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Molecular pathogenesis of the obligate intracellular bacterium *Coxiella burnetii*

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Abstract

The agent of Q fever, *Coxiella burnetii*, is an obligate intracellular bacterium that causes acute and chronic infections. The study of *C. burnetii* pathogenesis has benefited from two recent fundamental advances: improved genetic tools and the ability to grow the bacterium in extracellular media. In this Review, we describe how these recent advances have improved our understanding of *C. burnetii* invasion and host cell modulation, including the formation of replication-permissive *Coxiella*-containing vacuoles. Furthermore, we describe the Dot/Icm (defect in organelle trafficking/intracellular multiplication) system, which is used by *C. burnetii* to secrete a range of effector proteins into the host cell, and we discuss the role of these effectors in remodelling the host cell.

Coxiella burnetii is the Gram-negative obligate intracellular pathogen that causes Q fever, a condition which presents as either acute or chronic disease (BOX 1). Phylogenetic analyses have shown that isolates causing acute and chronic disease fall into distinct groups, supporting the prediction that there are genetically distinct *C. burnetii* pathotypes (BOX 2). As an intra-cellular pathogen, this organism has evolved a range of mechanisms to invade and survive within host cells. *C. burnetii* has a tropism for professional phagocytes and invades such cells using classic phagocytic mechanisms that rely on specific receptor–ligand interactions[1]. Inside the host cell, invading bacterial pathogens typically subvert phagosomal maturation using a variety of mechanisms[2]. However, *C. burnetii* does not follow this paradigm, but instead actively directs the maturation of a phagolysosome-like compartment known as the *Coxiella*-containing vacuole (CCV)[3–5].

Box 1

Clinical presentation and epidemiology of Q fever

Q fever is a zoonosis that is most often transmitted by aerosolized soil or animal products contaminated with *Coxiella burnetii*. Cattle, sheep and goats are the most common

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reservoirs for this pathogen, but it also infects many wild animal species and arthropods, and is therefore considered a ubiquitous zoonotic contaminant[107]. Although infection rarely results in mortality, it does cause significant morbidity. Furthermore, the bacterium is stable for long periods in the environment and has therefore been classified as a category B select agent by the US CDC because of the potential for use as a bioterrorism agent[108].

The infectious dose for *C. burnetii* can be as low as a single organism in the lungs, and acute Q fever usually occurs 2–6 weeks after exposure, although the infection remains asymptomatic in >50% of cases. Symptomatic disease is characterized by high fever, severe headache (retro-orbital), myalgia, malaise, pneumonia and hepatitis, and usually lasts 1–2 weeks[109]. In symptomatic cases, the acute illness spontaneously resolves within 1–6 weeks and is effectively mitigated by antibiotics such as tetracycline and third-generation cephalosporins[110,111]. By contrast, some studies have estimated that in 1–2% of cases, acute Q fever develops into a chronic infection that manifests as endocarditis, hepatitis or as an incompletely defined post-Q fever fatigue syndrome[112–114]. Diagnosis of acute Q fever is difficult because obtaining a culture of the organism is challenging and requires a BL3 containment laboratory. Diagnosis relies on serology using immunofluorescence assays and ELISAs (enzyme-linked immunosorbent assays).

Most outbreaks involve a limited number of people, but the potential impact on a large population was recently highlighted by infections in the Netherlands[115]. Between 2007 and 2010, more than 4,000 cases of acute Q fever were reported in this country, with an annual summer seasonal peak. The outbreak was mainly restricted to the south of the country, coincident with intensive dairy goat farming in this region, and the source of the infections seemed to be linked to several goat herds in close proximity to population centres. The outbreaks provided a clear demonstration of the threat to public health if adequate diagnostic, therapeutic and epidemiological tools are not developed and available. Following the introduction of veterinary control measures in 2009 (including the vaccination of goat and sheep dairy herds, culling of pregnant animals on infected farms[116] and improved hygiene procedures), the number of reported Q fever cases in the Netherlands has returned to the low historical rates (fewer than ten cases per year). In addition, a dramatically improved diagnostics and epidemiology network is now established, and public health officials are anticipating a slow influx of chronic disease cases over the next decade, as a result of the ~15% infection rate estimated for the regional populations in which the outbreaks occurred[117].

Box 2

Pathotype-specific virulence

Pathotype-specific virulence of *Coxiella burnetii* is a long-standing hypothesis that could account for the difference between the propensity of different isolates to cause either acute or chronic disease[118–122]. Restriction fragment-length polymorphism (RFLP) and pulse-field gel electrophoresis (PFGE) analysis of DNA isolated from 32 *C. burnetii* strains led to the differentiation of isolates into six genomic groups (group I to group VI;

see the table (group III is not shown)) [119] that showed a distinct pattern of association with acute and chronic disease. Interestingly, isolates from group I, II and III are associated with acute infections, whereas groups IV and V consist of isolates associated with chronic infections (group VI contains the *C. burnetii* str. Dugway isolates, which have low virulence in guinea pigs). Other genotyping approaches have taken advantage of the fully sequenced *C. burnetii* genome using either multispacer sequence typing (MST) or multiple-locus variable number of tandem repeats analysis (MLVA). MST analysis suggested that the plasmids QpH1 and QpDV are associated with acute disease isolates, and QpRS is associated with chronic disease isolates, indicating a correlation between genotype and disease manifestation[118]. Similarly to MST analysis, MLVA analysis demonstrated that there are genetic differences between isolate groups from acute and chronic disease, suggesting that isolates cluster into distinct groups according to disease severity[118,121]. Consistent with this, there are no polymorphic ORFs between the isolates in restriction group I and the reference isolate *C. burnetii* str. RSA 493 (also in group I), but 87 polymorphic ORFs were identified between group V isolates and *C. burnetii* str. RSA 493, and at least half of these were annotated as hypothetical proteins[123]. These results are intriguing because isolates from group V are associated with chronic infections, suggesting that novel virulence factors contribute to distinct acute and chronic pathotypes[119].

In a mouse infection model, isolates from group I induce persistent high-level cytokine secretion throughout the duration of infection, whereas group IV and group V isolates induce only moderate secretion at the peak of clinical disease [124]. In the guinea pig model, isolates from group IV fail to induce fever, whereas group I isolates induce high fever and cause death at the highest challenge dose. In the severe combined immunodeficiency (SCID) mouse, which cannot mount an acquired immune response, group I isolates cause severe cachexia and death, whereas group IV and group V isolates induced only transient weight loss. Although this evidence clearly suggests that genetic pathotype differences exist, host factors are also likely to be involved in the development of chronic Q fever, as patients who develop a chronic infection often exhibit elevated interleukin-10 (IL-10) production[125], and mice that constitutively express IL-10 have been proposed as a suitable chronic model for Q fever[126]. It is likely that both pathotype-specific differences and host factors are involved in the development of chronic Q fever.

Isolate (strain name)	Genome size (bp)	Plasmid present	Source	Restriction group	MST group	Acute disease potential[124]
C. burnetii str. Nine Mile I RSA 493	1,995,281	QpH1	Tick, USA, 1935	I	16	High virulence
C. burnetii str. Henzerling RSA 331	2,016,427	QpH1	Human blood, Italy, 1945	II	18	Not determined
C. burnetii str. CbuK_Q154	2,102,380	QpRS	Human heart valve USA, 1976	IV	8	Low virulence
C. burnetii str. CbuG_Q212	2,008,870	None	Human heart valve, Canada, 1981	V	21	Intermediate virulence
C. burnetii str. Dugway	2,158,758	QpDG	Rodent, USA, 1958	VI	20	Low virulence

Isolate (strain name)	Genome size (bp)	Plasmid present	Source	Restriction group	MST group	Acute disease potential[124]
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C. burnetii is readily transmitted between hosts and environmental reservoirs, a characteristic that is partially attributed to the ability of the bacterium to survive in the environment for long periods of time. The environmental stability of the organism seems to be linked to its ability to transition between distinct developmental stages. Small cell variants (SCVs) are metabolically inactive and resistant to numerous harsh environmental conditions. The switch from SCV to large cell variant (LCV) occurs after the invasion of host cells and during acidification of the phagosome, which triggers *C. burnetii* to become metabolically active[6,7]. Until recently, the only characterized virulence factor of *C. burnetii* was lipopolysaccharide (LPS), which was identified through the observation that serial passage in a non-immunologically competent host results in the transition from a smooth to a rough phenotype and the loss of virulence[8]. Virulent smooth cell variants contain LPS with a complete O antigen and are referred to as phase I organisms, whereas avirulent rough cell variants lack the terminal O antigen sugars and are referred to as phase II organisms. The identification and characterization of other virulence factors has been facilitated by two recent advances: the development of axenic culture media and improvements in the tools available for genetic manipulation (BOX 3).

Box 3

Recent progress in the development of extracellular growth media and genetic tools

The first successful introduction of exogenous plasmid DNA into *Coxiella burnetii* was reported more than 10 years ago and involved the transformation of eukaryotic host cell-propagated *C. burnetii* with a shuttle vector containing a 5.8 kb autonomous replication sequence (ARS)[127,128]. The generation of new genetic tools has benefited from the development of axenic media to propagate *C. burnetii*. This advance was made after a breakthrough metabolic study demonstrated that the organism has enhanced metabolic potential *in vitro* in the presence of acid activation buffer (pH 4.5)[129]. The second major breakthrough came after the discovery that terminal oxidases are associated with micro-aerophilic respiration[130]. This led investigators to test the metabolic potential of *C. burnetii* at low oxygen levels, which revealed that 2.5% oxygen is optimal for growth and metabolism[130]. In turn, these two observations led to the development of acidified citrate cysteine medium (ACCM), which allows for an ~3 log increase in the number of genome copies after 6 days of culture[130].

Shortly after the development of ACCM, genetic transformation and the isolation of single colonies on solid medium were achieved using an improved medium (ACCM-2)[75]. The ability to isolate single colonies allowed the *Himar1*-based transposon system to be used for the generation of mutant libraries[131]. The original *C. burnetii Himar1* mutants were isolated after electroporation and infection of Vero cells, a technical and laborious process[131]. A second important genetic tool was a stably maintained shuttle vector

plasmid for *C. burnetii* containing the plasmid RSF1010 origin of replication (adapted from use in *Legionella pneumophila*)[127]. As an alternative approach to transformation with plasmids, a site-specific Tn*7* transposition system was recently developed[132]. Finally, an easy-to-use, site-directed mutagenesis strategy has now been developed, which will accelerate the testing of virulence gene candidates in *C. burnetii* mutants[64]. This complement of genetic tools and extracellular growth methods will now allow for the fulfilment of Koch's postulates to verify the functions of a number of genes, including the candidate virulence factor genes.

In this Review, we describe our current understanding of the molecular pathogenesis of *C. burnetii*, including a discussion of a newly identified virulence factor, the type IV Dot/Icm (defect in organelle trafficking/intra-cellular multiplication) secretion system. We also describe how advances in extracellular cultivation techniques, genetic modification of clonal populations and the use of animal models of acute disease are helping to further our understanding of this intracellular pathogen.

The *Coxiella burnetii* infection cycle

Adhesion and invasion

After aerosol transmission, *C. burnetii* targets alveolar macrophages and passively enters these cells by actin-dependent phagocytosis[9,10]. The identification of a type IV secretion system (T4SS) in *C. burnetii* suggested that this bacterium uses an active trigger mechanism (mediated by the secretion of T4SS effector proteins) to induce uptake after initial binding to the host cell[11]. However, recent studies using T4SS mutants have revealed that these bacteria have no defect in their ability to invade either phagocytic cells (monocytes and macrophages) or non-phagocytic cells, supporting the idea that entry into phagocytic cells proceeds via passive actin-dependent phagocytosis and that entry into non-professional phagocytes proceeds via an active zipper mechanism[12,13]. Non-phagocytic cells do not usually engulf large particles; however, intracellular pathogens can invade these cells after direct contact through the zippering of bacterial ligands to host cell receptors.

Virulent *C. burnetii* binds to phagocytic cells using as the main receptor and enters the cell $\alpha_V\beta_3$ integrin via RAC1-dependent phagocytosis, which requires membrane ruffling[14,15] (FIG. 1). Interestingly, $\alpha_V\beta_3$ integrin is typically involved in the removal of apoptotic cells via phagocytosis, and this is generally associated with an inhibition of inflammation[16]. Thus, the ability to use $\alpha_V\beta_3$ integrin for invasion might be exploited by *C. burnetii* as a mechanism to avoid the induction of an inflammatory response. Consistent with this, *C. burnetii* is characterized as a stealth pathogen that enters cells without alerting the immune system. Although the identity of the bacterial ligand for $\alpha_V\beta_3$ integrin has not been determined, a class of likely candidates would be membrane-associated proteins that contain the integrin-binding domain arginine-glycine-aspartic acid (RGD)[11]. $\alpha_V\beta_3$ integrin is poorly expressed on resting monocytes, so it is likely that *C. burnetii* uses other receptors to invade these cells[17].

Although the identity of the receptor on non-professional phagocytes is currently unknown, the mechanism of *C. burnetii* entry into these cells also appears to be dependent on actin rearrangement[9]. As the β_3-subunit is poorly expressed on bronchial epithelium, it seems unlikely that $\alpha_V\beta_3$ integrin is the primary receptor for uptake in non-professional phagocytes, assuming that invasion of non-professional phagocytic cells is an important component of respiratory infection[18]. Histopathological analysis was carried out on the lungs of both animal models of pneumonia and human patients with atypical pneumonia caused by *C. burnetii*, and this analysis identified monocytes and macrophages as the primary infection sites, but epithelial and endothelial cell infection was also evident[19]. In a variety of tissue culture models, including mouse fibroblasts (L929), African green monkey kidney cells (Vero), human monocytes (THP-1) and mouse macrophages (J774), *C. burnetii* cells of different phases exhibit distinct uptake kinetics, whereby avirulent phase II bacteria are more readily internalized than fully virulent phase I bacteria[9,10]. Although this is an interesting phenomenon, the mechanisms by which these variants enter host cells is not discussed in depth here, as phase II *C. burnetii* does not cause infections in immunocompetent animal models or humans[20]. However, both phase I and phase II *C. burnetii* replicate with similar kinetics and in indistinguishable, proteolytically active CCVs in tissue culture cells[21], which is important to note because many studies that examine intracellular trafficking (see below) have used the avirulent phase II variant (as it requires only BL2 containment)[22].

Both phase I and phase II variants engage the $\alpha_V\beta_3$ integrin receptor in monocytes, but dramatic reorganization of the filamentous (F)-actin cytoskeleton is observed with phase I bacteria only[14]. Cytoskeletal changes during invasion are a common effect of several other invasive pathogens that promote phagocytosis through the secretion of effectors which activate host GTPases[23,24]. Although the T4SS of *C. burnetii* is not required for uptake by the host cell, it remains to be determined whether T4SS effectors contribute to the reorganization of the actin cytoskeleton during infection[12,13]. Interestingly, actin-cytoskeletal ruffling induced via SRC tyrosine kinases is observed following the binding of phase I *C. burnetii*[25]. This membrane ruffling requires contact between *C. burnetii* and host cells and can be induced using purified LPS from phase I bacteria[25,26]. The ability to induce these ruffles is also dependent on the expression of Toll-like receptor 4 (TLR4) on the host cell surface[27]. These observations suggest that LPS induces membrane ruffling and the T4SS effectors temporally control downstream signalling cascades after internalization, although this has not yet been demonstrated experimentally.

From phagosome to CCV

Phagocytosis results in the formation of the phagosome, which matures into a phagolysosome following a series of highly ordered and regulated fusion and fission events[1]. Shortly after internalization at the cell surface, the nascent phagosome develops into an early phagosome and acquires the small GTPase RAB5. This GTPase stimulates fusion with early endosomes, resulting in acidification of the lumen to approximately pH 5.4 (through an incompletely defined process) and acquisition of the early-endosomal marker protein EEA1 (REF. 28). One of the most striking features of the maturing phagosome is that its size remains constant, indicating that membrane components are constantly removed

after fusion events[1]. The late phagosome lacks RAB5 but acquires the GTPase RAB7, lysosome-associated membrane glycoprotein 1 (LAMP1), LAMP2 and the vacuolar ATPase, which pumps protons into the maturing phagosome to further decrease the lumenal pH to about pH 5 (REF. 28). Finally the phagosome fuses with lysosomal compartments to acquire cathepsins and hydrolases, and the vacuolar ATPase further reduces the pH to around pH 4.5 (REFS 1,28).

The maturation of the CCV essentially follows the canonical endosomal pathway described above, but there are a number of important differences that rely on specific bacterial proteins. Thus, these differences are considered pathogenic mechanisms, although how they benefit *C. burnetii* remains to be determined. After internalization of the bacterium, the nascent CCV is similar in size to a nascent phagosome and sequentially recruits RAB5 and RAB7, indicating that it traffics through the typical endosomal cascade described above[22] (FIG. 1). However, maturation of the CCV involves expansion of this compartment to a size that almost completely fills the host cell cytoplasm, an aspect that differs vastly from the unchanging size of the maturing phagolysosome[1,29] (FIG. 1). Simultaneous functional inhibition of both RAB5 and RAB7 through dominant-negative mutations impairs expansion of the CCV, whereas inhibition of RAB5 alone (but not RAB7 alone) blocks *C. burnetii* entry into host cells[22]. Furthermore, formation of the large CCV is dependent on active *C. burnetii* protein synthesis, probably because of a requirement for the synthesis and subsequent secretion of effectors across the CCV membrane into the cytoplasm[5,12]. However, in the absence of protein synthesis, small CCVs still acquire LAMP proteins and acidify, suggesting that the acquisition of LAMPs and acidification are passive processes, similar to the normal maturation process of phagosomes[30].

Most intracellular pathogens subvert the endosomal cascade and arrest maturation of the phagosome at an early stage to avoid fusion with lysosomes[2]. However, in *C. burnetii*, lysosomal enzymes, including cathepsin D (CTSD) and lysosomal acid phosphatase (ACP2), accumulate in the CCV, although this is delayed until 2 hours after infection (compared with only 15 minutes after infection for phagosomes harbouring inert particles)[4]. It has been suggested that the delay is caused by interactions of the CCV with the autophagy pathway[22]. The T4SS of *C. burnetii* is unlikely to be involved, as translocation of effectors does not occur until at least 8 hours after infection, suggesting that the bacterial proteins which interact with the autophagy pathway remain to be identified[13]. Autophagy is implicated in host defence through innate and adaptive immunity to intracellular pathogens[31], and as early as 5 minutes after internalization, the CCV is decorated with the autophagy marker microtubule-associated protein light-chain 3 (LC3)[3,22] (FIG. 1). However, the role of autophagy in *C. burnetii* pathogenesis is unclear; a recent genome-wide RNAi screen failed to show that autophagy is essential for *C. burnetii* growth. On the other hand, if autophagy is induced in the host cell before infection with *C. burnetii*, the intracellular bacterial load and the size of the CCV increase[32,33]. One potential benefit of the autophagy interaction is that autophagy vesicles are often loaded with nutrients and membranes from their degraded cargo, and these components might serve as fuel for the SCV-to-LCV conversion[22,32]. The conversion to LCVs coincides with the arrest of CCV endosomal maturation; the percentage of LCVs (as a proportion of the total number of SCVs

and LCVs) 1 hour after infection approaches 80%, and by 16 hours the CCV contains only LCVs[4,6] (FIG. 1).

Between 8 hours and 2 days after infection, the CCV enlarges drastically and can occupy nearly the entire volume of the host cell[34]. The large CCV forms as a result of homotypic fusion of multiple smaller CCVs, and it can continue to expand through heterotypic fusion with autophagic, endocytic and lysosome vesicles[5,30]. Maintenance of the large CCV not only requires protein production by the bacterium[30], but also depends on the actin cytoskeleton, as treatment of infected cells with actin-depolymerizing agents results in the formation of small CCVs only[35]. In addition, *C. burnetii* infection activates the host cell kinase families protein kinase C, protein kinase A and myosin light chain kinase, which are required for the establishment and maintenance of the CCV[36]. Furthermore, during this stage of the infection, the CCV interacts with the early secretory pathway (as suggested by the accumulation of RAB1B on the CCV membrane), an interaction which is required for formation of the large CCV[37] (FIG. 1). Interaction with the ER through the early secretory pathway might provide a source of lipids for the formation of the large CCV. These interactions are probably orchestrated by *C. burnetii* effectors, as bacterial protein production is required for the formation of the large CCV; however, the identity and specific functions of these putative effectors remain to be determined[37]. Interestingly, the CCV membrane contains a comparable amount of cholesterol to that in the plasma membrane, which is twice that of a normal lysosomal compartment[38]. Furthermore, host genes involved in cholesterol synthesis are upregulated during *C. burnetii* infection, and inhibition of cholesterol metabolism negatively affects CCV formation, suggesting that *C. burnetii* directly influences cholesterol metabolism, potentially through interactions with the ER[38–40]. Although these events are reasonably well characterized, the *C. burnetii* virulence factors that direct formation of the CCV remain to be identified.

Mature CCV

Approximately 6 days after infection, the CCV is heavily loaded with LCV bacteria, which start differentiating back into SCVs[6] (FIG. 1). The mature CCV maintains essentially the same features as the early CCV: the pH remains low (it has a pH of about 4.5–5, the same pH as the phagolysosome in uninfected cells[41]) and the vacuole contains all of the same markers. In addition, the mature CCV retains fusogenic capacity, which relies on *C. burnetii* protein synthesis[5]. Surprisingly, host cell viability is not affected by the drastic expansion of the CCV, which occupies much of the cytoplasm and with a volume much larger than the space required by *C. burnetii*[42]. In addition, the generation time and genome stability of the host cell is unchanged[43]. *C. burnetii* prolongs host cell viability in two ways: it actively inhibits apoptotic signalling pathways, and it induces pro-survival factors. For example, *C. burnetii* prevents the induction of apoptosis following exogenous activation with the pro-apototic drug staurosporine, and this inhibitory effect is dependent on protein production by the bacterium[42]. The anti-apoptotic activity of *C. burnetii* could be the result of the interplay between beclin 1 (BECN1) and BCL2. BECN1 is an autophagy initiation protein that interacts with the anti-apoptotic protein BCL2, and both are present on the CCV. Their interaction prevents the release of cytochrome *c* from the mitochondria and thereby leads to apoptosis inhibition, a phenomenon associated with *C. burnetii* infection and a known

function of BCL2 (REFS 44,45). A second anti-apoptotic activity initiated after *C. burnetii* infection is the sustained activation of the pro-survival signalling proteins ERK1 (also known as MAPK3), ERK2 (also known as MAPK1) and the AKT family[46].

Promoting the survival of infected cells seems to be essential for the establishment and maintenance of chronic *C. burnetii* infections. There is evidence to suggest that during host cell cytokinesis the large CCV is segregated to only one of the daughter cells, leaving the other cell uninfected[47]. This would provide the bacterium with host cells to infect, thereby promoting a chronic infection. The ability to prevent apoptosis and stimulate pro-survival pathways would also be beneficial for persistent infection, as this activity maintains the host cell to allow continued replication of the bacterium. During acute infection, the infectious dose can be as low as 1–10 *C. burnetii* cells, which suggests that there are other mechanisms in place that promote the spread of replicating bacteria to infect other susceptible cells[48]. A recent report demonstrated that *C. burnetii* is also able to induce apoptosis through the release of cytochrome *c* in a mechanism that is dependent on bacterial protein synthesis[49]. Therefore, the prevention of apoptosis seems to be exploited by *C. burnetii* to cause persistent infections, whereas induction of apoptosis seems to enable spread of the infection to nearby susceptible cells. This strategy is also used by other intracellular pathogens; for example, *Mycobacterium tuberculosis* inhibits apoptosis early during infection, but induces cell death at a later stage of infection[50].

Dot/Icm secretion and host subversion

Sequencing of the *C. burnetii* genome identified three secretion systems: a type I secretion system (T1SS)[51], a T2SS-related pilus biogenesis machinery[52] and a conjugation-related T4SS[53]. Currently, little is known about the role of the T1SS and T2SS in the pathogenesis of *C. burnetii*. By contrast, the availability of *Legionella pneumophila* as a surrogate host for the expression of putative *C. burnetii* T4SS effectors has provided insight into the role of this secretion system, and this is the focus of the discussion below.

The T4BSS (the Dot/Icm secretion system)

T4SSs translocate effector substrates from the bacterial cytosol directly into the cytosol of eukaryotic host cells and are intimately involved in the pathogenesis of many bacteria. These multicomponent protein machines can be subdivided into several substructures, including a pilus, a core transport complex and a type IV coupling protein complex[54] (FIG. 2). Furthermore, T4SSs are separated into two subgroups, T4ASS and T4BSS, on the basis of homology to the *Agrobacterium tumefaciens* and *L. pneumophila* systems, respectively[55]. The T4BSS, which is also known as the Dot/Icm system, resembles the conjugation machinery encoded on IncI plasmids. Studies with *L. pneumophila* have identified several core complexes that define the topology of the Dot/Icm apparatus, such as the subcomplex consisting of DotC, DotD, DotF (also known as IcmG), DotG (also known as IcmE) and DotH (also known as IcmK), which bridges the inner and outer bacterial membranes and is functionally the core transport complex[56] (FIG. 2). A second subcomplex comprises the coupling protein DotL (also known as IcmO) (which recruits IcmSW-dependent substrates to the secretion apparatus), DotM (also known as IcmP), DotN (also known as IcmJ), IcmS and IcmW[57] (FIG. 2). The *C. burnetii* str. Nine Mile I isolate encodes homologues for 24 of

the 27 Dot/Icm proteins found in *L. pneumophila*, and this high degree of similarity between the two systems suggests that they are structurally similar[11]. The *C. burnetii* genome lacks *dotV* (also known as *icmC*), *icmR* and *dotJ* (also known as *icmM*), but has a gene duplication of *dotI* (also known as *icmL*; resulting in the genes *icmL.1* and *icmL.2*); the similarity of DotJ and DotI suggests that this DotI duplication can substitute for the absence of DotJ, a functional homologue of IcmR[11,58] (FIG. 2). The conservation between these two T4BSSs became apparent when it was shown that *dotB, icmS, icmW* and *icmT* from *C. burnetii* can complement the intracellular growth defect associated with an *L. pneumophila* strain in which these genes are mutated[59]. This was the first indication that *C. burnetii* encodes a functional Dot/Icm system and led to the use of *L. pneumophila* as a surrogate host to identify and characterize *C. burnetii* effector proteins[60,61] (TABLE 1). Using this system, transcriptional analysis demonstrated that several *C. burnetii dot/icm* genes are actively transcribed at early time points after infection[62], and similarly to in *L. pneumophila*, the Dot/Icm system localizes to the poles of *C. burnetii* cells during infection[63]. Using transposon and site-specific mutants, several studies have now confirmed the dependence of *C. burnetii* on the Dot/Icm system for intracellular survival[64,12,13]. Current research is now focused on identifying the roles of the T4SS effectors to provide insight into the pathogenic mechanisms of this intracellular bacterium.

Identification and characterization of Dot/Icm substrates

Initially, all *C. burnetii* effector screens used *L. pneumophila* as a surrogate host[65,66]. However, the development of *C. burnetii* shuttle vectors (BOX 3) enabled the adaptation of fluorescence-based β-lactamase (TEM1) translocation assays for *C. burnetii*, which were used to confirm the results observed using *L. pneumophila*[61,67]. Recent genetic and bioinformatic screens have identified 60 *C. burnetii* Dot/Icm substrates (the best characterized of which are shown in TABLE 1), and ~60 further substrates have recently been added to this list[13,60,61,65,67,68] (Robert A. Heinzen, personal communication). To date, the target and function of the majority of the ~120 Dot/Icm substrates remain undefined. A large number of the encoding genes (23 of the 60 substrate genes initially characterized) have a GC content that is significantly different from the average GC content of the *C. burnetii* genome (42.7%)[61]. The obligate intracellular nature of *C. burnetii* and the presence of eukaryotic-like domains in many substrates suggest that the genes encoding these substrates were acquired by interdomain horizontal gene transfer from a eukaryotic source[51,69]. Furthermore, similarly to *L. pneumophila* effectors, many of the *C. burnetii* T4SS substrates contain a carboxy-terminal motif or recognition sequence that is required for translocation[61,66,67].

Bacterial pathogens use the T4SS as part of their virulence programme to control the activity of various effector proteins in order to ensure the proper progression of infection. For example, the *L. pneumophila* effector protein LubX acts as an E3 ubiquitin ligase that targets the effector SidH for degradation several hours after infection, representing a mechanism of temporal control[70]. *C. burnetii* effector functions are likely to be controlled by similar temporal mechanisms, including transcriptional control, translocation efficiency and protein stability in host cells. This hypothesis was supported by the recent observation that *C. burnetii* Dot/Icm effectors are not translocated until 8 hours after infection and also

require acidification of the CCV for translocation[13,71]. Although this expression pattern is in agreement with the ability of *dot/icm* mutants to passively traffic through the endocytic pathway to the phagolysosome, it does not explain how *C. burnetii* is able to manipulate the autophagy pathway immediately after uptake into host cells, nor how the bacterium delays lysosomal fusion[12,13]. Thus, it is possible that T4SS-independent effectors are involved in the manipulation of the autophagy pathway and the delay in lysosomal fusion.

Another mechanism of control is executed at the transcriptional level by the two-component regulatory system PmrAB, which has been shown to directly regulate the Dot/Icm secretion system in both *L. pneumophila* and *C. burnetii*[72]. The PmrAB system regulates virulence genes in other pathogenic bacteria, such as *Pseudomonas aeruginosa*, in response to low Mg^{2+} levels, high Fe^{3+} levels and low pH[73]. Bioinformatic analysis revealed that genes encoding five of the structural Dot/Icm proteins (*dotP* (also known as *icmD*), *icmQ*, *icmV*, *icmW* and *dotD*) contain a PmrA-binding sequence located upstream of the −10 region of their promoters. Furthermore, 20 putative PmrA-regulated *C. burnetii* genes have been confirmed as Dot/Icm substrates, indicating that this system might represent a global virulence regulator in *C. burnetii*[61,65,72]. Bioinformatic analysis also suggests that other unknown mechanisms exist for regulating non-PmrA-regulated effectors, as many newly identified effectors do not contain PmrA-binding sequences but are still secreted. Although *L. pneumophila* and *C. burnetii* are phylogenetically related, only ten of the identified Dot/Icm substrates from *C. burnetii* display significant similarity to any of the >250 *L. pneumophila* T4SS substrates. This is not surprising considering that *L. pneumophila* and *C. burnetii* replicate in distinct vacuolar compartments during co-infection[74], and suggests that *L. pneumophila* and *C. burnetii* Dot/Icm effectors have specific functions that target diverse vesicle trafficking pathways to create a distinct permissive replicative environment for each bacterium.

Plasticity and redundancy in Dot/Icm substrates from different pathotypes

The Dot/Icm effectors exhibit striking heterogeneity among the sequenced C. *burnetii* isolates belonging to different pathotypes[13]. Only 19 of the currently identified effectors, including three plasmid-encoded effectors, are fully conserved among the acute and chronic isolates (TABLE 1). The fact that these effectors have been maintained in in all *C. burnetii* isolates suggests that they have essential roles for intracellular survival. The plasticity of the remaining effectors might be mediated, in part, by extensive recombination between abundant insertion sequences (ISs) found within the *C. burnetii* genome[51]. In isolates causing chronic and avirulent disease, the majority of homologues for the *C. burnetii* Nine Mile I effectors are either truncated or found as pseudogenes. This suggests that the truncated homologues are nonfunctional, a hypothesis supported by the observation that ectopic expression of CBU1532 (encoded by *C. burnetii* str. Nine Mile I) leads to rounding of the host cell, whereas the alternative-start homologue CBUD0454 (encoded by *C. burnetii* str. Dugway) does not induce this phenotype[13]. Thus, we speculate that several group-specific Dot/Icm effectors participate in pathotype-specific virulence, a hypothesis that is now testable with site-specific mutagenesis methods[64,75].

Compared with other secretion systems, the Dot/Icm system from *L. pneumophila* has an astounding number of substrates. Dot/Icm is capable of translocating at least 8.5% of the *L. pneumophila* proteome (which corresponds to approximately 250 out of 2,943 ORFs). Why would a pathogen need so many effectors? An intriguing recent study suggests that at least 30% of *L. pneumophila* Dot/Icm effectors are not involved in the establishment of mammalian infection, but have been acquired instead to enable adaptation to multiple hosts[76]. Although a survey of the *C. burnetii* proteome for secretion substrates is incomplete, it seems that a substantial proportion of the proteome — to date, 5.8% — does serve as T4SS substrates[13,60,61,65,67,68] (Robert A. Heinzen, personal communication). *C. burnetii* is capable of infecting and colonizing a wide range of mammalian and arthropod hosts, and a diverse pool of T4SS substrates might facilitate this wide host range. In addition, *L. pneumophila* encodes several examples of multiple effectors that target the same host pathway to orchestrate intracellular bacterial survival. Evidence suggests that *C. burnetii* uses a similar strategy, as three effectors have been identified that inhibit apoptosis (AnkG, CaeA and CaeB)[77,78] (TABLE 1).

Diverse cytosolic functions for Dot/Icm effectors

The majority of the identified Dot/Icm substrates are annotated as hypothetical proteins. However, most encode one or more eukaryotic-like domains implicated in protein–protein interactions, and these domains provide clues to protein functionality. These domains include ankyrin repeats, coiled-coil domains and tetratricopeptide repeats[13,61,66] (TABLE 1). Other substrates have eukaryotic-like domains that are implicated in post-translational modifications, such as putative serine/threonine kinase domains, F-box domains and Fic domains[13,61,67] (TABLE 1). Eukaryotic proteins carrying these motifs are involved in a variety of host processes, including apoptosis, ubiquitylation, lipid metabolism and membrane trafficking, suggesting that *C. burnetii* effectors are involved in modulating many of these cellular processes[65,79–83]. For example, the T4SS effectors AnkG, CaeA and CaeB all prevent intrinsic apoptosis[77,78] (FIG. 3; TABLE 1). Modulation of apoptosis by AnkG is dependent on the ability of AnkG to bind the host mitochondrial matrix protein p32 (also known as C1QBP); the mechanism of action of p32 during apoptosis has not yet been established, but the protein is thought to regulate the opening of the permeability transition pore[78]. CaeB localizes to the mitochondria and inhibits mitochondrial membrane permeabilization, thus preventing the release of pro-apoptotic proteins[77] (FIG. 3). These two effectors target the same cellular pathway, albeit through different mechanisms. Although CaeA localizes to the nucleus, the exact mechanism of apoptosis inhibition by this protein remains to be determined[77]. As discussed above, for *C. burnetii*, the benefits of inhibiting apoptosis include the maintenance of host cell viability, despite the mature CCV occupying almost the entire volume of the cell.

Functional assays serve as a starting point for characterizing the many effector functions of *C. burnetii*, and ectopic expression of effector proteins in eukaryotic cells has demonstrated that many effectors target specific host organelles[13,61,66,67]. For example, at least three effectors (CaeA, CBU1314 and CBU1976) are targeted to the nucleus[13,61], and this probably depends on the presence of nuclear localization motifs (TABLE 1). As *C. burnetii* infection has been shown to alter host gene transcription[84], it is reasonable to suggest that

these putative nuclear effectors are involved in this process (FIG. 3). In addition, several vesicle-associated effectors target the CCV and autophagosome compartments[13,66]. Recently, researchers have identified a family of CCV-binding proteins, several of which are essential for intracellular replication (Robert A. Heinzen, personal communication). These effectors might serve as CCV stabilizers or as docking points for signalling molecules, and/or they might promote the fusogenic activities of the CCV (FIG. 3). Because *C. burnetii* alters the fusogenic capacity of the CCV, identifying effectors that disturb the secretory pathway in eukaryotic cells is crucial. *C. burnetii* actively modulates vesicle trafficking pathways by recruiting membranes to the CCV, and there is evidence that the early secretory pathway contributes to CCV biogenesis[37]. Dot/Icm effectors that interfere with the host secretory pathway have been identified for *L. pneumophila* using a SEAP (secreted embryonic alkaline phosphatase) assay[65,85–87]. In *C. burnetii*, an effector (CBU0635) that disrupts mammalian secretory pathways has been identified using the same assay and localizes next to the Golgi apparatus[13] (FIG. 3; TABLE 1). Further analysis will be required to determine the mechanism of action of CBU0635 and its contribution to *C. burnetii* virulence.

Ubiquitin-related F-box-containing proteins represent an interesting group of Dot/Icm effectors. Similarly to *L. pneumophila*, *C. burnetii* encodes several paralogues of F-box-containing proteins; three of them have been confirmed to be Dot/Icm effectors, and one (CpeC) is associated with ubiquitin[61,67]. On the basis of their function in eukaryotic cells, this group of proteins might promote proteasome-mediated protein degradation[70,88]. For example, AnkB (an F-box protein) of *L. pneumophila* targets non-essential host proteins (through ubiquitylation) for degradation by the 26S proteasome, providing a source of amino acids for bacterial growth[88]. A second possible mechanism involves the degradation of host proteins that impede bacterial replication. Whether *C. burnetii* F-box proteins have similar roles remains to be determined.

Our knowledge of the functions of the Dot/Icm effectors from *C. burnetii* is still in its infancy. However, the recent description of a site-directed mutagenesis system for *C. burnetii*[64] should aid in the characterization of all ~120 identified effectors and help to establish how they contribute to pathogenesis.

Immune evasion strategies

C. burnetii has several immune evasion strategies that are associated with the structure of its LPS. Virulent phase I *C. burnetii* produces LPS with a complete O antigen and exhibits serum resistance (the bacterium only moderately activates complement and prevents surface deposition of complement factor C3b)[89]. Furthermore, the structure of this so-called smooth LPS masks bacterial recognition by the pattern recognition receptor TLR2 (REF. 90), which recognizes ligands within LPS. By contrast, avirulent phase II *C. burnetii* produces a rough LPS, which lacks the terminal O antigen sugars and is readily detected by TLR2; this detection induces the production of interleukin-12 (IL-12) and tumour necrosis factor (TNF), and activates macrophages to mediate bacterial clearance[91]. Conversely, phase I *C. burnetii* does not induce maturation of primary dendritic cells (DCs) and induces the production of relatively low levels of IL-12 and TNF[90]. This suggests that the LPS of phase

I bacteria, containing the full-length O antigen, shields TLR2 ligands on the bacterial cell surface[90]. Other studies have shown that LPS from both phase I and phase II *C. burnetii* induces the production of TNF in macrophages, but this induction is likely to be due to contaminating TLR2 ligands, as purified LPS (which could contain contaminating TLR ligands) but not chromatography-purified lipid A (the LPS component recognized by TLR4) induces TLR2 signalling[15,91,92].

Early studies showed that *C. burnetii* LPS is ~1,000-fold less endotoxic than the LPS from enteric species such as *Escherichia coli*[93]. It was subsequently discovered that *C. burnetii* uses a second LPS-dependent intrinsic immune evasion strategy: rather than serving as an agonist of TLR4 signalling, *C. burnetii* lipid A acts as a TLR4 antagonist[91]. The lipid A portion of *C. burnetii* LPS (both phase I and phase II variants) is composed of a tetra-acylated structure, a form of lipid A that is associated with antagonism of TLR4 signalling in several other bacterial pathogens, including *Yersinia pestis*[94–96]. Elucidating the role of TLR4 in *C. burnetii* pathogenesis was complicated by the observation that this cell surface receptor seems to be involved in actin rearrangement and phagocytosis of virulent phase I *C. burnetii*[27]. However, studies suggest that the ability of TLR4 to distinguish between the LPS of phase I and phase II variants is not dependent on the structure of lipid A, but depends on novel signalling mechanisms involving O antigen-mediated phagocytosis (the O antigen is not present in phase II LPS)[27,91]. Further studies will be required to resolve these findings, especially because the transcriptional profiles of host cells infected with either phase I or phase II *C. burnetii* do not show expression of genes required for TLR4 signalling[97].

The ability of *C. burnetii* to evade detection by pathogen recognition receptors prevents the activation of infected macrophages and provides a replication-permissive intracellular niche. A number of studies have shown that the activation of infected cells via interferon- (IFN) inhibits *C. burnetii* replication[98–101] owing primarily to the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS)[102]. *C. burnetii* prevents the production of ROS via the secretion of an acid phosphatase (CBU0335); the mechanism of action has yet to be confirmed, but it is likely that this protein prevents assembly of the NADPH oxidase complex, although this mechanism would be insufficient to protect against the high level of ROS that is induced by IFNγ[103,104]. RNS production requires *de novo* synthesis of inducible nitric oxide synthase (iNOS), which occurs after the induction of pro-inflammatory cytokines such as those produced after TLR stimulation[105]. Indeed, in contrast to *E. coli* LPS, which stimulates macrophages to secrete nitrates, exposure of macrophages to *C. burnetii* does not lead to nitrate secretion, which is not surprising considering that *C. burnetii* invades cells without stimulating TLRs[97].

Host cell death is often induced after infection as an immune defence mechanism[50]. This serves two purposes: it eliminates the infected cell, and it allows the infected apoptotic blebs to be engulfed by DCs and the subsequent presentation of antigens through major histocompatibility complex (MHC) class I molecules, which induces protective immunity to intracellular pathogens[50]. Thus, the ability of *C. burnetii* to inhibit apoptosis represents an immune evasion strategy. Autophagy is another mechanism used by the innate immune system to remove intracellular pathogens. However, as mentioned above, *C. burnetii*

actively recruits autophagy components to the CCV, and the induction of autophagy actually promotes *C. burnetii* intracellular replication[3,32,106].

Conclusions

C. burnetii is ubiquitous in the environment, has a wide host range and has the potential to cause epidemic outbreaks, owing in part to its aerosol transmission and extreme stability. Thus, understanding the pathogenic mechanisms of this organism is a high research priority. From our understanding of the intracellular trafficking pathway and the host processes that are activated or suppressed during infection, it is clear that *C. burnetii* has adapted to a low-pH CCV and inhibits activation of its primary host cells, macrophages, by evading recognition by the innate immune system.

Research on *C. burnetii* is currently undergoing a renaissance. The long-standing idea that LPS is the sole virulence determinant is now considered invalid, and there is a growing appreciation for the essential role of a functional T4SS to promote intracellular growth. The *C. burnetii* T4SS transports more than 100 established substrates, but those that are essential for intracellular replication remain to be determined. Recent reports suggest that, compared with *L. pneumophila*, *C. burnetii* has several essential effectors that are secreted via the T4SS, demonstrating that there is less redundancy in *C. burnetii* than in *L. pneumophila*. High-throughput screening of random and defined mutant libraries is ongoing and will surely identify the next series of essential genes for intracellular growth *in vitro* and *in vivo*. Finally, the development of site-specific mutagenesis methods now allows the roles of predicted pathways and factors to be validated in order to gain an increasingly refined understanding of the molecular pathogenesis of Q fever.

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Glossary

Axenic culture media	Media used for the growth of intracellular bacteria in the absence of host cells
Category B select agent	An infectious disease agent of the second highest priority, as defined by the US CDC guidelines for potential misuse. These agents are usually readily transmitted by aerosol, are stable in the environment and cause moderate morbidity and low mortality
BL3 containment	A level of biocontainment that includes a separation from general traffic areas by double doors, airlocks and negative air flow. Access is limited to trained personnel and requires users to wear personal protective equipment. All research activities with

	potential exposure of the agent to the atmosphere are conducted within biosafety cabinets
Integrin	A highly conserved family of heterodimeric surface glycoproteins involved in binding to extracellular matrix components such as fibronectin and vitronectin through arginine-glycine-aspartic acid (RGD) domains
BL2 containment	A level of biocontainment that involves the use of standard laboratory space in which work using an infectious agent is carried out within a biosafety cabinet
SRC tyrosine kinases	A family of kinases that was originally identified through homology to Rous sarcoma virus oncogene v-*src* and is involved in signal transduction from cellular receptors
Cachexia	Loss of weight, fatigue and weakness that are associated with severe inflammatory disease and cannot be reversed by nutritional supplementation
***Himar1*-based transposon system**	A eukaryotic horn fly element that is extensively used to create mutations in bacteria and relies on only an AT dinucleotide for insertion
***C. burnetii* str. Nine Mile I**	The original *Coxiella burnetii* strain isolated from ticks in 1935. This strain was later serially passaged in embryonated hen eggs and guinea pigs to obtain the avirulent isolate *C. burnetii* str. Nine Mile II
Two-component regulatory system	A bacterial signal transduction system involving a sensor kinase that responds to an environmental stimulus by phosphorylating a response regulator, which controls the transcription of downstream genes
Insertion sequences	Mobile genetic elements consisting of short inverted repeats flanking one or more ORFs
Ankyrin repeats	Eukaryotic protein domains consisting of repeating segments of 33 amino acids that form a helix–turn–helix motif and mediate protein–protein interactions. These domains are some of the most commonly found domains in eukaryotic proteins
Coiled-coil domains	Structural motifs that are found in proteins and consist of 2–5 α-helices wrapped around each other in a left-handed manner to form a superhelix
Tetratricopeptide repeats	Structural motifs that mediate protein–protein interactions and are composed of a degenerate ~34 amino acid sequence that is often arranged in a tandem array

F-box domains	Structural motifs composed of approximately 50 amino acids and that contain tryptophan-aspartic acid repeats. These domains function as protein–protein interaction domains. F-box proteins were first characterized as components of ubiquitin ligase complexes
Fic domains	(Filamentation induced by cyclic AMP domains). Protein domains that mediate ampylation of proteins and regulate protein function

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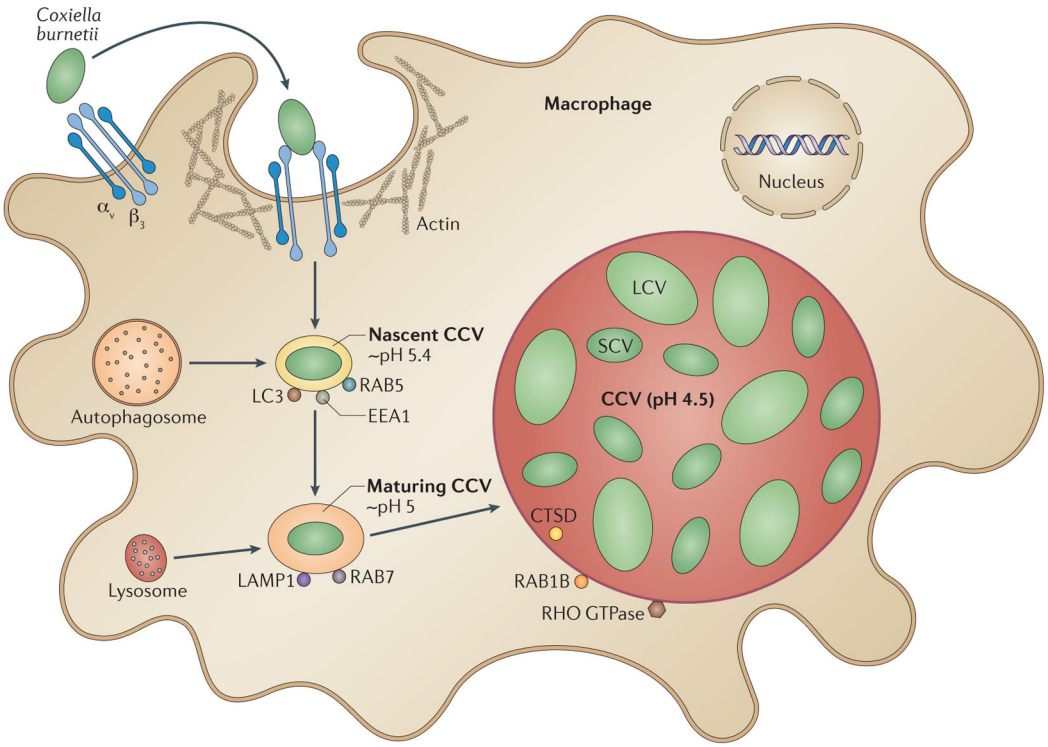

Figure 1. The intracellular trafficking pathway of *Coxiella burnetii*

Coxiella burnetii binds to macrophages through $\alpha_V\beta_3$ integrin, which triggers phagocytosis of the bacterium through an actin-dependent mechanism. The nascent *Coxiella*-containing vacuole (CCV) acquires RAB5 and EEA1 as early as 5 minutes after internalization and acidifies to approximately pH 5.4, which is characteristic of normal phagosomal development. By contrast to phagosomes, the CCV also acquires microtubule-associated protein light-chain 3 (LC3; an autophagosomal marker), a process that is dependent on bacterial protein synthesis. The nascent CCV develops through fusion and fission events with early endosomes and then late endosomes, leading to the disappearance of RAB5 and EEA1 and the acquisition of RAB7 and lysosome-associated membrane glycoprotein 1 (LAMP1) 40 to 60 minutes after internalization, in concurrence with a further acidification to pH 5, which is characteristic of normal phagosomal development. Lysosomal enzymes, including cathepsin D (CTSD), start accumulating in the CCV by 2 hours after internalization, at which point the vacuole is at ~pH 4.5; this is delayed significantly from normal phagolysosomal acquisition of CTSD. This pause in CCV development might allow conversion of the bacteria from small cell variants (SCVs) to the metabolically active large cell variants (LCVs). Between 8 hours and 2 days after internalization, the CCV expands to occupy an increasingly dominant portion of the cytoplasmic space of the host cell. This process is dependent on bacterial protein synthesis and involves the recruitment of both RHO GTPase and RAB1B to the CCV membrane. RHO GTPase is likely to be involved in maintenance of the large vacuole, whereas the recruitment of RAB1B from the ER might facilitate the acquisition of additional membranes to create this spacious CCV.

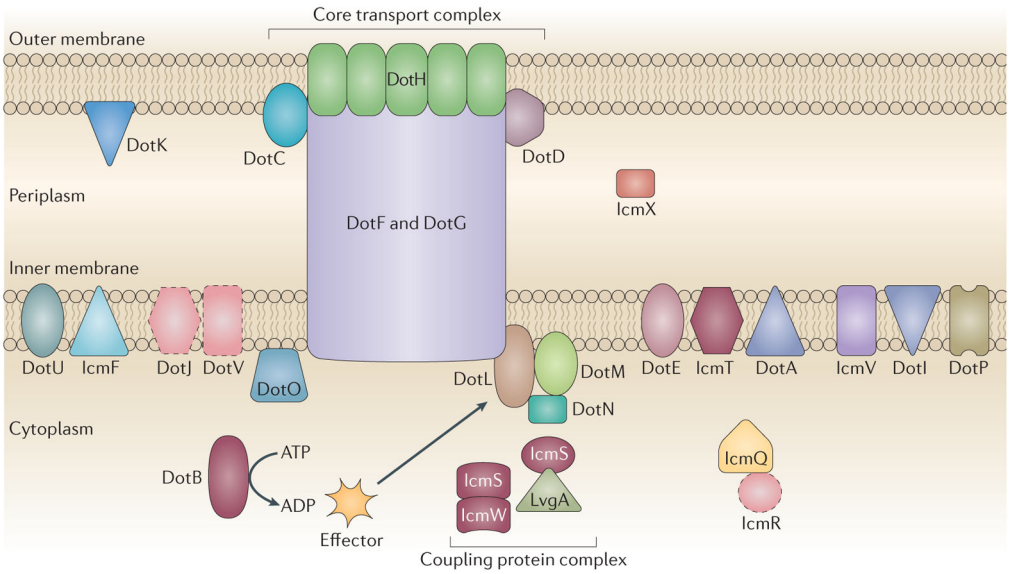

Figure 2. The type IVB secretion systems of *Coxiella burnetii* and *Legionella pneumophila*

Coxiella burnetii encodes 24 of the 27 *Legionella pneumophila* type IVB secretion system (T4BSS) components. The *C. burnetii* system lacks three homologues, DotJ (defect in organelle trafficking J; also known as IcmM), DotV and IcmR (intracellular multiplication R), which are depicted with a dashed outline. Studies using *L. pneumophila* identified a subcomplex termed the core transport complex, which links the inner and outer membranes and is composed of DotC, DotD, DotF, DotG and DotH. As this complex is also conserved in the T4ASS, it is hypothesized that *C. burnetii* assembles a similar complex. A second subcomplex consists of the coupling protein DotL (providing a link between the substrates and the transport complex), DotM, DotN, IcmS and IcmW. The ATPase activity of DotB is required for secretion, but the function of this activity remains to be determined. *C. burnetii* proteins that can substitute for the corresponding proteins in the *L. pneumophila* system are shown in maroon.

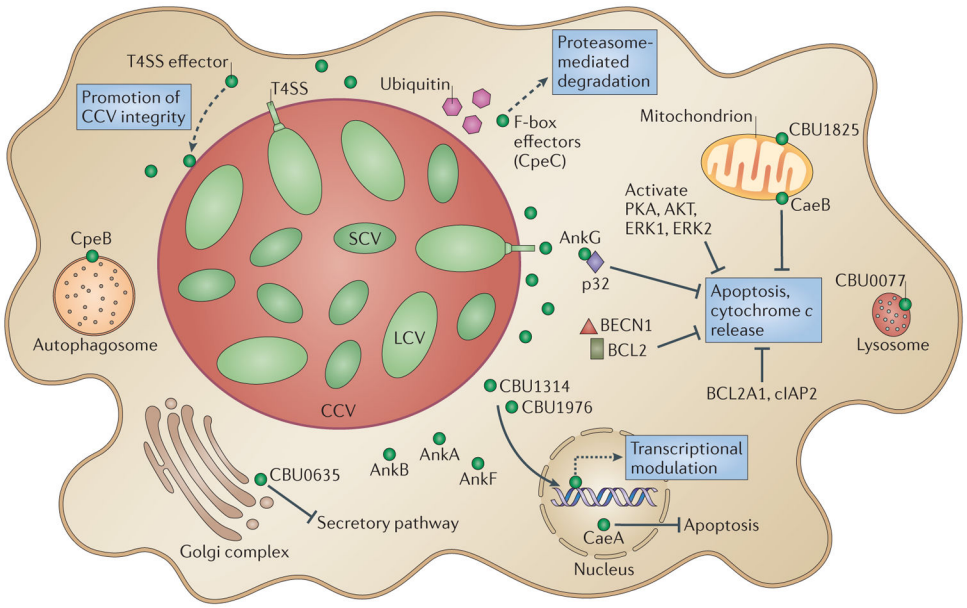

Figure 3. Roles of type IV secretion system effectors during *Coxiella burnetii* intracellular infection

A new family of type IV secretion system (T4SS) effectors has been identified that localizes to the *Coxiella*-containing vacuole (CCV) and might have diverse functions, such as CCV stability and fusogenicity. Located next to the Golgi apparatus, CBU0635 interferes with the host secretory pathway on ectopic expression. At least three T4SS effectors localize to the nucleus (CaeA, CBU1314 and CBU1976) when ectopically expressed in host cells, and these proteins might be involved in the transcriptional modulation that occurs during *Coxiella burnetii* infection. Exogenously stimulated apoptosis is modulated by *C. burnetii*, and an ankyrin repeat-containing protein (AnkG) has anti-apoptotic activity through an interaction with p32, a host protein that is involved in pathogen-induced apoptosis. A second T4SS effector, CaeB, alters mitochondrial membrane permeability during exogenously stimulated apoptosis. *C. burnetii* infection also increases the synthesis of anti-apoptotic proteins BCL2-related protein A1 (BCL2A1; also known as BFL1) and cIAP2 (also known as BIRC3) and causes activation of the pro-survival kinases AKT, ERK1 and ERK2. Other effectors might also be involved in the modulation of apoptosis. Several T4SS effectors have F-box domains and are potentially involved in promoting proteasome-mediated degradation of proteins; these effectors include CpeC, which localizes to ubiquitin-rich compartments in host cells. The effector CpeB has been localized to the autophagosome, and AnkA, AnkB and AnkF localize to the cytoplasm. Dashed arrows represent predicted effector functions that have not yet been experimentally demonstrated. All T4SS effectors are shown as green circles. BECN1, beclin 1; LCV, large cell variant; PKA, protein kinase A; SCV, small cell variant.

Table 1

Coxiella burnetii Dot/Icm substrates with predicted effector functions

***C. burnetii* str. Nine Mile I locus**	**Encoded protein**	**Genetic differences between homologues***			**Encoded protein domains**	**Protein localization‡**	**Protein function**	**Refs**
		***C. burnetii* str. CbuK_Q154**	***C. burnetii* str. CbuG_Q212**	***C. burnetii* str. Dugway**				
CBU0072	AnkA	–	A	AS	Ankyrin	Cytoplasm	ND	60
CBU0144/0145	AnkB	AS	–	–	Ankyrin	Cytoplasm	ND	60
CBU0447§	AnkF	–	–	–	Ankyrin	Cytoplasm	ND	60
CBU0781	AnkG	NT	–	–	Ankyrin	Microtubules	Anti-apoptotic	60,78
CBUA0006§	CpeA	–	–	–	ND	Puncta	ND	67
CBUA0013§	CpeB	–	–	–	ND	Autophagosome	ND	67
CBUA0014	CpeC	A	A	A	F-box	Ubiquitin-rich structures	ND	67
CBUA0015	CpeD	A	A	A	Kinase, coil	ER (partial)	ND	67
CBUA0016	CpeE	A	A	A	ND	No distinct localization	ND	67
CBUA0023§	CpeF	–	–	–	ND	No distinct localization	ND	67
CBU1532	CaeB	AS, CT	AS	AS	ND	Mitochondria	Anti-apoptotic	13,77
CBU1524	CaeA	–	–	AS	ND	Nucleus	Anti-apoptotic	13,77
CBU1314§	Not yet named	–	–	–	Coiled-coil	Nucleus	ND	61
CBU1543§	Not yet named	–	–	–	Coiled-coil	Vesicles	ND	61
CBU1556§	Not yet named	–	–	–	Coiled-coil	Cytoskeleton-like filaments	ND	61
CBU0801§	Not yet named	–	–	–	ND	Vesicles	ND	61
CBU1825§	Not yet named	–	–	–	ND	Mitochondria	ND	13
CBU1379	Not yet named	–	–	FS	Protein kinase	No distinct localization	ND	61
CBU1457§	Not yet named	–	–	–	Tetratricopeptide	ND	ND	61
CBU2078§	Not yet named	–	–	–	Fic	ND	ND	61
CBU0077§	Not yet named	–	–	–	ND	Lysosome	ND	13
CBU0635§	Not yet named	NT	–	–	ND	Golgi, vesicles	Inhibits the secretory pathway	13

Dot/Icm, defect in organelle trafficking/intracellular multiplication. ND, not determined.

[*] Genetic differences between homologues and the corresponding gene in *Coxiella burnetii* str. Nine Mile I: absent (A), an alternative start (AS), a carboxy-terminal truncation in the encoded protein (CT), a frame shift (FS), an amino-terminal truncation in the encoded protein (NT) or no genetic differences (−).

[‡] Determined by ectopic expression in host cells.

[§] Conserved between pathotypes.