. Green dots indicate host proteins implicated in *Coxiella* infection events. Black arrows indicate events that do not require the translocation of *Coxiella* effectors; red arrows indicate processes mediated by *Coxiella* effectors. CCV: Coxiella-containing vacuole; EE: Early endosome; LE: Late endosome; Mito: Mitochondria; Nu: Nucleus; RE: Recycling endosome; SNARE: Soluble NSF attachment receptor.

of two-component systems. Interestingly, insertions in 32 annotated pseudogenes and 11 intergenic regions predicted to encode sRNA, resulted in strong replication defects, suggesting these genomic regions produce products important for host cell colonization. Alternatively, these Tn insertions could exert polar effects on, or disrupt promoter regions of, adjacent genes involved in intracellular growth.

A seminal finding of Martinez *et al.* [50] was identification of OmpA (CBU1260) as a *Coxiella* invasin mediating internalization by nonphagocytic cells. Structure predictions suggest OmpA exposes four unstructured loops at the surface of *Coxiella* with deletion analysis implicating loop 1 as essential for internalization. Purified OmpA, or antibodies targeting the extracellular domains of OmpA, neutralize *Coxiella* infectivity, supporting a receptor/ligand model of host cell entry. Interestingly, macrophages efficiently internalize the *Coxiella ompA* Tn mutant; however, the organisms display intracellular replication defects. OmpA may benefit *Coxiella* growth in macrophages by modulating innate immune signaling as demonstrated for OmpA-like proteins of other intracellular pathogens [135].

A second Tn screen was conducted by Newton *et al.* [60] using *Himar1* modified to express mCherry fluorescent protein. Over 3237 arrayed insertion mutants were recovered and the Tn insertion points of 460 mutants determined. Using a visual fluorescence microscopy screen incorporating LAMP-1 staining for demarcation of the CCV, mutants were isolated that displayed no intracellular replication (21 genes, 6 intergenic regions), moderate intracellular replication defects (24 genes, 1 intergenic region), a multivacuole phenotype (1 gene) or a filamentous phenotype (seven genes). Again, *dot/icm* genes were highly represented (16 of 21) among inactivated genes that confer a null growth phenotype, with the two-component system *pmrAB* also included in this group. Additional regulators, such as the GacS-like sensor kinase CBU1761, were identified as needed for optimal intracellular growth. Mutation of genes encoding the previously described Dot/Icm substrates CBU1461, CBU1751 (Cig57) and CBU1754 [88,89,94] resulted in moderate intracellular replication defects whereas insertions interrupting production of CBU0021 produced an unusual multivacuole phenotype without an overall replication defect. A novel effector protein (CBU1780) was also identified (null replication phenotype). Mutants in 15 additional effector genes showed no growth deficiency, suggesting functional redundancy among this effector cohort.

The Tn mutant screens of Martinez *et al.* [50] and Newton *et al.* [60] are notable in identifying the same factors as critical for successful host cell parasitism, such as the Dot/Icm T4BSS and its regulator PmrAB and the effectors CBU0021 and CBU1780. However, disparate results were also obtained, primarily with respect to mutated genes conferring moderate to strong replication defects in the study by Martinez *et al.* [50] with no discernable phenotype in the study by Newton *et al.* [60]. These discordant results could be attributable to the use of different microscopic scoring systems,

host cell lines (Vero versus HeLa) and/or lengths of infection. Also, it is unclear whether some replication defects might be attributable to a defect in overall metabolic fitness that manifests both extra- and intra-cellularly. Nonetheless, both screens are landmark studies that revealed novel determinants of intracellular growth and/or CCV development.

Table 1 summarizes the characteristics of mutated *Coxiella* effector genes and their associated phenotypes. **Figure 2** illustrates steps in *Coxiella* infection and CCV formation, and depicts host cell processes potentially modulated by *Coxiella* effector proteins.

Regulating type 4 translocation

Current data support a model whereby effector translocation requires both host cell membrane contact and pathogen metabolism. Evidence for host membrane contact is based on the observation that verified effector proteins are not detected by mass spectrometry in ACCM-2 following *Coxiella* culture [136] and electron micrographs showing protrusions emanating from the *Coxiella* surface that appear to penetrate the CCV lipid bilayer **(Figure 3)**. The presence of Dot/Icm proteins with Walker box ATPase motifs, such as DotB, suggests substrate translocation is an energy-requiring process that requires active pathogen metabolism [84]. *Coxiella* is metabolically active only at moderately acidic pH [64]. As such, disruption of host factors, such as Rab5 and Rab7, that regulate endolysosomal maturation and acidification, also inhibit Dot/Icm-mediated secretion [108]. Of note, effector translocation is detected earlier in mouse macrophages (4 h postinfection) than HeLa cells (8 h postinfection), suggesting the CCV maturation/acidification process occurs more rapidly in the former cell type [108]. Inhibiting transcription with rifampicin upon infection inhibits effector release, but it is unclear whether this effect is Dot/Icm specific or due to a general decline in metabolic fitness [108]. Induction of the T4BSS as late as one day postinfection initiates *Coxiella* replication, showing secretion is not required during transit through the endolysosomal cascade for productive infection [80]. This result, and data showing nonreplicating *Coxiella* remain viable in LAMP-1-positive vacuoles of host cells treated with bacteriostatic antibiotics for 2 days [65], indicate *Coxiella* is inherently resistant to digestion by lysosomal hydrolases. Intimate membrane contact and energy-dependent translocation of preformed effectors is invoked for the *L. pneumophila* T4BSS [137]; however, unlike *Coxiella, Legionella* effector translocation must occur coincident with pathogen internalization to promote development of an ER-derived vacuole that avoids lysosome fusion and supports growth [137,138]. The environmental cue(s) that activates the *Coxiella* T4BSS is unknown, but could involve inorganic ions or pH as demonstrated for other specialized secretion systems [139,140].

A critical regulator of the *Coxiella* T4BSS is the two-component system PmrAB [50,60,105]. Genetic inactivation of the system by three independent groups resulted in strong intracellular growth defects [50,60,105]. Using a luciferase reporter assay and a *Coxiella* Δ*pmrA* mutant, Beare *et al.* [105] demonstrated PmrA is a critical transcriptional regulator of Dot/Icm operons and effector genes containing predicted PmrA regulatory elements. Coincidently, functional PmrA is required for effector translocation [60,105]. RNA sequencing of the Δ*pmrA* mutant revealed several *pmrA*-regulated genes, including a few new effector genes, lacking PmrA regulatory elements. Cross-talk between PmrAB and other *Coxiella* regulatory factors may control transcription of these genes. Of much interest are the host cell signal(s) that activate the sensory kinase PmrB.

In addition to a C-terminal translocation signal, many Dot/Icm substrates require recognition of an internal signal by the chaperone (adaptor) complex IcmSW for optimal transport [92,141]. IcmS dependency was demonstrated for 7 of 11 *Coxiella* ARD effectors in the surrogate *L. pneumophila* system [92]. Tn mutagenesis of *icmS* and *icmW* results in a moderate [50,60] and strong [60] intracellular growth defects, respectively. A working model of IcmSW engagement suggests chaperone binding maintains effectors in an unfolded state that facilitates recognition of the C-terminal signal sequence by the translocation apparatus [142]. Whether IcmSW targets effector subsets with common functions or temporal constraints remains to be determined.

Conclusion

The phagolysosome-like niche of *Coxiella* is unique among intracellular bacteria and represents a fascinating example of pathogen–host adaptation. The CCV can expand to occupy the majority of host cell cytoplasm and accommodate hundreds of replicating *Coxiella* without causing obvious cytoxicity. In fact, persistently infected mouse L929 cells display a normal cell cycle and doubling time [143]. Habitation of a vacuole that

fully matures through the endolysosomal pathways ensures sufficient acidity to drive *Coxiella* metabolism. In contrast, metabolic quiescence associated with the relatively neutral extracellular environmental likely contributes to prolonged maintenance of *Coxiella* as an infectious form. The mature *Coxiella* vacuole is clearly a specialized compartment that engages multiple vesicular trafficking pathways for acquisition of membrane for vacuole enlargement and presumably nutrients for pathogen growth. The newfound ability to genetically manipulate *Coxiella* now establishes *Coxiella* as a model system of macrophage parasitism. Most predominantly, genetics have unraveled the essential nature of the Dot/Icm T4BSS in macrophage colonization and are beginning to elucidate functions of the associated effector pool. Effector mutations that result in defects in endosome fusion, autophagosome interactions and apoptosis/inflammasome inhibition have been identified, although their biochemical activities remain unresolved. Tn mutagenesis screens have revealed numerous *Coxiella* genes assisting host invasion and intracellular replication, including OmpA, the first identified *Coxiella* invasin.

Future perspective

The burgeoning tractability of *Coxiella* as host–pathogen model system has enticed new researchers into the field for what should be the golden age of *Coxiella* virulence factor discovery. However, *Coxiella* researchers would benefit from further refinement of existing tools and development of new technologies. Saturation-level Tn mutagenesis of *Coxiella* strains that cause clinical disease in animal models of Q fever would allow high-throughput Tn-insertion sequencing strategies for identification of the global gene repertoire required for animal infection [144]. Tn-based promoter traps could also define genome-wide transcriptional responses under diverse environmental conditions. Existing Cre-*lox* technology should be explored for generation of attenuated, nonrevertable variants of *Coxiella* pathotypes for use in pathogen–host interaction studies at biosafety level 2. Restricted use of antibiotic resistance genes as selectable markers make development of nonantibiotic based selection schemes and markerless allelic exchange methods desirable. From the host cell side, genome editing using CRISPR technology now simplifies generation of knockout cell lines to identify host cell functions participating in infection and effector activity [145]. Macrophage transcriptional responses to infection by different *Coxiella* pathotypes [15,16] will shed light on strain-specific host cell interactions.

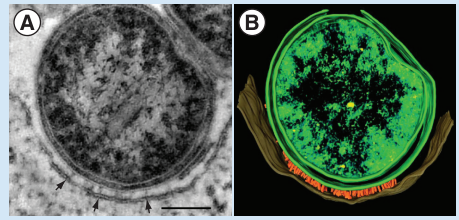

Figure 3. Association of *Coxiella* cell envelope projections with the *Coxiella*-containing vacuole membrane. (A) Transmission electron micrograph and **(B)** 3D pseudocolored tomograph of a THP-1 macrophage infected for 48 h with *Coxiella*. Cell envelope-associated needle-like structures (~20 nm in length) at points of *Coxiella*–coxiella-containing vacuole membrane contact (arrows) are evident that are likely components of the T4BSS. Bar =100 nm.

Defining T4BSS effector function is central to understand Q fever pathogenesis. Unfortunately, the field has only scratched the surface in understanding effector activities, the relevance of effector gene polymorphisms, strain specificity in effector cohorts and potential effector redundancies. Contributing to these gaps in knowledge is an incomplete grasp of the host cell systems co-opted for *Coxiella* parasitism. Apoptosis and inflammasome inhibition are clearly strategies to sustain the macrophage host for *Coxiella*'s lengthy infectious cycle, with subversion of several vesicular trafficking pathways providing the CCV membrane and nutrients. However, the complex effector pool of *Coxiella* undoubtedly modulates additional eukaryotic systems for pathogen benefit such as the cytoskeleton [146], ubiquitination [147] and nuclear processes via the activity of an emerging class of effectors known as nucleomodulins [148,149]. Elucidating signal transduction events that trigger effector translocation, the temporal reliance on effector groups during infection and whether effector–effector regulation occurs [150,151], are of much interest to the field.

Despite progress in genetic dissection of effector functions using tissue culture models of infection, LPS still remains the only genetically defined virulence factor evaluated using immunocompetent rodent models (mice and guinea pigs) [33,152]. To date, the attenuated NMII strain has been exclusively

EXECUTIVE SUMMARY

Coxiella: a wide-ranging zoonotic pathogen

- *Coxiella burnetii* has worldwide distribution with a broad range of animal hosts. Shedding by domestic livestock is primarily responsible for aerosol transmission of Q fever to humans.
- Pathoadaptation appears to drive strain variation within the genus *Coxiella* with some genotypes showing distinct animal reservoirs and disease associations.

Making a home in host cells

- *Coxiella* is unique among intracellular bacteria in replicating within a macrophage vacuole that phenotypically resembles a large phagolysosome. The *Coxiella*-containing vacuole (CCV) fuses with endolysosomal, autophagic and secretory vesicles.
- *Coxiella* exerts a potent antiapoptotic effect on host cells that benefits its slow intracellular growth rate.

Type 4 home remodeling

- Host cell-free growth revolutionized the study of *Coxiella*, in large part by accelerating the development of advanced genetic tools.
- Conventional mutation/complementation strategies are now available to directly examine *Coxiella* gene function, including Dot/Icm and effector-encoding genes.

Mining for effectors

- Expression in *Legionella pneumophila* of a random library of *Coxiella* proteins N-terminally fused to the CyaA cytosolic translocation reporter revealed multiple *Coxiella* Dot/Icm substrates.
- Strong bioinformatics predictors of T4BSS substrates include eukaryotic-like motifs and C-terminal positively charged E-blocks, as well as upstream PmrA regulatory sequences on encoding genes.
- Full-length effector genes conserved between *Coxiella* strains presumably perform functions required for macrophage colonization.

The hard part: effector function

- To date, mutation of 24 of 46 Dot/Icm effector genes confer defects in intracellular growth and/or CCV formation.
- Ectopic expression in mammalian cells and/or gain of function in *L. pneumophila* has shed light on antiapoptotic functions of the effectors AnkG, CaeA and CaeB.
- Like antiapoptosis effectors, inhibition of the inflammasome by the effector IcaA is another strategy employed by *Coxiella* to prolong host cell viability.
- Synthetic inhibition or potentiation of yeast growth have identified CBU0885 (CetCb4), CBU1676 (Cem9) and CBU0388 (CetCb2) as potential modulators of the mammalian MAP kinase pathway.
- Cumulative data suggest the effectors CBU0021 (CvpB, Cig2) and CBU0665 (CvpA) modulate vesicular trafficking events.

Blasting the chromosome with Himar1

- Screening of *Coxiella* Himar1 Tn mutant libraries has provided a genome-wide view of genes required for optimal intracellular growth and/or CCV formation.
- High-content screening for internalization defects revealed OmpA as a *Coxiella* invasin mediating internalization by epithelial cells.

Regulating type 4 translocation

- Regulation of the T4BSS by *Coxiella* is multifactorial with transcriptional control involving the two-component sensory system PmrAB and post-translational control involving the chaperone pair IcmSW and a C-terminal translocation signal.
- CCV acidification that activates *Coxiella* metabolism is required for effector translocation by the Dot/Icm system.

used to generate mutants using transformation. Because of biocontainment constraints, many *Coxiella* laboratories are restricted to using this strain and therefore have employed infection of immune-deficient mice to glean information on *Coxiella* pathogenesis [153,154]. New invertebrate models of infection have also shown promise in assessing virulence attributes of NMII mutants [155]. Nonetheless, effector mutations may only manifest during infection of mammals with intact innate and adaptive immune responses, requiring mutant generation and subsequent animal testing of clinically relevant strains of *Coxiella*.

Supplementary data

To view the supplementary data that accompany this paper, please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/fmb-2016-0044

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