Chapter 9
Coxiella burnetii Secretion Systems

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Contents

Abstract The ability of bacteria to transport proteins across their membranes is integral for interaction with their environment. Distinct families of secretion systems mediate bacterial protein secretion. The human pathogen, *Coxiella burnetii* encodes components of the Sec-dependent secretion pathway, an export system used for type IV pilus assembly, and a complete type IV secretion system. The type

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IVB secretion system in *C. burnetii* is functionally analogous to the *Legionella pneumophila* Dot/Icm secretion system. Both *L. pneumophila* and *C. burnetii* require the Dot/Icm apparatus for intracellular replication. The Dot/Icm secretion system facilitates the translocation of many bacterial effector proteins across the bacterial and vacuole membranes to enter the host cytoplasm where the effector proteins mediate their specific activities to manipulate a variety of host cell processes. Several studies have identified cohorts of *C. burnetii* Dot/Icm effector proteins that are predicted to be involved in modulation of host cell functions. This chapter focuses specifically on these secretion systems and the role they may play during *C. burnetii* replication in eukaryotic host cells.

Keywords Bacterial secretion • Dot/Icm • Effector proteins • Type IV secretion system • Virulence determinant

9.1 Introduction

Secreted and surface proteins of bacteria are essential for bacterial physiology, virulence, and recognition by host immune mechanisms. Transport of these proteins from the cytoplasm to the bacterial cell envelope or outside of the bacterial cell is dependent on the function of specific secretion systems. These systems recognize defined protein signatures and actively move these proteins across lipid membranes. Bacterial pathogens often have one or more specialized secretion systems that act on specific subsets of bacterial proteins involved in interaction with eukaryotic host cells. In these instances, numerous gene products function in concert to form a highly specialized molecular apparatus designed to deliver macromolecules into host cells. The importance of these secretion systems in promoting virulence makes them important candidates for research studies and attractive targets for new antimicrobial therapies.

Coxiella burnetii is a Gram-negative facultative intracellular pathogen and the causative agent of Q fever in humans (Madariaga et al. 2003). *C. burnetii* invades host cells and over several days directs the formation of a unique vacuolar compartment that resembles a lysosome (Voth and Heinzen 2007). Following uptake, the nascent *C. burnetii* vacuole sequentially acquires markers of early and late endosomes, eventually forming a single spacious compartment referred to as the *C. burnetii*-containing vacuole (CCV), which demonstrates robust fusion with host endocytic vesicles and expands to occupy much of the host cytoplasm. The CCV lumen has a pH of approximately 4.7 and contains lysosomal hydrolases, such as cathepsin D (Heinzen et al. 1996). The lysosomal membrane proteins LAMP-1 and LAMP-2 are present on the CCV and this compartment is rich in cholesterol (Heinzen et al. 1996; Howe and Heinzen 2006). In addition, proteins usually found on autophagosomes, such as microtubule-associated protein light-chain 3 (LC3) and Rab24, are present on the CCV membrane (Beron et al. 2002). It is predicted that bacterial proteins delivered into the lumen of this vacuole and across the vacu-

ole membrane will be important determinants of host pathogenesis. Thus, a more complete understanding of *C. burnetii* secretion should provide important insight into the mechanisms by which this pathogen creates and maintains this specialized vacuole.

During infection, the bacteria exist as two morphologically distinct forms. A metabolically inert small cell variant is stable in the environment and capable of infecting a wide range of host cells (McCaul and Williams 1981). Once the spacious CCV is established the small cell variant morphs into a large cell variant capable of replication to high numbers within the CCV (Coleman et al. 2004). Like several other intracellular bacterial pathogens, *C. burnetii* is able to block the intrinsic death pathway and prevent the induction of host cell apoptosis in infected cells, which is important for survival within cells over long periods of time (Voth et al. 2007; Luhrmann and Roy 2007).

There is evidence to suggest that establishment of the CCV is distinct from the default transport pathway that delivers endocytic cargo to lysosomes. Because the CCV is highly fusogenic, fusing promiscuously with other endocytic vesicles and phagosomes residing in the cell, it suggests that *C. burnetii* overrides the host mechanisms that regulate lysosome biogenesis (Howe et al. 2003a). Inhibition of *C. burnetii* protein synthesis by chloramphenicol treatment impedes fusogenicity and prevents formation and maintenance of the CCV, indicating that continued synthesis of bacterial proteins is required to maintain this specialized organelle (Howe et al. 2003b), predicting a role for secreted bacterial products in this process.

The genomes of several strains of *C. burnetii* have been sequenced and reveal genes predicted to encode proteins used by *C. burnetii* to survive and replicate within host cells (Seshadri et al. 2003; Beare et al. 2009), including homologues to known secretion systems and putative substrates of these systems. The importance of these secretion systems in the ecology and evolution of pathogenesis are current areas of investigation in the field of *C. burnetii* biology.

9.2 Secretion Systems Encoded by *Coxiella burnetii*

The ability of intracellular bacteria to subvert host mechanisms and survive within mammalian cells is often dependent on the function of a subset of bacterial proteins that interact with host cell factors. To function in this manner, these virulence factors are translocated across bacterial membranes, and in many cases, the host compartmental membrane that contains the bacteria. Gram-negative pathogens use sophisticated secretion systems for this purpose and much work has been devoted to understanding how these molecular machines function (Cambronne and Roy 2006). The genome of *C. burnetii* predicts functional versions of the Sec-dependent, type I, type IV pilus, and type IV secretion systems (described below); and is missing canonical components defining the type II and type III secretion systems, type V autotransporters, and type VI secretion (Seshadri et al. 2003; Beare et al. 2009).

Table 9.1 *C. burnetii* homologs of Gram-negative inner membrane transport components

	ORF name	
	***C. burnetii* Nine Mile strain**	***C. burnetii* Dugway strain**
Sec dependent pathway		
SecY	CBU0258	CBUD1834
SecE	CBU0224	CBUD1868
SecG	CBU1449	CBUD0543
SecD	CBU1142	CBUD1240
SecF	CBU1141	CBUD1238
YajC	CBU1143	CBUD1241
YidC	CBU1920	CBUD0201
SecA	CBU0147	CBUD1958
SecB	CBU1519	CBUD0465
SecM	None	None
SRP		
Ffh	CBU0450	CBUD1624
4.5S	None	None
SRP receptor		
FtsY	CBU1903	CBUD0216
Tat (twin arginine translocation) pathway		
TatA	None	None
TatB	None	None
TatC	None	None
TatE	None	None
Signal peptidase		
SPase I (LepB-1)	CBU1099	CBUD1201
SPase I (LepB-2)	CBU1504	CBUD0480
SPase II (LspA)–lipoprotein SPase	CBU0397	CBUD1671

9.2.1 Sec-Dependent Secretion

Targeted export of proteins across the bacterial inner membrane by the Sec-dependent general secretion pathway is a ubiquitous process in eubacteria. Most of the Sec system components, including signal recognition particle (SRP)-mediated membrane insertion components, are found in the *C. burnetii* genome (Table 9.1; Seshadri et al. 2003; Lima et al. 2009; Mori and Ito 2001). Consistent with this pathway being operational in *C. burnetii* there are numerous genes encoding proteins with predicted amino (N)-terminal signal peptides that function in directing unfolded proteins to the canonical Sec pathway for transport across the bacterial inner membrane (Seshadri et al. 2003). Several of these signal sequence-containing proteins have potential roles in virulence, including phospholipase D (CBU0968), carboxypeptidase (CBU1261), and acid phosphatase (CBU0335).

Many, but not all bacteria utilize a second dedicated secretion apparatus called the Twin-arginine translocase (Tat) for delivering folded peptides across the inner membrane (De Buck et al. 2008; Lee et al. 2006). In some pathogens, such as *Pseudomonas aeruginosa* and *Legionella pneumophila*, the Tat pathway is required for the transport of specific virulence factors across the inner membrane (Voulhoux et al. 2001; Rossier and Cianciotto 2005). Once exported to the periplasm these proteins typically engage the type II secretion system for translocation across the bacterial envelope. Unlike *L. pneumophila*, the *C. burnetii* genome lacks homologues to the known functional components (*tatA*, *tatB*, *tatC*, *tatE*) of the twin-arginine translocase. Thus, the Tat pathway does not appear to be operational in *C. burnetii*.

9.2.2 Type I Secretion

The type I secretion apparatus spans the bacterial cell envelope and is similar to ATP-binding cassette (ABC) transporters. It is composed of a specific outer membrane protein (OMP), an ABC protein, and a membrane fusion protein (MFP) (Thanabalu et al. 1998). The *C. burnetii* genome has a gene *tolC* (CBU0056) that is predicted to encode a protein homologous to the type I secretion system outer membrane channel protein TolC, and is therefore potentially competent for type I secretion. Although the function of *C. burnetii* TolC is unknown, a TolC homolog of *L. pneumophila* is involved in multidrug efflux function and required for virulence, suggesting TolC may play a similar role in *C. burnetii* (Ferhat et al. 2009).

9.2.3 Type IV Pilus System

The type II secretion (T2S) system works in conjunction with the general secretion pathway to ultimately deliver proteins across the Gram-negative outer membrane (Cianciotto 2005). Proteins destined for type II translocation have an amino terminal signal sequence and are capable of Sec or Tat dependent export to the periplasm, where they then engage the type II secretion apparatus. The apparatus is composed mainly of a multimeric outer membrane complex formed from pore-forming secretin proteins. Some components of the T2S system are evolutionarily-related to Gram-negative type IV pili, which uses a modified version of type II secretion for pilus biogenesis (Hobbs and Mattick 1993; Sauvonnet et al. 2000).

The full repertoire of genes encoding members of a prototypical T2S system is lacking in the *C. burnetii* genome, including the major and minor pilin genes *pulGHIJK*; however, several *C. burnetii* genes encode proteins with homology to components of the bacterial type IV pili (T4P) system. These include the conserved

ATPase PilB, the outer membrane secretin PilQ, the prepilin peptidase T4P PilD, and the transmembrane protein PilC. These components are also conserved in most T2S systems. In addition, the genome of *C. burnetii* encodes homologs to *pilE*, *pilF*, and the major type IV prepilin gene *pilA*. Of note is the lack of a *pilT* homolog in *C. burnetii*, which encodes an ATPase essential for pilus retraction. It is interesting to speculate that *C. burnetii* may secrete a subset of signal sequence-containing proteins through the type IV pilus, as has been reported for *Francisella novicida* (Hager et al. 2006). Several signal sequence-containing proteins of *C. burnetii* are potential virulence protein candidates for T4P-related secretion based on the presence of one or more eukaryotic-like motifs within the mature protein sequence. An example is the predicted protein encoded by *enhC*, which has homology to EnhC (Enhanced entry protein C) of *L. pneumophila*, a factor that is involved in early stages of host cell infection (Liu et al. 2008). The *enhC* product has a predicted Sec-dependent signal sequence, and 21 tandemly-arranged tetratricopeptide repeats (TPRs)–a motif found in eukaryotic adaptor proteins that functions in signal transduction. Whether the *C. burnetii* EnhC protein resides on the bacterial cell surface and participates in pathogen uptake remain unanswered questions.

9.2.4 Type IV Secretion System

Type IV secretion systems of Gram-negative bacteria are related to the conjugal transfer systems that participate in exchange of broad-host range plasmids between bacterial cells (Segal et al. 1998; Christie and Vogel 2000; Vogel et al. 1998). Virulence-associated type IV secretion systems are found in several bacterial pathogens including *Agrobacterium tumefaciens*, *Bordetella pertussis, Helicobacter pylori*, *Bartonella henselae*, *Brucella suis*, *Rickettsia prowazekii*, *Xanthomonas* spp., *L. pneumophila*, and *C. burnetii*, and function in the secretion and delivery of bacterial effector proteins into host cells (Alvarez-Martinez and Christie 2009). Bacterial type IV secretion systems are classified as either type IVA or type IVB based on homology to the *A. tumefaciens virB/virD4* system or the *L. pneumophila* Dot/Icm system, respectively (Christie and Vogel 2000). The *L. pneumophila* Dot/Icm system is ancestrally related to the *tra/trb*-encoded Inc plasmid system and has been shown to mediate transfer of the IncQ plasmid RSF1010 between *L. pneumophila* strains, as well as to *E. coli* (Segal et al. 1998; Vogel et al. 1998). During host infection, *L. pneumophila* uses the Dot/Icm type IV secretion system to deliver a large repertoire of effector proteins into host cells that are important in establishing a niche for intracellular survival and replication of the bacteria (Hubber and Roy 2010). The *C. burnetii* genome encodes an intact type IVB secretion system highly homologous to the *L. pneumophila* Dot/Icm system (Segal and Shuman 1999; Seshadri et al. 2003). This locus contains homologues to most of the known *L. pneumophila dot/icm* genes and is organized similarly with few exceptions. No other type IV-related

systems are encoded by *C. burnetii*. Because the *C. burnetii* Dot/Icm system is the only virulence determinant to date that has been shown to be essential for bacterial replication inside eukaryotic host cells, a more detailed description of this apparatus and the effectors translocated by the system are provided in the following sections.

9.3 Genomic Analysis of the *Coxiella burnetii* Dot/Icm System

L. pneumophila, the causative agent of Legionnaire's disease, is a Gram-negative intracellular pathogen that survives within and kills free-living amoebae and human macrophages (Rowbotham 1980; Horwitz and Silverstein 1980; Nash et al. 1984). The intracellular success of *L. pneumophila* is dependent on the function of a type IVB secretion system called Dot/Icm (Defect in organelle trafficking/Intracellular multiplication) (Marra et al. 1992; Berger and Isberg 1993). The Dot/Icm system is a major virulence determinant of *L. pneumophila* and is required for the creation of a vacuole that evades endocytic maturation and develops into an endoplasmic reticulum-derived organelle that supports replication of *L. pneumophila* within eukaryotic host cells (Kagan and Roy 2002; Horwitz 1983; Swanson and Isberg 1995).

The *dot/icm* genes in *L. pneumophila* are located on two chromosomal loci and encode 24 protein components that assemble to form a membrane-bound secretion apparatus that functions in the translocation of effector proteins across both the bacterial envelope and the host cell vacuolar membrane (Swanson and Isberg 1995; Vogel et al. 1998; Segal et al. 1998). Early evidence from *C. burnetii* genomic sequencing revealed putative gene products with high amino acid homology to Dot/Icm proteins of *L. pneumophila* (Seshadri et al. 2003; Sexton and Vogel 2002). *C. burnetii* is the only other animal pathogen in which the Dot/Icm secretion system has been identified; however, genome sequencing of a phytopathogen *Xanthomonas* sp. suggests the presence of a plasmid-encoded *dot/icm* locus (Thieme et al. 2005). The *C. burnetii dot/icm* genes are found on a 35 kb region of the chromosome and there is synteny with the genes in *L. pneumophila* (Fig. 9.1). Comparison of Dot/Icm proteins reveals that most of the components found in the *L. pneumophila* system are present in the *C. burnetii* system (Seshadri et al. 2003). *C. burnetii* appears to lack an *icmM* gene and instead has two genes that encode proteins with similarity to the *icmL* product. Given that there is a high degree of similarity between the *L. pneumophila icmL* and *icmM* products, the second *C. burnetii icmL* gene is likely to encode a protein that functions similarly to the *L. pneumophila icmM* product. Initially, *C. burnetii* was thought to also lack the *icmR* gene, which encoded a protein that forms a complex with the *icmQ* product in *L. pneumophila*. However, *C. burnetii* has a gene immediately upstream of *icmQ* that encodes a 49 amino acid protein called CoxigA (CBU1634a) that is functionally homologous to the *L. pneumophila* IcmR protein. CoxigA is a member of the FIR family of proteins, which are

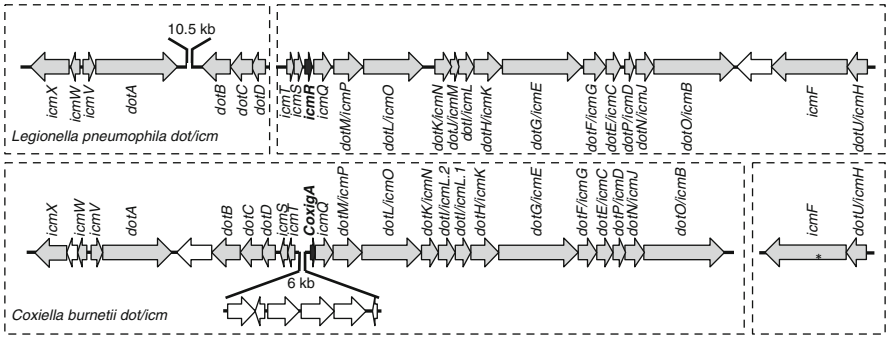

Fig. 9.1 Genomic organization of the genes encoding Dot/Icm systems. The *L. pneumophila* and *C. burnetii dot/icm* genes represented as *shaded arrows* are aligned to demonstrate synteny. Genes that are not required for Dot/Icm-dependent processes are shown as *white arrows*. Genes that are linked together on the chromosome are contained within the dashed boxes. The location and size of intervening sequences are indicated. The asterisk indicates the location of a stop codon within *C. burnetii icmF* gene

Functional Homologues of IcmR identified in other species of *Legionella*. FIR proteins are encoded by a family of hypervariable genes that are located in a similar genomic position upstream of *icmQ* (Feldman and Segal 2004; Feldman et al. 2005).

9.4 Transcriptional Regulation of the *dot/icm* Genes

Analysis of RNA transcribed from a subset of *C. burnetii dot/icm* genes has demonstrated transcription of three linkage groups, which would correspond to three operons consisting of *icmW-CBU1651-icmX*, *icmV-dotA-CBU1647* and *icmT-icmS-icmD-icmC-icmB-CBU1646* (Morgan et al. 2010b) (see Fig. 9.1). Examination of the relative transcript levels showed a general trend towards increased transcription of these *dot* and *icm* genes during the initial 16 h of *C. burnetii* infection of a eukaryotic host cell, and a subsequent drop in transcript levels by 24 h post-infection (Coleman et al. 2004; Morgan et al. 2010b). Changes in the RNA levels for *icmT* were subtle, yet corresponded to IcmT protein levels increasing two to three fold during the initial 24 h of infection and then remaining constant (Morgan et al. 2010b). These data suggest that the *dot/icm* locus is upregulated in response to infection and that this may facilitate the establishment of the replicative vacuole and a productive infection.

Presumably, a number of different regulatory factors work in concert to coordinate the expression of both components of the Dot/Icm apparatus and the Dot/Icm effectors that are delivered into host cells by the type IV system. A cohort of regulatory

proteins has been shown to tightly control the expression of virulence-related genes in *L. pneumophila*. Most virulence genes seem to be upregulated as *L. pneumophila* enter stationary phase, including genes encoding the Dot/Icm system. The two-component response regulators PmrA, CpxR and LetA, the stationary phase sigma factor RpoS and the ppGpp synthetase RelA, have all been shown to play an important role in regulating virulence in *L. pneumophila* (Zusman et al. 2007; Altman and Segal 2008; Gal-Mor and Segal 2003; Lynch et al. 2003; Shi et al. 2006; Rasis and Segal 2009; Bachman and Swanson 2001). Homologues of each of these Dot/Icm regulators are present in *C. burnetii*. The stationary phase sigma factor RpoS of *C. burnetii* shares 50% identity with *L. pneumophila* RpoS and can functionally complement an *Escherichia coli rpoS* null mutant (Seshadri and Samuel 2001). Interestingly, the *C. burnetii* RpoS protein is abundant in the replicating large cell variants of *C. burnetii* and undetectable in the stationary small cell variants, suggesting that the genes regulated by RpoS in *C. burnetii* may expressed at a higher level during exponential phase and down regulated in stationary phase, which would be consistent with the pattern of *dot/icm* gene expression observed. A consensus promoter sequence for RpoS, however, has not been clearly defined, and the role of this sigma factor in Dot/Icm regulation in *C. burnetii* remains to be determined (Melnicakova et al. 2003).

There is evidence that regulation of the *C. burnetii* Dot/Icm system is mediated directly by the response regulator PmrA. The *C. burnetii pmrA* gene (CBU1227) encodes a protein in which the third alpha helix in the predicted structure is identical to that predicted for the *L. pneumophila* PmrA protein. Because this region is a determinant of the helix-loop-helix motif that mediates DNA recognition it was anticipated that these two response regulators would recognize a similar consensus sequence in the promoters of PmrA-regulated genes (Zusman et al. 2007). Bioinformatical analysis confirmed the presence of PmrA regulatory sequences upstream of five putative promoter regions controlling the transcription of operons that express 23 *dot/icm* genes. Gel mobility shift assays confirmed that the *C. burnetii* PmrA protein bound to the regulatory sequence upstream of these genes. Furthermore, a PmrA-regulated transcriptional fusion to *lacZ* was introduced into a *L. pneumophila pmrA* mutant strain to show that the *C. burnetii* PmrA protein could restore transcriptional activity (Zusman et al. 2007).

In *L. pneumophila*, PmrA also regulates the transcription of a large number of Dot/Icm effector proteins, and the presence of the PmrA consensus sequence has been used as a signature to identify Dot/Icm effectors (Zusman et al. 2007; Chen et al. 2010). Analysis of the *C. burnetii* genome identified 68 genes containing this upstream consensus regulatory element. It has been suggested that *C. burnetii* genes that have been called *cig* for coregulated with i*cm* genes, would be attractive candidates for encoding Dot/Icm effectors (Zusman et al. 2007). As detailed in the following sections, subsequent independent studies have now confirmed that several of the *cig* genes encode Dot/Icm effectors.

9.5 Dot/Icm Apparatus Assembly

The high degree of similarity between the *L. pneumophila* Dot/Icm components and those encoded by *C. burnetii* predicts that the two systems produce structurally analogous secretion systems. There is a great deal of interest in understanding how the individual Dot/Icm components assemble to build the machinery that translocates effector proteins into host cells. Partial modeling of the *L. pneumophila* Dot/Icm secretion apparatus has been achieved using both biochemical and genetic approaches. Protein-protein interactions, identification of the subcellular localization of individual Dot/Icm proteins and examination of the stability of each component of the system in systematic knockout backgrounds have provided some insight into the relationships between individual key components of the type IVB secretion system (Vincent et al. 2006). A core subcomplex, consisting of the inner membrane proteins DotG and DotF with the outer membrane protein DotH and the outer membrane lipoproteins DotC and DotD, combines to span both bacterial membranes (Vincent et al. 2006). It is predicted that DotC and DotD facilitate the outer membrane insertion of DotH to form the outer membrane pore and DotF and DotG can form both homo- and heterodimers in the inner membrane to interact with the DotC-DotD-DotH outer membrane complex. Given that the *C. burnetii* homologues share approximately 50% similarity to the *L. pneumophila* core subcomplex it is expected that this structure is also a key component of the *C. burnetii* Dot/Icm apparatus.

Surprisingly, despite being evolutionarily distinct and possessing more than double the number of proteins, the Dot/Icm type IVB apparatus produces a core structure that appears to be related to the VirB apparatus in *A. tumefaciens*, which is a type IVA system (Vincent et al. 2006). The VirB apparatus consists or a core complex containing the inner membrane protein VirB8 interacting with the membrane spanning protein VirB10 and the outer membrane proteins VirB7 and VirB9 (Krall et al. 2002). Most recently, the crystal structure of *L. pneumophila* DotD has been reported, revealing a structure surprisingly distinct from VirB7, the presumed type IVA counterpart (Nakano et al. 2010). Rather, the C-terminus of *L. pneumophila* DotD displays similarity to the N-terminal subdomain of secretins, the outer membrane components of type II and type III secretion systems (Nakano et al. 2010). This suggests DotD may create a channel in the bacterial outer membrane through which effectors potentially travel.

Proteins of the *C. burnetii* Dot/Icm secretion system have been visualized by immunofluorescence and electron microscopy. Antibodies to IcmT, IcmV and DotH clearly demonstrated polar localization of these proteins, particularly in the large cell variant of *C. burnetii* (Morgan et al. 2010a). Electron micrographs indicate that the poles of *C. burnetii* can interact with the CCV membrane, which is where protein translocation by the Dot/Icm system should be occurring; however, the functional relevance of these observations has yet to be determined. It is unclear whether polar localization of the Dot/Icm apparatus is important for the efficient translocation of substrates; however, this is not a unique observation as a range of bacterial pathogens

express virulence factors and secretion system components preferentially at the bacterial poles (Morgan et al. 2010a; Jaumouille et al. 2008; Carlsson et al. 2009; Jain et al. 2006).

9.6 Functional Analysis of the *Coxiella burnetii* Dot/Icm System

Several lines of evidence indicate that the *C. burnetii* Dot/Icm system is functional and has similar features to the *L. pneumophila* Dot/Icm secretion system. Reverse transcription-PCR indicates that *dot/icm* genes are expressed during *C. burnetii* infection of Vero cells, including the genes *dotB*, *icmQ*, *icmS*, and *icmW* (Zamboni et al. 2003). These genes are of particular interest because they represent type IV secretion components that are not present in the conjugal DNA transfer system and likely have specific roles in translocation of virulence factors in *L. pneumophila* and *C. burnetii*. In addition, genetic complementation was used to test functional similarity by asking whether expression of individual *C. burnetii dot/icm* genes in a corresponding *L. pneumophila* mutant having the gene of interest deleted could restore *L. pneumophila* growth in eukaryotic cells. It was found that the *C. burnetii* genes encoding *dotB*, *icmS*, *icmW*, and *icmT* restored intracellular replication when expressed in a corresponding *L. pneumophila* deletion strain (Zamboni et al. 2003; Zusman et al. 2003). In contrast, *L. pneumophila icmB*, *icmJ*, *icmO*, *icmP, icmQ* mutants could not be complemented by the corresponding *C. burnetii* gene product (Zamboni et al. 2003; Zusman et al. 2003).

Genetic complementation studies have been further verified by studies examining protein-protein interactions. The IcmS and IcmW proteins are small proteins that appear to form a stable complex in the *L. pneumophila* cytosol (Zuckman et al. 1999; Ninio et al. 2005). These proteins interact directly with many different effectors and are believed to function as chaperones that facilitate the recognition of translocation signals in the effectors that enable these proteins to be recognized as substrates by the secretion apparatus (Cambronne and Roy 2007; Ninio et al. 2005). As was shown for the *L. pneumophila* proteins, it was found that the *C. burnetii* IcmW and IcmS proteins interact with each other (Zusman et al. 2003). Additionally, cross species interactions between the *C. burnetii* and *L. pneumophila* IcmW and IcmS proteins supported the observation that the *C. burnetii* genes encoding either component could complement function in a corresponding *L. pneumophila* mutant (Zusman et al. 2003). The cytoplasmic IcmQ protein is essential for Dot/Icm function and through homotypic interactions is capable of forming pores in lipid membranes *in vitro* (Dumenil et al. 2004; Raychaudhury et al. 2009). The activity of IcmQ is regulated by the IcmR protein, which is capable of forming a protein complex with IcmQ (Coers et al. 2000; Raychaudhury et al. 2009). The *C. burnetii* IcmQ protein did not interact with the *L. pneumophila* IcmR protein, which explains why the *C. burnetii icmQ* gene did not complement the *L. pneumophila icmQ* deletion mutant (Zamboni et al. 2003; Zusman et al. 2003). In summary, these studies indicate the

two systems are closely related and that some of the Dot/Icm components can function in both systems. The observation that the proteins that comprise the membrane-bound translocation machine are not interchangeable between the two systems suggests that divergent evolution has resulted in structural constraints that prevent these *C. burnetii* components from interacting correctly with components of the *L. pneumophila* Dot/Icm apparatus.

The host cell processes targeted by *C. burnetii* and the specific function of *C. burnetii* effectors are anticipated to be quite unique. Unlike other intracellular bacteria, *C. burnetii* does not mediate phagosome arrest or escape, and therefore does not require effector proteins that function in this regard. Study of the *C. burnetii* intracellular lifestyle and interactions with host cells can provide some clues as to functions that may be encoded by Dot/Icm effectors. Specific traits of the developing CCV are dependent on *C. burnetii* protein synthesis. Blocking *C. burnetii* protein synthesis, with the application of chloramphenicol, obstructs the capacity of the CCV to promote fusion of endocytic vesicles and lysosomes, and prevents formation of the characteristic large spacious vacuole in which the bacteria reside (Howe et al. 2003a, b). Chloramphenicol treatment also significantly reduces the capacity of *C. burnetii* to block the intrinsic death pathway and inhibit host cell apoptosis (Luhrmann and Roy 2007; Voth et al. 2007). The ability of *C. burnetii* to actively perturb vesicular fusion and apoptosis signaling are examples of putative functions for specific Dot/Icm effector proteins.

To determine whether the *C. burnetii* Dot/Icm system plays an important role in host cell infection, a mutant deficient in this apparatus was isolated. Using random transposon mutagenesis, an insertion in the *dotI/icmL.1* gene of *C. burnetii* was isolated (Carey et al. 2011). The *icmL::Tn* mutant grows similarly to the isogenic parental strain in axenic medium, indicating that the Dot/Icm system is not required for replication in defined medium. When the *icmL::Tn* mutant was used to infect mammalian host cells, a complete defect in *C. burnetii* intracellular growth was observed. The *icmL::Tn* mutant phenotype is similar to the effect chloramphenicol treatment has on wild type *C. burnetii* replication in cultured host cells (Carey et al. 2011). These findings indicate that the Dot/Icm secretion system is essential for successful infection of host cells by *C. burnetii* and indicates that the effectors translocated by this secretion system must play an important role in creating a host cell compartment that permits *C. burnetii* replication.

9.7 Effectors of the Dot/Icm Secretion System

In *L. pneumophila* there have been over 200 different proteins identified that are translocated into host cells by the Dot/Icm secretion system (Burstein et al. 2009; Huang et al. 2011). Most of these Dot/Icm effectors are dispensable for intracellular replication of *L. pneumophila,* highlighting genetic and functional redundancy among the effector repertoire. Despite this, several *L. pneumophila* Dot/Icm effectors

have been shown to have a profound influence on specific processes that are subverted during host cell infection, including subversion of vesicular trafficking, modulation of host GTPase function and localization, and promoting the ubiquitination of host and bacterial proteins (see review articles (Newton et al. 2010; Ensminger and Isberg 2009; Shin and Roy 2008)).

Using strategies that were successful in identification of *L. pneumophila* Dot/Icm effectors, several studies have reported the identification of *C. burnetii* effectors that are translocated into host cells by the Dot/Icm system (Pan et al. 2008; Voth et al. 2009; Chen et al. 2010) (Table 9.2). Protein fusions have been the primary method used to detect protein translocation into host cells by the Dot/Icm system. The basis of this detection scheme is to fuse a putative effector protein to an enzyme with a novel activity that can indicate delivery of the hybrid into the host cytosol. The calmodulin-activated adenylate cyclase, CyaA, from *Bordetella pertussis* is a commonly used reporter to examine bacterial translocation of effector proteins into host cells (Sory and Cornelis 1994; Chen et al. 2010). After being transported to the host cytosol, the Cya protein binds to calmodulin and then efficiently converts ATP into cAMP. Thus, the production of cAMP is measured after infection to determine the efficiency by which the effector hybrid has been translocated into the host cytosol by the Dot/Icm system. Another reporter system that provides both visual and quantitative means to measure protein translocation is the TEM-1 β-lactamase fluorescence-based system (BlaM) (Charpentier and Oswald 2004). The BlaM-based system takes advantage of a fluorescent substrate, CCF2-AM, composed of a coumarin and fluorescein moiety. Excitation of the coumarin moiety at 409 nm results in FRET to the fluorescein moiety and subsequent fluorescence emission at 520 nm. Translocated BlaM fusion proteins cleave the β-lactam ring linking coumarin and fluorescein disrupting the FRET reaction, which shifts fluorescence emission to 447 nm. This change in the fluorescence signal in the cytosol of infected host cells indicates translocation of the BlaM-effector protein fusion by the Dot/Icm system (Charpentier and Oswald 2004).

Both Cya and BlaM reporter assays have been used to demonstrate Dot/Icm-dependent translocation of *C. burnetii* effector proteins expressed in *L. pneumophila* (Chen et al. 2004; de Felipe et al. 2008). Recent advances in *C. burnetii* genetics have also provided means to express reporter fusion proteins in *C. burnetii,* which has confirmed that effectors translocated by the *L. pneumophila* Dot/Icm system are also translocated by *C. burnetii* during host cell infection (Chen et al. 2010). In addition, translocation of the endogenously-produced effector proteins AnkG and CBU0077 has been demonstrated during *C. burnetii* infection of host cells by immunoblot analysis of fractionated host cells that were infected with *C. burnetii* (Pan et al. 2008; Carey et al. 2011). Lastly, the *C. burnetii icmL:Tn* mutant does not show any translocation of a plasmid-expressed BlaM-CBU0077 fusion protein after infection of host cells, demonstrating clearly that the Dot/Icm system is essential for translocation of effector proteins (Carey et al. 2011).

Table 9.2 ***C. burnetii*** **effectors.** Shown are genes in the four sequenced strains of *C. burnetii* that or *C. burnetii*

Nine Mile RSA493	Size kDa (bp)	Dugway 5 J108-111	Size kDa (bp)	K Q154	Size kDa (bp)
CBUA0014 (CoxU3)	44.5 (1,254)	DNA Absent		DNA Absent	
CBU0041 (CoxCC1)	82.7 (2,133)	CBUD2061	82.7 (2,133)	CbuK1986	82.7 (2,133)
CBU0071	36.4 (975)	CBUD2035 (*ankP*)	76.7 (2,043)	**CbuK1982**	76.0 (2,013)
CBU0072 (*ankA*)	44.3 (1,164)	CBUD2034	53.0 (1,407)	**CbuK1982**	44.3 (1,164)
CBU0077	29.6 (792)	CBUD2029	29.6 (792)	CbuK1977	29.6 (792)
CBU0080	18.7 (480)	**CBUD2028**	23.1 (600)	CbuK1976	38.1 (987)
CBU0129 (CoxCC2)	28.5 (348)	**CBUD1978**	24.4 (630)	**Frameshift–no ORF**	
CBU0144/0145 (*ankB*)	41.9 (1,140)	**CBUD1960**	41.0 (1,116)	CbuK1907	39.9 (1,080)
CBU0175 (CoxK1)	28.9 (741)	CBUD1925	28.9 (741)	CbuK0362	28.9 (741)
CBU0295	56.8 (1,467)	CBUD1787	56.8 (1,467)	**CbuK0462**[a]	34.2 (888)
CBU0329	23.2 (597)	CBUD1750	68.4 (1,764)	**CbuK0525**	43.0 (1,107)
CBU0410 (CoxCC3)	68.4 (1,737)	CBUD1664	68.4 (1,737)	**CbuK1657**	53.1 (1,467)
CBU0414 (CoxH1)	30.4 (786)	CBUD1656	48.4 (1,248)	**CbuK1651**	34.6 (894)
CBU0425	51.9 (1,365)	CBUD1648[a]	54.0 (1,248)	**CbuK1643**	14.5 (378)
CBU0447 (*ankF*)	21.3 (555)	CBUD1627	21.3 (555)	CbuK1411	21.3 (555)
CBU0635	55.3 (1,488)	CBUD0646	55.3 (1,488)	**CbuK1621**	38.4 (1,029)
CBU0781 (*ankG*)	38.6 (1,017)	CBUD0829	38.6 (1,017)	CbuK0651	25.7 (669)
CBU0794 (CoxCC4)	53.1 (1,395)	**CBUD0861**[a]	46.0 (1,200)	CbuK0662[a]	39.5 (1,029)
CBU0801 (*rimL*, CoxH2)	16.9 (438)	CBUD0868	16.9 (438)	CbuK0670	16.9 (438)
CBU0814 (CoxU1)	12.6 (321)	**CBUD0882**	47.9 (1,266)	CbuK0684	70.2 (1,854)

encode effectors of the Dot/Icm system based on translocation assays in either *L. pneumophila*

G Q212	Size kDa (bp)	Features of Protein	Identification Method	Reference
DNA Absent		F-box, coiled-coil motif, encoded on the QpH1 plasmid	Putative PmrA binding motif Homology to Dot/Icm substrate	Chen et al. (2010)
CbuG1970	82.7 (2,133)	2 coiled-coil motifs	Putative PmrA binding motif	Chen et al. (2010)
DNA absent		6 Ank repeats	Ankyrin repeat domain containing protein	Voth et al. (2009)
DNA Absent		4 Ank repeats	Ankyrin repeat domain containing protein	Pan et al. (2008); Voth et al. (2009)
CbuG1938	29.6 (792)	4 TM domains	Proximity to CBU0080	Carey et al. (2011)
CbuG1937[a]	34.3 (888)	Helix-Loop-Helix motif in CbuK1976 and CbuG1937.	Screen for translocation signals	Carey et al. (2011)
CbuG1886	51 (1,311)	coiled-coil motif	Putative PmrA binding motif	Chen et al. (2010)
CbuG1870	36.0 (984)	7 Ank repeats	Ankyrin repeat domain containing protein	Pan et al. (2008); Voth et al. (2009)
CbuG1837	28.9 (741)	Predicted serine/ threonine kinase	Homology to Dot/Icm substrate	Chen et al. (2010)
CbuG1711	51.3 (1323)	3 SLRs in CBU0295, CBUD1787 and CbuG1711.	Screen for translocation signals	Carey et al. (2011)
CbuG1677	738 (28.9)		Proximity to CBU0328	Carey et al. (2011)
CbuG1603	68.4 (1,737)	coiled-coil motif	Putative PmrA binding motif	Chen et al. (2010)
CbuG1597[a]	45.1 (1161)		Homology to Dot/Icm substrate	Chen et al. (2010)
CbuG1587	56.2 (1,482)		Screen for translocation signals	Carey et al. (2011)
CbuG1564	21.3 (555)	3 Ank repeats	Ankyrin repeat domain containing protein	Pan et al. (2008); Voth et al. (2009)
CbuG1369	55.3 (1,488)	6 TM domains	Screen for translocation signals	Carey et al. (2011)
CbuG1220	38.6 (1,017)	2 Ank repeats, coiled-coil motif	Ankyrin repeat domain containing protein	Pan et al. (2008); Voth et al. (2009)
CbuG1207	55.4 (672)	coiled-coil motif, CbuG1207 has a C-terminal repeat	Putative PmrA binding motif	Chen et al. (2010)
CbuG1199	14.5 (372)	Ribosomal protein S18 alanine acetyltransferase	Homology to Dot/Icm substrate	Chen et al. (2010)
CbuG1185[a]	31.6 (834)	F-box motif, RCC1/ BLIPII domain, 1 TM domain	Putative PmrA binding motif Homology to Dot/Icm substrate	Chen et al. (2010)

(continued)

Table 9.2 (continued)

Nine Mile RSA493	Size kDa (bp)	Dugway 5 J108-111	Size kDa (bp)	K Q154	Size kDa (bp)
CBU0881 (CoxCC5)	26.3 (666)	DNA Absent		DNA Absent	
CBU0937 (CoxDFB1)	53.4 (1,452)	CBUD1137	53.4 (1,452)	CbuK0900	53.4 (1,452)
CBU1025	88.8 (2,361)	CBUD1019 (*ankH*)	96.6 (2,574)	**CbuK0815**	64.2 (1,713)
CBU1045 (CoxDFB2)	42.7 (825)	CBUD0997	65.5 (1,749)	**CbuK0793**	17.4 (444)
CBU1107	35.5 (918)	CBUD1210	122.3 (3,177)	**CbuK0977**	18.1 (468)
CBU1108	25.8 (657)	As above		**As above**	
CBU1213[a–]	74.9 (1,974)	CBUD1298 (*ankI*)	82.8 (2,181)	DNA Absent	
CBU1217 (CoxU2)	57.8 (1,494)	**CBUD1301**	44.0 (1,025)	CbuK1077	55.9 (1,440)
CBU1253/1254	66.6 (1,752)	**CBUD1338 (*ankJ*)**	75.6 (1,986)	**CbuK1113**	35.3 (915)
CBU1314 (CoxCC6)	24.4 (645)	CBUD1402	24.4 (645)	CbuK1177	24.4 (645)
CBU1379 (CoxK2)	78.0 (2,031)	**CBUD1462**	59.2 (1,344)	CbuK1237	76.1 (1,980)
CBU1406[a] (CoxDFB3)	17.6 (468)	CBUD0588	32.1 (837)	CbuK1480	32.1 (834)
CBU1425 (CoxDFB4)	15.9 (453)	CBUD0572	15.9 (453)	CbuK1498	15.9 (453)
CBU1457 (CoxTPR1)	78.3 (2,031)	CBUD0496[a]	71.0 (1,848)	CbuK1685[a]	74.4 (1,932)
CBU1460 (CoxCC7)	29.6 (786)	**CBUD0501**	27.3 (714)	**CbuK1689**	19.7 (519)
CBU1461 (CoxCC8)	71.4 (1,899)	**CBUD0503**	79.6 (2,103)	CbuK1690	86.0 (2,271)
CBU1524 (CoxCC9)	25.1 (660)	CBUD0462	36.8 (996)	CbuK1751	24.3 (642)
CBU1525	43.9 (1,146)	CBUD0461	115.4 (3,033)	**CbuK1752**	52.5 (1,752)
CBU1532	16.5 (438)	CBUD0454	49.7 (1,332)	**CbuK1760**[a]	28.1 (762)
CBU1543 (CoxCC10)	22.3 (567)	CBUD0444	23.3 (594)	CbuK1769	23.3 (594)

G Q212	Size kDa (bp)	Features of Protein	Identification Method	Reference
CbuG1121	26.3 (666)	coiled-coil motif	Putative PmrA binding motif	Chen et al. (2010)
CbuG1068	53.4 (1,452)	coiled-coil motif	Interaction with DotF	Chen et al. (2010)
CbuG0983[a]	14.9 (390)	14 Ank repeats	Ankyrin repeat domain containing protein	Voth et al. (2009)
CBUG0959	65.5 (1,749)		Interaction with DotF	Chen et al. (2010)
DNA Absent			Proximity to CBU1108	Carey et al. (2011)
DNA Absent			Homology to CBU1525	Carey et al. (2011)
CbuG0789	82.8 (2,181)	4 Ank repeats, *cig*	Ankyrin repeat domain containing protein	Voth et al. (2009); Zusman et al. (2007)
CbuG0795	33.8 (864)	2 internal repeat domains	Homology to Dot/Icm substrate	Chen et al. (2010)
CbuG0758[a]	25.8 (669)	2 Ank repeats	Ankyrin repeat domain containing protein	Voth et al. (2009)
CbuG0696	27.2 (717)	coiled-coil motif	Putative PmrA binding motif	Chen et al. (2010)
CbuG0633	72.2 (1,860)	Putative serine/ threonine protein kinase	Homology to Dot/Icm substrate	Chen et al. (2010)
CbuG0608[a]	27.0 (693)		Interaction with DotF	Chen et al. (2010)
CbuG0591	15.9 (453)	N-terminal signal sequence and domain common to Rickettsial 17 kDa surface antigen proteins	Interaction with DotF	Chen et al. (2010)
CbuG0554[a]	77.6 (2,013)	3 SLRs and coiled-coil motif.	Putative PmrA binding motif	Chen et al. (2010)
CbuG0550	14.9 (387)	coiled-coil motif	Putative PmrA binding motif	Chen et al. (2010)
CbuG0549	61.4 (1,617)	1 TM domain, coiled-coil motif, 1 Ank repeat,	Putative PmrA binding motif	Chen et al. (2010)
CbuG0468	24.3 (642)	coiled-coil motif	Proximity to CBU1525 Putative PmrA binding motif	Carey et al. (2011), Chen et al. (2010)
CbuG0485[a]	36.1 (948)		Screen for translocation signals	Carey et al. (2011)
CbuG0477	49.7 (1,332)	5 TM domains, coiled-coil motif	Proximity to CBU1525	Carey et al. (2011)
CbuG0467	20.5 (519)	1 TM domain	Putative PmrA binding motif	Chen et al. (2010)

(continued)

Table 9.2 (continued)

Nine Mile RSA493	Size kDa (bp)	Dugway 5 J108-111	Size kDa (bp)	K Q154	Size kDa (bp)
CBU1556 (CoxCC11)	64.6 (1,704)	CBUD0430	64.6 (1,704)	CbuK1785	64.6 (1,704)
CBU1569 (CoxCC12)	63.1 (1,644)	CBUD0419	63.1 (1,644)	**CbuK1796**	45.6 (897)
CBU1599 (CoxCC13)	67.9 (1,803)	**CBUD0392**	49.8 (1,326)	**CbuK1827**	66.6 (1,773)
CBU1636 (CoxCC14)	45.0 (1,176)	**CBUD0362**	20.8 (555)	**CbuK1859**	29.8 (795)
CBU1751 (CoxDFB5)	48.8 (1,263)	CBUD0250	48.8 (1,263)	CbuK0255	48.8 (1,263)
CBU1757 (*ankM*)	73.4 (1,938)	CBUD0245	96.8 (2,556)	**CbuK0249**	34.2 (900)
CBU1769 (CoxH3)	22.8 (618)	CBUD0236	22.8 (618)	CbuK0236	22.8 (618)
CBU1776	23.7 (609)	CBUD0231	36.2 (924)	CbuK0231	36.2 (924)
CBU1823 (CoxH4)	40.3 (1,050)	CBUD0053	40.3 (1,050)	**CbuK0119**	28.5 (747)
CBU1825 (CoxDFB5)	13.3 (345)	CBUD0054	16.1 (420)	CbuK0120	16.2 (420)
CBU1963	72.9 (1,899)	CBUD2063	81.4 (2,115)	CbuK2014[a]	76.8 (1,995)
CBU2052 (CoxCC15)	34.0 (903)	CBUD2147	33.7 (897)	CbuK2099	33.7 (897)
CBU2056	25.4 (663)	CBUD2151	25.6 (669)	CbuK2102	25.6 (669)
CBU2059	52.6 (1,389)	CBUD2154	61.0 (1,593)	**CbuK2105**	22.7 (600)
CBU2078 (CoxFIC1)	39.9 (1,047)	CBUD2174	39.9 (1,047)	CbuK2125	39.9 (1,047)
DNA absent		**CBUD1552**	23.1 (630)	CbuK1330 (*ankN*)	58.0 (1,593)
DNA absent		CBUD1108 (*ankO*)	83.9 (2,223)	DNA absent	

Putative pseudogenes are indicated in *bold type*, and *gray shading* represents a gene that is *TM* predicted transmembrane domain, *SLR* Sel1-like repeat

[a] ORF has 3′ truncation, therefore the resulting protein, lacking the C-terminus, is possibly not

G Q212	Size kDa (bp)	Features of Protein	Identification Method	Reference
CbuG0454	64.7 (1,707)	4 TM domains, coiled-coil motif	Putative PmrA binding motif	Chen et al. (2010)
CbuG0443	38.2 (1,002)	2 coiled-coil motifs	Putative PmrA binding motif	Chen et al. (2010)
CbuG0414	59.2 (1,566)	1 coiled-coil motif	Putative PmrA binding motif	Chen et al. (2010)
CbuG0386	15.9 (459)	1 coiled-coil motif (only in CBUD0362 and CbuK1859)	Putative PmrA binding motif	Chen et al. (2010)
CbuG0133	48.8 (1,263)	2 coiled-coil motifs	Interaction with DotF	Chen et al. (2010)
CbuG0139	90.9 (2,397)	8 Ank repeats, *cig*	Ankyrin repeat domain containing protein	Voth et al. (2009)
CbuG0151	22.8 (618)	Putative alpha/beta hydrolase	Homology to Dot/Icm substrate	Chen et al. (2010)
DNA absent			Proximity to CBU1780	Carey et al. (2011)
CbuG0196	26.2 (690)	*cig*	Homology to CBU1963Homology to Dot/Icm substrate Putative PmrA binding motif	Carey et al. (2011), Zusman et al. (2007); Chen et al. (2010)
CbuG0197	16.3 (420)		Homology to CBU1823 Interaction with DotF	Carey et al. (2011), Chen et al. (2010)
CbuG1971[a]	24.0 (612)		Downstream of CBU1957	Carey et al. (2011)
CbuG2057[a]	24.8 (645)	Coiled-coil motif	Proximity to CBU2056 and CBU2059 Putative PmrA binding motif	Carey et al. (2011), Chen et al. (2010)
CbuG2060[a]	13.5 (348)		Screen for translocation signals	Carey et al. (2011)
CbuG2063	22.7 (600)		Screen for translocation signals	Carey et al. (2011)
CbuG2079	39.9 (1047)	Fic domain	Fic domain containing protein	Chen et al. (2010)
CbuG1487	58.2 (1,590)	7 Ank repeats	Ankyrin repeat domain containing protein	Voth et al. (2009)
DNA absent		8 Ank repeats	Ankyrin repeat domain containing protein	Voth et al. (2009)

conserved in all four genomes

translocated

9.7.1 *Coxiella burnetii Effectors with Ankyrin Repeats*

Genome sequencing of *L. pneumophila* and *C. burnetii* has revealed a number of bacterial genes encoding proteins with motifs that are common in eukaryotic organisms but rare in bacteria. Analysis of the *L. pneumophila* genome identified 30 predicted proteins with significant similarity to eukaryotic proteins and a further 32 proteins with eukaryotic domains or motifs (Chien et al. 2004; Cazalet et al. 2004). Given the GC bias of these genes and the eukaryotic homology, it is believed the genes encoding these eukaryotic-like proteins have been acquired by interdomain horizontal transfer (de Felipe et al. 2005). Many of these *L. pneumophila* eukaryotic-like proteins are translocated by the Dot/Icm secretion system and are presumed to have effector functions.

Similarly, the *C. burnetii* genome encodes a wide variety of genes predicted to produce proteins with eukaryotic-like motifs. The gene products can be categorized into two groups. The first are proteins with motifs or domains that are generally considered to function as scaffolds that promote specific protein-protein interactions. These include scaffolds created by ankyrin repeats, tetratricopeptide repeats, coiled-coil motifs and leucine rich repeats. The second category includes proteins predicted to have enzymatic functions that can affect eukaryotic signal transduction processes. Examples include *C. burnetii* proteins with GTPase domains, motifs that promote protein ubiquitination such as the F-box domain, and kinases and phosphatases that have the potential to modify proteins and lipids.

Within the category of proteins having eukaryotic-like scaffolding domains are the ankyrin repeat-containing proteins called Anks (Pan et al. 2008; Voth et al. 2009). An ankyrin repeat is a motif of 33 amino acids that forms antiparallel α-helices with connecting loop regions. The tertiary structure of this pattern enables the loop regions to be available for specific protein-protein interactions, and eukaryotic proteins with ankyrin repeats are abundant and influence a wide range of cellular processes from transcription regulation to cytoskeletal motility (Mosavi et al. 2004). Interestingly, several other intracellular bacterial pathogens with virulence-associated secretion systems also encode proteins with ankyrin repeats, including *Anaplasma phagocytophilum*, *Erlichia chaffeensis* and *Rickettsia* spp. (Rikihisa and Lin 2010; Ogata et al. 2005). Thus, the ankyrin repeat domain has likely been subverted by these pathogens to create novel effectors that can interact with eukaryotic proteins.

The genome sequence of *Coxiella burnetii* Nine Mile RSA 493 revealed 13 genes encoding Ank proteins (Seshadri et al. 2003). These *C. burnetii* Ank proteins were tested for Dot/Icm-dependent translocation into host cells using *L. pneumophila* as a surrogate host (Pan et al. 2008). Using the Cya reporter, this study showed Dot/Icm-dependent translocation of AnkA, AnkB, AnkF and AnkG (Pan et al. 2008)(Table 9.2). The proteins AnkE, AnkH, AnkI and AnkM showed less efficient translocation, and no detectable translocation of AnkC, AnkD, AnkJ, AnkK and AnkL was observed (Pan et al. 2008). To validate that a positive signal obtained using Cya reporters expressed in *L. pneumophila* represented a *bone fide* translocation event that would occur during infection, translocation of the endogenous AnkF protein during *C. burnetii* infection was demonstrated by immunoblot analysis.

After the genomes of the *C. burnetii* Dugway strain and the human endocarditis isolates K and G were sequenced it was observed that there is a high degree of variability in the predicted sequences for the Ank proteins (Beare et al. 2009). The Dugway isolate encodes 11 Ank proteins (AnkA, AnkC, AnkD, AnkF, AnkG, AnkH, AnkI, AnkK, AnkM, AnkO and AnkP) that are longer than the Ank proteins encoded by the Nine Mile isolate, with AnkO being unique to Dugway. With the exception of five Ank proteins encoded by the Nine Mile isolate (AnkA, AnkC, AnkF, AnkG and AnkK) most of the *ank* genes possessed mutations that would either result in frameshifts or deletions that would make these proteins over 20% shorter than the corresponding version of the protein encoded by the other sequenced strains of *C. burnetii*. This suggests that pseudogenization may be occurring in the Nine Mile strain, reducing the number of functional Ank proteins that are produced. It is interesting to note that most of the strains produce truncated versions of AnkB, AnkJ and AnkL, suggesting the function of these Ank proteins may not be important for survival in specific hosts, and there are four Ank proteins (AnkC, AnkF, AnkG and AnkK) that are conserved among the sequenced genomes, which could play important roles in host persistence of *C. burnetii* in both pathogenic and non-pathogenic conditions (Beare et al. 2009).

When the Ank proteins produced by the other sequenced strains of *C. burnetii* were tested for translocation by the *L. pneumophila* Dot/Icm secretion system using the CyaA reporter assay, it was found that the only Ank proteins where translocation could not be detected were AnkC, AnkD, AnkK and AnkL (Voth et al. 2009) (Table 9.2). Additionally, it was shown that efficient Dot/Icm-dependent translocation of AnkA, AnkF, AnkG and AnkP required the chaperone protein IcmS (Voth et al. 2009). This is consistent with findings that the *C. burnetii* IcmS protein can restore function to a *L. pneumophila icmS* mutant, and that a subset of the *C. burnetii* effectors require the IcmS-IcmW chaperone complex for efficient recognition by the Dot/Icm system (Zamboni et al. 2003; Ninio et al. 2005; Cambronne and Roy 2007). In addition, it was shown that the signal sequence required for Dot/Icm-dependent translocation was located near the C-terminus of the Ank proteins, which is consistent with the mapping studies performed on several *L. pneumophila* Dot/Icm substrates (Voth et al. 2009; Nagai et al. 2005). The location of this translocation signal has obvious repercussions for Dot/Icm substrates that have acquired frameshift mutations or deletions that create C-terminal truncations, as these truncated proteins would lack the translocation signal. The AnkB proteins predicted for the *C. burnetii* K isolate and the Nine Mile AnkI protein were both shown to have C-terminal truncations in comparison to the longer proteins encoded by the Dugway isolate, and that the loss of these C-terminal sequences prevented these proteins from being translocated by the Dot/Icm secretion system (Voth et al. 2009).

Based on previous data suggesting that *C. burnetii* effectors may have an important role in inhibiting host cell apoptosis, several of the Ank proteins encoded by the Nine Mile strain were tested for anti-apoptotic properties. The Ank proteins were ectopically produced in eukaryotic cells, and apoptosis was assessed after cells were treated with staurosporine, which is a potent agonist of apoptosis. The highly conserved AnkG was shown to protect eukaryotic cells from apoptosis induced by staurosporin

treatment (Luhrmann et al. 2010). Most strikingly, this anti-apoptotic activity was also observed for *L. pneumophila* producing the *C. burnetii* AnkG protein. Whereas mouse dendritic cells normally undergo apoptosis in response to *L. pneumophila* infection, when dendritic cells were infected with *L. pneumophila* expressing AnkG, the cells remained healthy and supported *L. pneumophila* intracellular replication (Luhrmann et al. 2010; Nogueira et al. 2009). This indicates that the quantities of AnkG that are delivered by the Dot/Icm system during infection are sufficient to block a cell autonomous host response that promotes cell death and limits pathogen replication. Thus, studies on AnkG provide proof of principle that *C. burnetii* proteins translocated into host cells by the Dot/Icm system have effector activities that help create a permissive environment for replication.

9.7.2 Screens for Additional Dot/Icm Effector Proteins

The repertoire of effectors in *C. burnetii* is predicted to include more proteins than the Ank family of effectors. Approaches to identify additional effectors in *C. burnetii* have included targeted screens examining proteins with domains that are similar to regions found in *L. pneumophila* effectors, examining proteins that have PmrA regulatory sequences upstream of their coding regions, examining proteins that interact with the DotF protein, proteomic approaches to identify secreted proteins, and random genetic screens to identify proteins having a Dot/Icm dependent translocation signal (Carey et al. 2011; Chen et al. 2010; Samoilis et al. 2010). These studies have revealed a large number of *C. burnetii* proteins that have the potential to function as effectors based on their ability to be translocated into host cells by the Dot/Icm system (Table 9.2). Future studies are needed to determine the biochemical functions of these proteins to better understand how they may be manipulating host cell functions during infection. Determining the activities of these proteins will provide novel insight into the mechanisms by which *C. burnetii* modulates vesicular transport in the cell and evades intrinsic cell processes that mammalian hosts have evolved to restrict the replication of intracellular pathogens.

9.7.3 Effector Plasticity Between Coxiella burnetii Isolates

The discovery of a large repertoire of effector proteins has revealed that there is a substantial degree of variation of individual effectors when the genomes of different *C. burnetii* isolates are compared. Only four of the Ank proteins (AnkC, AnkF, AnkG and AnkK) and 1 of the 18 Dot/Icm substrates identified by the random screen approach (CBU0077) are conserved in the four sequenced genomes (Table 9.2). Interesting, the non-pathogenic Dugway isolate has the largest genome and fewest pseudogenes and IS elements, which has led to the suggestion that this isolate represents an earlier stage of pathoadaptation than the other isolates that have

been sequenced (Beare et al. 2009). One possible explanation for this phenomenon is that most strains of *C. burnetii* have a commensalistic relationship with their hosts, and many of the effector proteins are involved in modulating host responses to limit host pathology. This would explain why Dugway and strains of *C. burnetii* associated with chronic infection tend to show less pseudogenization than strains such as the Nine Mile isolate that cause acute disease. The benefit to the loss of effectors may be more rapid replication within a host organism, which would likely necessitate more efficient transmission between infected and uninfected hosts. Consistent with the hypothesis, the growth rate of the Nine Mile isolate in cultured mammalian cells is faster than that observed for Dugway or the isolates from chronic infection (Roman et al. 1991). It is possible modern large-scale ranching practices have provided habitats that promote efficient transmission of *C. burnetii*, and that this is leading to changes in the effector repertoire where proteins that are involved in maintaining a commensalistic relationship are being lost, resulting in strains that replicate faster and are more pathogenic. Thus, examining *C. burnetii* effector plasticity may provide an interesting model system to determine how changes in host dynamics may result in evolutionary adaptations that enhance virulence.

9.8 Concluding Remarks

Many questions remain about the biogenesis and structure of the *C. burnetii* Dot/Icm apparatus and the process by which effector protein translocation occurs. Recent advances have provided a growing list of *C. burnetii* effectors, however a comprehensive approach is required to understand all the functions mediated by the Dot/Icm system and the individual effectors. The development of genetic tools for *C. burnetii* will inevitably aid this process, as we now have the ability to analyze isogenic mutants deficient in a single effector protein or in a functional Dot/Icm system. Additionally, the ability to introduce functional alleles encoding an effector protein from isolates such as Dugway into the Nine Mile strain will provide gain-of-function phenotypes that will aid in determining the role of these individual effector proteins. Thus, additional studies on the Dot/Icm effectors from *C. burnetii* will provide novel information on the infection strategies this organism has evolved to survive and grow inside mammalian host cells.

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