Bacterial Type IV Secretion Systems: Versatile Virulence Machines

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Abstract

Many bacterial pathogens employ multicomponent protein complexes to deliver macromolecules directly into their eukaryotic host cell to promote infection. Some Gram-negative pathogens use a versatile type IV secretion system (T4SS) that can translocate DNA or proteins into host cells. T4SSs represent major bacterial virulence determinants and have recently been the focus of intense research efforts designed to better understand and combat infectious diseases. Interestingly, although the two major classes of T4SSs function in a similar manner to secrete proteins, the translocated "effectors" vary substantially from one organism to another. In fact, differing effector repertoires likely contribute to organism-specific host cell interactions and disease outcomes. In this review, we discuss the current state of T4SS research, with an emphasis on intracellular bacterial pathogens of humans and the diverse array of translocated effectors used to manipulate host cells.

Keywords

type IV secretion; effector; Dot/Icm substrate; intracellular pathogen

Type IV secretion systems

Type IV secretion systems (T4SSs) are specialized macromolecule delivery machines ancestrally related to bacterial conjugation systems. Many highly infectious intracellular bacteria employ these systems to deliver DNA or proteins into eukaryotic cells where they carry out a variety of activities essential for replication and avoidance of the immune response. These multicomponent systems allow bacterial proteins to traverse both membranes and the periplasm of the Gram-negative bacterial surface. T4SSs are broadly classified as type IVA (T4ASS) or IVB (T4BSS), depending on whether the structural components resemble the VirB/D4 complex of the plant pathogen *Agrobacterium tumefaciens* or the conjugal transfer system of the self-transmissible IncI plasmid, respectively. Although the structural components differ, both systems deliver proteins, termed effectors, directly to the host cytosol via a central pore. For proper translocation, effectors often interact with chaperones used for effector recognition in the bacterial cytoplasm. Effector translocation signals required for secretion are still not strictly defined for some T4SSs despite increased interest in their identity. Interestingly, although they possess similar translocation machinery, T4SS-producing pathogens share very few common effector proteins. Diversity among effector repertoires is predicted to influence virulence and allow establishment of pathogen-specific environments for replication. Indeed, we are just beginning to understand the versatility of secreted effectors and their impact on

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host cell physiology. Below, we review the ever-expanding field of type IV secretion, focusing on intracellular bacterial pathogens and their respective effector repertoires.

A. Type IVA-producing pathogens – *Brucella, Bartonella*, and *Rickettsia*, and *Anaplasma*

The prototypical T4ASS was originally defined for *A. tumefaciens*. T4ASSs are also used by a group of highly infectious intracellular human pathogens that have the potential to cause serious disease with high levels of mortality if left untreated. The core set of structural components in this system is encoded by an operon containing 11 genes (Fig. 1). Increasing evidence from studies of the *Brucella* T4ASS indicates this system functions in a highly similar manner to that of *A. tumefaciens*. Additionally, several studies have shown a requirement for T4SS function in disease presentation. Below, we briefly introduce representative intracellular pathogens that use a T4ASS during infection.

Brucella spp. cause brucellosis, a disease that can present with flu-like symptoms or more serious indications such as neurological disorders. In the host, *Brucella* invades phagocytic and non-phagocytic cells, subverting the endocytic pathway to avoid trafficking to lysosomes [1]. The organism ultimately establishes and replicates in a vacuole decorated with endoplasmic reticulum (ER) components [2]. *Brucella* encodes a T4ASS required for replication and formation of this ER-derived compartment [3]. Activation of the *virB* operon is triggered during the stationary phase of bacterial growth by phagosome acidification [4]. Wild type *Brucella* acquires ER membrane structure at ER-exit sites in a T4SS-dependent manner [5]. Polar mutations in *virB1* prevent *Brucella* replication in eukaryotic cells and disruption of *virB10* prevents trafficking to the ER. *virB10* mutant-containing vacuoles acquire early endosomal markers including EEA1 similar to wild type *Brucella*, but fail to evade fusion with lysosomes, resulting in degradation [6]. Different from *A. tumefaciens*, *Brucella* contains a VirB12 gene and lacks *virD4* [7]. The functional consequences of this difference are unknown; however, VirB12 may not be absolutely required for T4SS function [7].

Bartonella spp. are zoonotic intracellular pathogens with a feline reservoir and infect humans via a bite or scratch, resulting in cat scratch fever, a necrotizing lymphadenopathy presenting with fever [8]. *B. henselae* infects human vascular endothelial cells via either endocytosis or invasome-mediated internalization and resides in a non-endocytic subcellular compartment [9]. The organism's T4ASS is required for virulence, as *B. tribocorum virB4* mutants do not cause bacteremia in mammals [10]. Recent studies show VirB/D4-mediated secretion is required for actin cytoskeletal rearrangement during uptake of *B. henselae* in an invasome required for infection [11,12]. An NF-κB-directed pro-inflammatory response also requires a functional T4ASS, as does the ability of *B. henselae* to prevent host cell apoptosis [9]. These events are likely mediated by bacterial effectors, such as the Bep family, that are secreted via a C-terminal BID domain [13].

Rickettsia spp. cause potentially fatal spotted fever (*R. rickettsii*), which is characterized by rash, headache, vomiting, and fever, and typhus (*R. prowazekii*), which has historically been a major cause of wartime deaths [14]. Rickettsiae are obligate intracellular bacteria that are transmitted to humans by an arthropod vector. Following transmission, the pathogen enters epithelial cells via induced phagocytosis [15]. Unlike the pathogens previously mentioned, rickettsiae escape the early phagosome and direct host actin polymerization to spread within and between cells [14]. Genome sequencing of *R. prowazekii* revealed a diverse set of T4ASS genes (Fig. 1); however, the number of VirB genes differs from those of *A. tumefaciens* and *Brucella*, and secreted effectors have not been reported [16]. Notable genetic differences include 3-4 copies of *virB6* and two copies each of *virB4, virB8*, and *virB9* (Fig. 1). Additionally, *virB1* is located in a different chromosomal region suggesting

operon expression may vary from that reported in *A. tumefaciens*. Functional implications of expressing multiple copies of VirB proteins are unknown.

Anaplasma phagocytophilum is an obligate intracellular pathogen that causes anaplasmosis following infection of human granulocytes. Disease symptoms include a flu-like illness with fever, malaise, anemia, and thrombocytopenia, and can be fatal if left untreated [17,18]. *A. phagocytophilum* invades host cells by lipid raft-mediated endocytosis, and establishes a membrane-bound inclusion lacking late endosomal and lysosomal markers. Inside the host cell, the pathogen inhibits NADPH oxidase activation, lysosomal fusion, and IFN-γ signaling [18]. Virulence factors are not clearly defined for *A. phagocytophilum*, but the organism encodes a T4ASS for secreting effector proteins into the host cytoplasm [19]. The genetic arrangement of *A. phagocytophilum* T4SS genes is similar to *Rickettsia spp.*, with multiple copies of *virB6* and an additional five copies of *virB2*. As with *Rickettsia spp.*, the functional impact of multiple *virB6* and *virB2* copies is unknown. Closely related *Ehrlichia chaffeensis* is a macrophage-tropic pathogen that also encodes a T4ASS, but effectors remain largely unidentified [17,18].

B. Type IVB-producing pathogens – *Legionella* and *Coxiella*

Legionella pneumophila is the causative agent of Legionnaires' disease, which presents as a pneumonia in humans. *L. pneumophila* contains 27 genes that collectively encode a T4BSS termed the Dot/Icm secretion system (Fig. 1). This T4SS was identified independently by two groups who named the components either Dot (**D**efective in **o**rganelle **t**rafficking) or Icm (**I**ntra**c**ellular **m**ultiplication) proteins [20,21]. Dot/Icm genes are related to the Tra/Trb genes of self-transmissible IncI plasmids [22]. The Dot/Icm T4SS can translocate both DNA and protein effectors, mediating delivery of mobilizable IncQ plasmids and over 300 protein effectors [23]. Similar to *Brucella, L. pneumophila* replicates in host cells within an ER-derived vacuole required for survival. Trafficking to this compartment requires a functional Dot/Icm T4SS [24], as Dot/Icm mutants cannot inhibit phagosome-lysosome fusion. T4SS-deficient *L. pneumophila* is also avirulent in animal models [25].

Coxiella burnetii, the etiologic agent of Q fever, causes flu-like acute disease and potentially fatal chronic endocarditis. The pathogen encodes a Dot/Icm T4SS highly similar to that of closely-related *L. pneumophila* [26] (Fig. 1). However, a few differences are apparent, including the absence of *lvgA* and *icmR*, a disrupted *icmF* gene, presence of the IcmR functional homolog CoxigA, and two copies of *icmL* in the *C. burnetii* genome. Despite T4SS similarity, *C. burnetii* displays a unique lifestyle among intracellular bacterial pathogens, replicating in a harsh lysosome-like parasitophorous vacuole (PV) required for replication. Not surprisingly, few secreted effectors are conserved between *C. burnetii* and *L. pneumophila*. T4SS conservation has proven immensely beneficial for studying *C. burnetii* effectors, as 62 *C. burnetii* Dot/Icm substrates have been identified using *L. pneumophila* as a genetically tractable surrogate host [27,28,29,30,31]. Importantly, recent studies showed the *C. burnetii* Dot/Icm T4SS is required for effector translocation, intracellular replication, vacuole formation, and inhibition of host cell apoptosis [27,32].

T4SS structural diversity

T4SSs are composed of between 12 (T4ASS) and 27 (T4BSS) proteins that collectively function to translocate effectors into the host cytosol. Proper assembly of this many components into a functional secretion complex is critical for efficient effector translocation. Although T4ASSs and T4BSSs both transport macromolecules into susceptible host cells, the proteins used to mediate this transfer differ. Prototypical T4ASSs are composed of 11 VirB proteins (VirB1-11) and VirD4, while the Dot/Icm T4BSS requires up to 27 proteins for efficient function. Both systems consist of a panel of proteins functioning in the bacterial

cytoplasm and periplasm, and each uses a central "core" of proteins to form the secretion channel or conduit spanning the inner and outer bacterial membranes through which translocated molecules must pass. Recent structural studies have provided a wealth of information defining organization of the secretion machine including the protein transport channel, inner and outer membrane components, and periplasmic proteins. Much of our current understanding of these complex systems has been gleaned from studies of the plant pathogen *A. tumefaciens*, which produces a T4ASS [33]. Although *A. tumefaciens* is not an intracellular pathogen, T4ASS structural aspects are predictably conserved among intracellular pathogens, which agrees with structural studies of the *Brucella* T4ASS [34]. Additionally, biochemical analyses and a recent structural study of *L. pneumophila* T4SS components [35,36] have provided a glimpse into T4BSS architecture. In this section, we review the current models of each delivery system.

A. Type IVA system

T4ASSs consist of 12 proteins, collectively termed the VirB/D4 family (Fig. 2). The central pore, or translocation channel, of this system consists of VirB6 – VirB10. Recent work has provided high resolution electron microscopy and structural evidence of the secretion pore [37,38]. This complex contains 14 copies each of VirB7, VirB9, and VirB10, which closely associate to compose a pore with a cytoplasmic diameter of 55 Å, a 20 Å diameter opening at the extracellular surface of the bacterium, and a 10 Å region of constriction in the channel. VirB10 serves a scaffolding function for the central channel and is the only component that inserts into both the inner and outer membranes of the organism [37,39]. VirB10 is also critical for proper substrate passage and pilus production [40]. A recent study showed that a single mutation in VirB10 blocks pilus production and allows unregulated release of an effector protein [41]. These results suggest VirB10, in addition to stabilizing the core complex of T4ASSs, also serves as a "gatekeeper", allowing proper passage of effectors when needed. VirB6 and VirB8 are membrane proteins that may regulate passage of macromolecules through the bacterial inner membrane into the periplasmic portion of the secretion channel [42,43,44]. Supporting this prediction, both proteins interact with bacterial cytoplasmic substrates to be transferred through the translocation channel [45].

Apart from the central core, six additional VirB/D4 proteins are required for efficient T4SS function and secretion of molecules. Extending from the translocation pore is an elongated pilus formed by VirB2 and VirB5 [46,47] that is not present in T4BSSs. VirB1 is a transglycosylase located in the periplasm that may provide openings in the peptidoglycan layer for efficient assembly of the VirB2/VirB5 pilus [48,49,50]. VirB3 is an inner membrane protein also predicted to be involved in pilus assembly [51]. In the bacterial cytoplasm, the ATPases VirB4, VirB11, and VirD4 provide the energy necessary to promote ATP-dependent transfer of substrates through the secretion channel [52]. All three proteins associate with the inner membrane, adapt homo-hexameric conformations, and collectively act as an energy-generating scaffold for effector translocation [34,53,54,55]. New evidence also shows that VirB4 interacts with VirB11 to control pilus structure during assembly of the secretion complex [56]. Furthermore, VirD4 likely serves as an effector recruitment protein, identifying and presenting proteins for translocation [52]. Interestingly, *Brucella* does not encode the VirD4 ATPase, but produces VirB12, which is absent in *A. tumefaciens*. The role of VirB12 in secretion is not completely understood, but the protein may be dispensable for proper function of the *Brucella* T4SS [7]. A *Brucella* VirD4 compensatory protein has not currently been described. (We also refer readers to two recent reviews of the detailed structural characteristics of T4ASSs [33,57]).

B. Type IVB system

T4BSS organization has been deciphered through studies of the *L. pneumophila* system. This T4SS consists of 22 structural proteins and 5 chaperones that interact with effector proteins in the bacterial cytoplasm (Fig. 2). These components are referred to as Dot or Icm proteins and were independently identified by the Isberg and Shuman laboratories, respectively [20,21]. These original discoveries of the *L. pneumophila* Dot/Icm T4SS showed the system efficiently transfers DNA. However, researchers have uncovered a versatile role for this T4SS in translocating many effector proteins. The Dot/Icm translocation pore is formed by DotC, DotD, DotF, DotG, and DotH, which are collectively analogous to the VirB6-10 complexes in type IVA systems. DotG has a similar proposed function to VirB10 [37] and interacts with inner and outer membrane components [35]. DotF interacts with DotG and contains a cytoplasmic portion that may be involved in substrate recognition [58]. DotF and DotG localization requires a complex containing DotC, DotD, and DotH. DotH oligomerizes in the bacterial outer membrane and is surface exposed in contact with the extracellular milieu [59]. DotH positioning in the outer membrane is aided by the lipoproteins DotC and DotD [35]. The DotD crystal structure was recently solved and demonstrates homology to secretin proteins [36]. DotD is predicted to adopt a 12 – 14 unit ring-like homo-oligomer that stabilizes interactions between DotC, DotF, DotG, and DotH.

Many membrane-associated Dot/Icm proteins have unknown functions. However, individual proteins were localized via elegant biochemical analyses by Vincent and Vogel [35]. DotE, I, J, P, V, IcmT, and IcmV are inner membrane proteins with undefined roles in translocation and DotO is an undefined cytoplasmic protein. DotA is an undefined inner membrane protein but is clearly critical for efficient effector delivery as DotA-deficient *L. pneumophila* is commonly used as a negative control for Dot/Icm substrate translocation [28,30,31,60]. Interestingly, DotA is secreted into the extracellular milieu at some point during the *L. pneumophila* infectious cycle [61]; however, the role of secreted DotA is unknown. DotU and IcmF are inner membrane proteins involved in stabilization of the secretion complex [62]. The IcmF gene is disrupted in closely related *C. burnetii*, but the functional implications of this mutation are unknown [63]. DotL forms a complex with DotM and DotN in the inner membrane and contains a cytoplasmic region predicted to act as a receptor for effectors to be secreted [64]. DotK is a lipoprotein that associates with the periplasmic face of the outer membrane and may tether the secretion complex to peptidoglycan due to an OmpA-like domain [65].

Six Dot/Icm proteins function in the bacterial cytoplasm to direct effectors to the secretion channel and promote the ATP-dependent transfer of proteins through the pore. DotB is a cytoplasmic ATPase analogous to VirB11 in T4ASSs. Because type IV secretion is an energy-dependent process, DotB activity is necessary for generating the required ATP. For proper function, DotB adopts a hexameric configuration similar to VirB11 [66,67]. The remaining five cytoplasmic Dot/Icm proteins serve as chaperones to identify and deliver effectors to the secretion apparatus for export into the host cytosol. These proteins work in pairs and form stable complexes required for efficient effector secretion. IcmQ and IcmR partner to form pores in membranes [68] while IcmS and IcmW interact to direct secretion of multiple effectors [69]. Indeed, IcmW has been used as bait to identify effectors targeted for Dot/Icm-mediated secretion [69]. Furthermore, IcmS and IcmW binding to effectors promotes conformational changes that expose C-terminal translocation signals [70]. Recently, LvgA was identified as a potential fifth chaperone that interacts with and stabilizes IcmS [71]. Interestingly, other *Legionella* species and *C. burnetii* do not encode IcmR, but contain functional homologs of IcmR predicted to compensate for this secretion factor [72]. Additionally, *C. burnetii* does not contain an obvious LvgA homolog.

Finally, IcmX is a periplasmic protein with no apparent membrane association. Similar to DotA, a modified form of IcmX is secreted into the extracellular milieu but the function of this protein is unknown [73].

Effector translocation signals

An area of T4SS function that has recently received increased attention is the identity of a motif or domain that serves as the signal for effector translocation. We currently do not fully understand the mechanism by which an effector is distinguished from non-effectors in the bacterial cytoplasm, then presented for delivery through the translocation pore, particularly regarding T4BSSs. It appears T4ASSs and T4BSSs recognize different translocation signals, although recent studies used the *L. pneumophila* T4BSS to identify T4ASS effectors from *Brucella* and *Anaplasma*. Two *Brucella* effectors, VceA and VceC, are translocated by *L. pneumophila*, demonstrating that a T4BSS can recognize translocation signals from T4ASS effectors [74]. Likewise, the *A. phagocytophilum* effector AnkA is secreted by *L. pneumophila* [75]. Thus, *L. pneumophila* is proving useful as a heterologous host for identifying effectors from genetically intractable pathogens such as *Anaplasma* [76]. Current translocation signal hypotheses have been derived from studying large groups of effectors and generally center on the C-terminal end of effectors. In support of this hypothesis, the Dot/Icm chaperones IcmS and IcmW promote a conformational change in some effectors that allows presentation of a C-terminal translocation region [70].

In the *A. tumefaciens* T4ASS, the translocation signal resides in a hydrophilic C-terminal region with a consensus R-X(7)-R-X-R-X-R-X-X(n) motif [77]. *Bartonella*-translocated effector proteins (Beps) contain an intracellular delivery domain termed the BID region that is conserved in conjugative relaxases that direct translocation of plasmid DNA. The BID domain and a short positively charged tail sequence together form a bipartite C-terminal translocation signal to mediate T4ASS-dependent translocation [13]. The *A. phagocytophilum* AnkA sequence supports the presence of overall positive charges in the C-terminal tail of translocated effectors, similar to *A. tumefaciens* [78]. In contrast, a requirement for hydrophobic C-terminal residues is supported by several studies examining T4BSS effectors from *L. pneumophila* and *C. burnetii*. Using the *L. pneumophila* effector RalF, Nagai *et al*. demonstrated that Dot/Icm substrates often contain a hydrophobic amino acid in the -3 or -4 amino acids at the effector C-terminus [60]. A separate study including a larger group of proteins confirmed this hydrophobic tendency and also implicated amino acids with small side chains in proximity to the hydrophobic residue(s) [79]. This C-terminal composition is also seen in *C. burnetii* effectors, with six plasmid-encoded effectors (CpeA-F) containing hydrophobic residues within the five C-terminal amino acids [30]. Removal of these five amino acids substantially impairs effector translocation by *C. burnetii*. Additionally, Burstein *et al*. developed an algorithm for *L. pneumophila* effector identification that scans proteins for hydrophobic residues at the -3 and -4 positions, a lack of negatively charged residues in the C-terminal six amino acids, and serine and threonine residues in the -3 to -11 position [80].

The Burstein *et al*. study also suggested the presence of glutamate and aspartate residues in the -8 to -18 positions. This observation corresponds to a recent hypothesis proposing the role of an E Block motif in *L. pneumophila* effector translocation [81]. The E Block is a region of 6-8 amino acid residues rich in glutamate or aspartate that resides in the -8 to -26 region of the effector C-terminus. By screening for this motif, the authors identified 49 new effectors and found an E Block in numerous previously identified effectors. These results, combined with the hydrophobic residue hypothesis, suggest a combination of properties may compose the translocation signal of T4SS substrates.

Effector protein repertoires

The ultimate purpose of T4SSs is to efficiently deliver a panel of diverse proteins or DNA directly into a host cell where they serve a wide variety of functions during infection. Effector proteins are critically important for the success of T4SS-producing pathogens and, therefore, have been the focus of intense research into their identity and function. This work is well warranted as T4SS effectors potentially represent novel therapeutic targets and allow researchers to better model host pathogen interactions. Several elegant studies have identified intriguing features of effectors secreted by T4ASSs and T4BSSs. Many effectors have been identified using bioinformatics to uncover eukaryotic-like domains/motifs. These regions are predicted to interact with host proteins following translocation into the cytosol and suggest horizontal gene exchange has occurred extensively between T4SS-producing organisms and their hosts [82]. This strategy has been used in many recent studies to identify *L. pneumophila* and *C. burnetii* effectors (described below). Effector identification has also been aided by development of effector-specific antibodies and sensitive enzymatic readouts of secretion, including beta-lactamase and adenylate cyclase reporter assays. These methods have been adapted to a diverse panel of intracellular pathogens and have provided many novel proteins for future characterization. Elucidation of effector function will rapidly advance our understanding of the molecular mechanisms of bacterial pathogenesis. Here, we review the most current effector discoveries and their role in infection. Effectors with defined activities or unique characteristics are listed in Table 1.

A. *Brucella*

The first *Brucella* effectors were identified by assessing translocation of proteins containing a *virB* promoter, suggesting coordinated expression with T4SS structural components [74]. This study showed translocation of VceA (**_virB_**-**c**o-regulated **e**ffector **A**) and VceC using a beta-lactamase readout of cytosolic delivery. VirB/D4-dependent secretion was confirmed using a VirB2-deficient strain of *B. abortus* and supported the presence of a C-terminal translocation signal as discussed above. Two recent reports identified five more novel *Brucella* T4SS substrates. de Barsy *et al.* [83] identified a translocated protein that specifically interacts with eukaryotic Rab2, a GTPase normally found on the *Brucella* replication vacuole membrane. The secreted protein was named RicA (**R**ab2 **i**nteracting **c**onserved protein **A**) and represents the first *Brucella* effector with a proposed function during intracellular growth. Shortly following this report, Marchesini *et al.* performed a bioinformatics screen of the *B. abortus* proteome and found 84 candidate effectors based on properties including eukaryotic-like domains, homology to known effectors, and protein-protein interaction motifs [84]. Using an adenylate cyclase reporter assay, six proteins were translocated into host cells and four required functional VirB10 and VirB11. Characterization of these and other effectors will enhance understanding of the *Brucella*-macrophage dynamic.

B. *Anaplasma*

Two *A. phagocytophilum* T4SS effectors have currently been identified. AnkA, which contains seven eukaryotic-like ankyrin repeat domains, traffics to the host nucleus during infection and binds to DNA, suggesting the effector modulates transcriptional events [85]. This prediction is supported by results showing AnkA binds to the *CYBB* promoter and influences expression of this component of the phagocyte oxidase complex [86]. AnkA contains an EPIYA motif that allows phosphorylation of the protein shortly following infection of eukaryotic cells. Phosphorylation is mediated by the host protein Src, and phosphorylated AnkA binds to SHP-1, potentially inhibiting anti-bacterial signaling [78]. In the host cytosol, AnkA interacts with Abi-1, which recruits Abl-1 to phosphorylate AnkA [87]. Collectively, these studies demonstrate the versatility of a single T4SS effector in an

intracellular pathogen infection. Furthermore, *A. phagocytophilum* encodes three other ankyrin repeat-containing proteins that represent candidate T4SS substrates [88]. Recently, Ats-1 (**A**naplasma **t**ranslocated **s**ubstrate-1) was identified as an *A. phagocytophilum* T4SS effector that influences mitochondrial-dependent apoptosis [89]. Ats-1 is delivered to the cytosol in a VirD4-dependent manner, then traffics to the mitochondrial matrix, where the protein is cleaved and inhibits Bax-induced apoptosis. This activity is mediated by Ats-1 disrupting docking of cytoplasmic Bax with the mitochondria, a prerequisite for mitochondrial-dependent apoptosis [90]. This activity is critical for *A. phagocytophilum* to maintain a viable host cell in which to replicate.

C. *Bartonella*

A series of studies by Dehio and colleagues defined a new family of *B. henselae* effector proteins termed Beps (**B**artonella-translocated **e**ffector **p**roteins). BepA-G each contain a Bep intracellular delivery (BID) domain required for cytosolic translocation and BepA-C contain a FIC (**f**ilamentation **i**nduced by **c**AMP) domain, which is an emerging effector motif often involved in manipulating the actin cytoskeleton [13,91]. Bep proteins are critical for multiple *B. henselae*-directed events as deletion of BepA-G results in impaired protection from apoptosis, increased IL-8 production, altered actin rearrangements during cellular entry, and sprout formation by endothelial cells [13,92]. Specific gene deletions have further defined individual Bep activity during endothelial cell infection. BepA specifically inhibits apoptosis, with BepA-deficient *B. henselae* unable to antagonize caspase activity in endothelial cells [93]. BepG regulates uptake of *B. henselae* as large aggregates into host cells via an invasome [12]. Additionally, BepC and BepF are able to trigger invasome-mediated uptake in a cofilin1-dependent manner [11]. Clearly, Beps are critical for *B. henselae* infection and further characterization of their activities will better define the *Bartonella*-host interaction.

D. *Legionella*

Of the pathogens in this review, *L. pneumophila* has by far the most identified and characterized T4SS effector proteins. Characterization of *L. pneumophila* effectors has demonstrated the applicability of this research to understanding basic eukaryotic cellular processes. In essence, *L. pneumophila* is teaching us novel aspects of cell biology that would likely not otherwise have been predicted. Amazingly, *L. pneumophila* translocates over 300 substrates via the Dot/Icm T4SS [23]. RalF was the first *L. pneumophila* effector identified [94] and has since served as a positive control for numerous secretion studies. *L. pneumophila* effectors are involved in many intracellular events necessary for the pathogen's lifestyle (Fig. 3). Effectors impact host cell survival, transcription, ubiquitination, and proper formation of the *L. pneumophila*-containing vacuole (LCV). Thus, *L. pneumophila* effectors will be discussed in terms of the general process they influence.

Generation and modification of the LCV—The LCV interacts with host ER proteins to generate a compartment decorated with rough ER. Establishment of this ER-derived niche allows the pathogen to avoid delivery to lysosomes where it would be destroyed. Dot/Icm mutants are unable to form an ER-derived vacuole and are degraded in lysosomes, indicating effector proteins are necessary for generating the LCV. Multiple studies have identified effectors that recruit ER components and interact with Rab GTPases that regulate ER to Golgi trafficking. RalF has a Sec7 homology domain and activates host ADP ribosylation factor-1 (ARF-1) at the LCV membrane [94]. This was the first evidence of a *L. pneumophila* effector modulating a eukaryotic protein involved in secretory transport. This initial work was followed by several groups that identified effectors interacting with Golgi- or ER-related proteins. YlfA (**Y**east **l**ethal **f**actor **A**) inhibits yeast growth and localizes to early secretory organelles and the ER [95]. LegC2 and LegC7 also co-localize with ER

proteins but appear to disrupt the organelle when overexpressed [96]. SidC, SidJ, and LegK2 are each required for recruitment of ER proteins to the LCV [97,98,99]. Furthermore, SidC is anchored to the LCV membrane via binding to phosphatidylinositol(4) phosphate (PI4P), indicating *L. pneumophila* coopts host phosphoinositides to generate a replicative niche [100]. LidA also interacts with host secretory machinery, localizing to ERGIC and Golgi structures and causing eventual organelle disruption [101].

A series of recent studies have collectively addressed interactions between *L. pneumophila* effectors and the small GTPase Rab1. Rab1 directs ER to Golgi transport by direct interaction with Golgi proteins and is present on the LCV at early times post-infection, suggesting *L. pneumophila* deploys Dot/Icm effectors shortly following uptake into a host cell [102,103]. SidM/DrrA is anchored to the LCV via PI4P binding [104,105] and specifically binds to Rab1, acting as a guanine exchange factor (GEF) to recruit the small GTPase to the vacuole [106,107]. Surprisingly, SidM/DrrA also functions as a Rab1 guanine dissociation inhibitor (GDI) displacement factor to release Rab from its GDI as a prerequisite for LCV recruitment [108,109]. The N-terminal region of SidM/DrrA also contains AMPylation activity, which prohibits access of Rab1 to GTPase activating proteins (GAPs) that would inactivate the protein. However, SidM/DrrA is not the only effector that regulates Rab1 LCV membrane levels. LidA interacts with Rab1 and enhances recruitment of the protein to the LCV [106]. AnkX is a phosphocholine transferase that modulates Rab1 activity independent of SidM/DrrA via an AMPylation-related FIC domain [110]. Two effectors, LepB and SidD, mediate inactivation of Rab1 to promote cycling off the LCV membrane. LepB displays GAP activity that inactivates Rab1, resulting in dissociation of the GTPase from the LCV [109]. SidD is a deAMPylase that reverses Rab1 AMPylation and triggers release from the vacuole [111]. Thus, at least five effectors work cooperatively to regulate Rab1 cycling to and from the LCV membrane, providing stringent regulation of a secretory transport protein during early stages of infection.

Aside from ER and Golgi interactions, other effectors are involved in proper LCV formation. SidK binds to VatA, a component of the host vacuolar ATPase following translocation into the cytosol, and this interaction inhibits acidification of the LCV, a condition that favors bacterial survival and replication [112]. A group of effectors termed Vips (**V**PS **i**nhibitor **p**roteins) disrupt vacuolar protein sorting (VPS) in yeast, suggesting they are involved in vesicular trafficking during mammalian cell infection [113]. VipA, D, and F each inhibit trafficking to lysosomes with VipA impacting Golgi transport and VipD altering formation of multivesicular bodies. Similarly, LegC3 and LegC7 cause VPS defects in yeast [96]. SetA and Ceg19 localize to late endosomal compartments [114], suggesting they play a role in preventing phagolysosomal fusion. AnkX also intercepts microtubule-dependent vesicular transport to alter phagolysosomal fusion and acidification [28]. Finally, AnkH and AnkJ are required for proper LCV formation in eukaryotic cells; however, their mechanism of action is undefined [115].

Release from protozoa—*L. pneumophila* spends a major portion of its life cycle inside a protozoan host, which serves as the pathogen's environmental reservoir. Therefore, it is not surprising that *L. pneumophila* produces and secretes effectors to control this niche. Two effectors have been identified that play a role in the pathogen's exit from protozoa. LepA and LepB are coiled coil domain (CCD)-containing proteins with limited homology to eukaryotic SNARE proteins [116]. *L. pneumophila* deficient in either effector is unable to properly escape from *Acanthamoeba* cells, possibly due to impaired regulation of exocytic machinery. Absence of these proteins also causes disregulated Dot/Icm-dependent hemolytic activity [117]. These studies suggest protozoa may represent an untapped source of host-specific *L. pneumophila* effectors.

Inhibition of apoptosis—At least two effectors promote cell survival during *L. pneumophila* infection, an event necessary for proper completion of the pathogen's intracellular growth cycle. The first identified was SdhA (**S**uccinate **d**e**h**ydrogenase **A**), which protects cells from mitochondrial- and caspase-dependent apoptosis [118]. Interestingly, cells infected with *L. pneumophila* lacking SdhA undergo rapid cell death [118,119], making this protein one of the few effectors with a readily observable defect in cellular growth. SidF also promotes eukaryotic cell survival via binding to the pro-apoptotic proteins BNIP3 and Bcl-rambo, effectively inhibiting their pro-death activities [120].

Regulation of host cell transcription—Bacterial pathogens often influence eukaryotic transcription as a stringent mechanism of controlling infection events. *L. pneumophila* targets NF-κB to regulate transcriptional responses using at least two effector proteins. NF-κB is modulated throughout *L. pneumophila* infection [121] and activation is needed for efficient inhibition of apoptosis during growth in macrophages [122,123]. Dot/Icm-translocated LegK1 is a serine/threonine kinase that directs NF-κB activation by phosphorylating IκBα [124], a protein that normally tethers NF-κB in the cytosol in an inactive form. LegK1 phosphorylation of IκBα releases NF-κB for nuclear translocation and transcriptional regulation. A subsequent study confirmed the NF-κB activating property of LegK1 and identified a second effector, LnaB (*Legionella* **N**F-κB **a**ctivator **B**), that contains a coiled coil domain and contributes to NF-κB activation [125].

Ubiquitination—A series of recent papers defined a ubiquitin-related activity for the Dot/Icm effector AnkB [126,127,128]. AnkB contains two ankyrin repeats and one eukaryotic-like F-box domain, a region typically involved in ubiquitination [115]. AnkB is required for *L. pneumophila* replication in mammalian cells and protozoa and for virulence in a mouse model of Legionnaires' disease, highlighting its importance in the pathogen's infectious cycle [115,127,129,130]. At the cellular level, AnkB is required for recruitment of ubiquitinated proteins, such as the focal adhesion protein parvin B [128], to the LCV. It is tempting to predict the recruited proteins are involved in vacuole formation and/or disruption of phagosome-lysosome fusion. Additionally, a recent study reported that LegU1 and LicA also interact with the ubiquitin machinery proteins Skp and Cullin, which form functional ubiquitination complexes [131]. LegU1 and LicA contain F-box regions, highlighting the importance of this motif in mediating ubiquitination by a bacterial pathogen. Furthermore, LegU1 associates with Skp and Cullin to recruit eukaryotic BAT3 (HLA-B-associated transcript 3) for ubiquitination and interacts with another *L. pneumophila* effector, Lpg2160, suggesting LegU1 is a multifunctional Dot/Icm substrate [131].

Effector-mediated turnover of bacterial and host proteins—An intriguing aspect of effector-mediated ubiquitination has recently been discovered for the Dot/Icm substrate LubX. LubX (*Legionella* **U**-**b**o**x** protein) encodes two U-box domains that are related to F-box motifs and are commonly found in eukaryotic E3 ubiquitin ligases [79]. Following delivery to the host cytosol, LubX mediates ubiquitination of eukaryotic Clk1 (Cdc2-like kinases 1) with one U-box domain binding to Clk1 while the other U-box directs ubiquitin modifications. Interestingly, Clk1 is not the only target of LubX, as the protein also mediates ubiquitination of the effector SidH, leading to proteasome-dependent degradation of the protein [132]. Thus, LubX is a multifunctional effector that regulates levels of another effector via ubiquitination and degradation. This novel activity may have important implications for other F-box-containing bacterial effectors and suggests effector targets are not always host proteins.

Targeting of host protein synthesis machinery—SidI targets host translation machinery, inhibiting eEF1A and eEF1Bγ [133,134]. SidI activity triggers a stress response involving activation of a heat shock regulatory protein. Similar to SidI, an Lgt family of *L. pneumophila* glucosyltransferases (Lgt1, 2, and 3) also targets host protein machinery [134,135,136], suggesting a panel of *L. pneumophila* proteins may alter the eukaryotic translational response to bacterial infection.

E. *Coxiella*

Recent progress has been made in the identification and characterization of T4SS effectors produced by *C. burnetii*. Due to inherent difficulties in genetically manipulating the bacterium, initial studies used closely-related *L. pneumophila* to identify Dot/Icm-translocated proteins. Pan *et al.* first demonstrated translocation of four ankyrin repeat-containing proteins (AnkA, B, F, and G) by the *L. pneumophila* T4SS and showed the presence of AnkF in the cytoplasm of infected cells [28]. This work was followed by a study showing that *C. burnetii* isolates of varying disease potential collectively encode 15 Anks, 11 of which are secreted in a Dot/Icm-dependent manner [31]. Recently, Luhrmann *et al.* identified the first function for a *C. burnetii* effector, showing that AnkG antagonizes host cell apoptotic death via binding to pro-apoptotic p32 [137], an event required to maintain a viable niche for replication [138]. While highly informative, these studies were not able to assess direct translocation of effectors by *C. burnetii*. However, recent studies used new genetic methods to show translocation of T4SS substrates by *C. burnetii* during infection [29,30] and two reports demonstrated the requirement of a functional Dot/Icm system for effector translocation and bacterial growth in eukaryotic cells [27,32]. To date, 62 *C. burnetii* effectors have been identified using *L. pneumophila* and/or by directly assessing translocation by *C. burnetii* [27,28,29,30,31]. These effectors have a variety of interesting characteristics, including ankyrin repeats, coiled coil domains, and kinase homology. Additionally, a group of effectors are encoded on the cryptic plasmid harbored by most *C. burnetii* isolates. Three of these plasmid-encoded effectors are present in all isolates examined thus far, suggesting a critical function for the plasmid in *C. burnetii* pathogenesis [30]. Functional characterization of these and other effectors will provide better understanding of *C. burnetii* isolate differences leading to disparate disease presentations.

Anti-bacterial targeting of T4SS components

An intriguing area of T4SS research is the potential for developing therapeutic strategies to inhibit secretion system or effector function, effectively disarming the invading pathogen. T4SS function elicits distinct immune responses and recent evidence suggests individual T4SS components and effectors are recognized by host sera following infection by *C. burnetii*, *B. abortus*, and *Anaplasma spp*. Beare *et al.* showed that sera collected from Q fever patients recognize the T4SS structural proteins IcmE and IcmK and also detect the secreted effector AnkG [139]. Likewise, VirB12 from *B. abortus* is detected by sera collected from infected mice and goats [140]. Multiple T4SS proteins from the *A. phagocytophilum*-related livestock pathogen *A. marginale* elicit an antigenic response, and this is likely true for human *Anaplasma* pathogens. VirB9 and VirB10 are recognized by sera from *A. marginale*-infected animals, while VirB2, VirB7, VirB11, and VirD4 are strongly immunogenic [141]. Collectively, these studies suggest host responses are directed towards T4SS proteins and these components may be used as diagnostic markers and targeted for vaccines and therapeutics.

A recent study demonstrated that inhibition of *B. suis* VirB8 leads to impaired infection [142]. Using a library of 29,567 inhibitors, the authors showed that 48 compounds specifically prevent VirB8 function through disruption of transcription, protein production, or protein-protein interactions using a bacterial two-hybrid assay. Additionally, a *L.*

pneumophila study reported the use of a bioactives library to discover inhibitors of the Dot/Icm T4SS [143]. It is tempting to predict these compounds may eventually be useful from a therapeutic standpoint, and these studies may serve as a blueprint for identifying new antibacterial agents.

Conclusions/future perspectives

T4SSs and their accompanying effector repertoires are recognized as essential determinants of bacterial virulence. Many Gram-negative pathogens rely on these DNA and protein delivery systems for efficient infection and manipulation of host cell physiology. As discussed, the number and type of secreted effectors varies greatly among pathogens and we are now beginning to understand the role of T4SSs in bacterial pathogenesis. However, several questions remain unanswered. First, what is the function of uncharacterized effectors? This question is currently under investigation by a number of very talented cellular microbiology laboratories and evidence from *L. pneumophila* studies has demonstrated that effector function opens many new doors into understanding a pathogen's lifestyle. Second, why are so many effectors secreted during infection by pathogens such as *L. pneumophila* and *C. burnetii*? *L. pneumophila* alone secretes over 300 effectors [23]. Is this a question of simple redundancy or does each effector have a distinct role? Finally, do newly identified T4SS genes comprise functional secretion systems? T4SS-encoding genes have been found in highly infectious pathogens such as *Rickettsia spp.* [16] and *Orientia tsutsugamuchi* [144], but functional aspects of these systems have not been tested. Additionally, it is unknown whether the *L. pneumophila* Lvh system is a functional system used for Dot/Icm-independent purposes [145,146]. Answers to these questions will undoubtedly allow better modeling of bacterial pathogenic mechanisms and provide new methods for combating infectious disease. As *L. pneumophila* has taught us, intracellular bacterial pathogens are often the most knowledgeable cell biology experts and should be studied to discover novel tenets of eukaryotic cell function.

Acknowledgments

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Executive summary

Type IV secretion systems

- T4SSs are multicomponent complexes that transport effector proteins from the bacterial cytoplasm directly to the eukaryotic cytosol

- *Brucella, Bartonella, Anaplasma,* and *Rickettsia* encode T4ASSs

- *Legionella* and *Coxiella* use T4BSSs

T4SS structural diversity

- T4ASSs are composed of 11 VirB proteins and most contain a VirD4 protein

- Dot/Icm T4BSSs are composed of up to 27 proteins

- Both T4SSs provides a central pore through which effectors are transported

- Chaperones regulate effector delivery to the translocation pore

Effector translocation signals

- Translocation signals reside in the C-termini of effectors

- Signals are composed of amino acids that influence hydrophobicity

- T4BSS effectors require less than 10 C-terminal amino acids for translocation

Effector protein repertoires

- Effector repertoires are incredibly diverse between bacterial pathogens

- Often interact with specific host proteins in the cytosol

- Regulate numerous infection events including apoptosis, cytokine response, host transcription, and secretory transport

- Often vital for proper formation of the pathogen's replication vacuole

Antibacterial targeting of T4SS components

- Sera from infected individuals recognize T4SS components

- Inhibitors of *Brucella* VirB8 antagonize infection

Conclusions/future perspectives

- Elucidation of T4SS and effector activity will allow enhanced modeling of host-pathogen interactions

- Targeting T4SS structural components and effectors may allow design of effective therapeutics that have little impact on the host

Type IVA

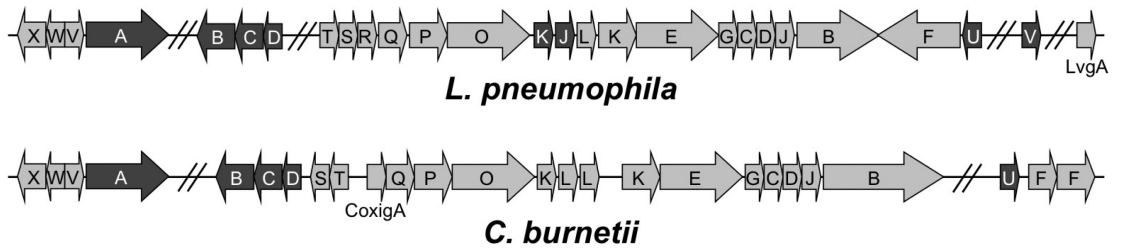**A. tumefaciens**

3-4 copies

Rickettsia spp.

Type IVB

L. pneumophila

C. burnetii
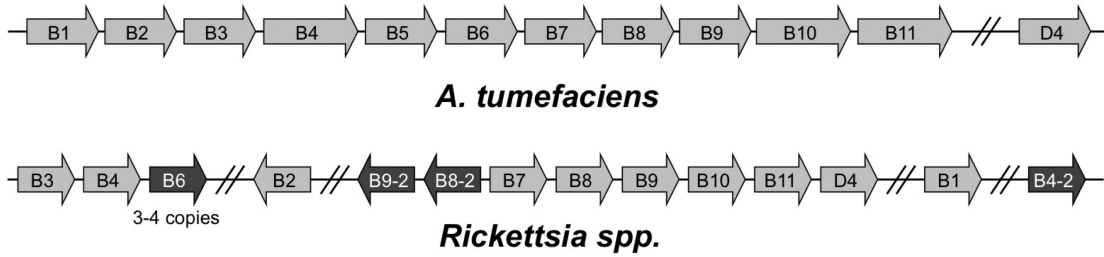
Figure 1. Genetic arrangement of T4SS-encoding genes
(A) The prototypical T4ASS structural proteins of *Agrobacterium* are encoded by *virB1-11* in an operon and *virD4*. Rickettsial organisms and *Anaplasma* encode a T4ASS, but contain multiple copies of certain genes, such as *virB6, virB8*, and *virB9*. **(B)** The Dot/Icm T4BSS is typified by *Legionella* and is encoded by up to 27 genes. Dark gray arrows denote *dot* genes and light gray indicate *icm* genes. *Coxiella* encodes a homologous system with a few differences, including two copies of *icmL*, absence of *icmR* and *lvgA*, presence of the IcmR functional homolog CoxigA, and a disrupted *icmF* gene.
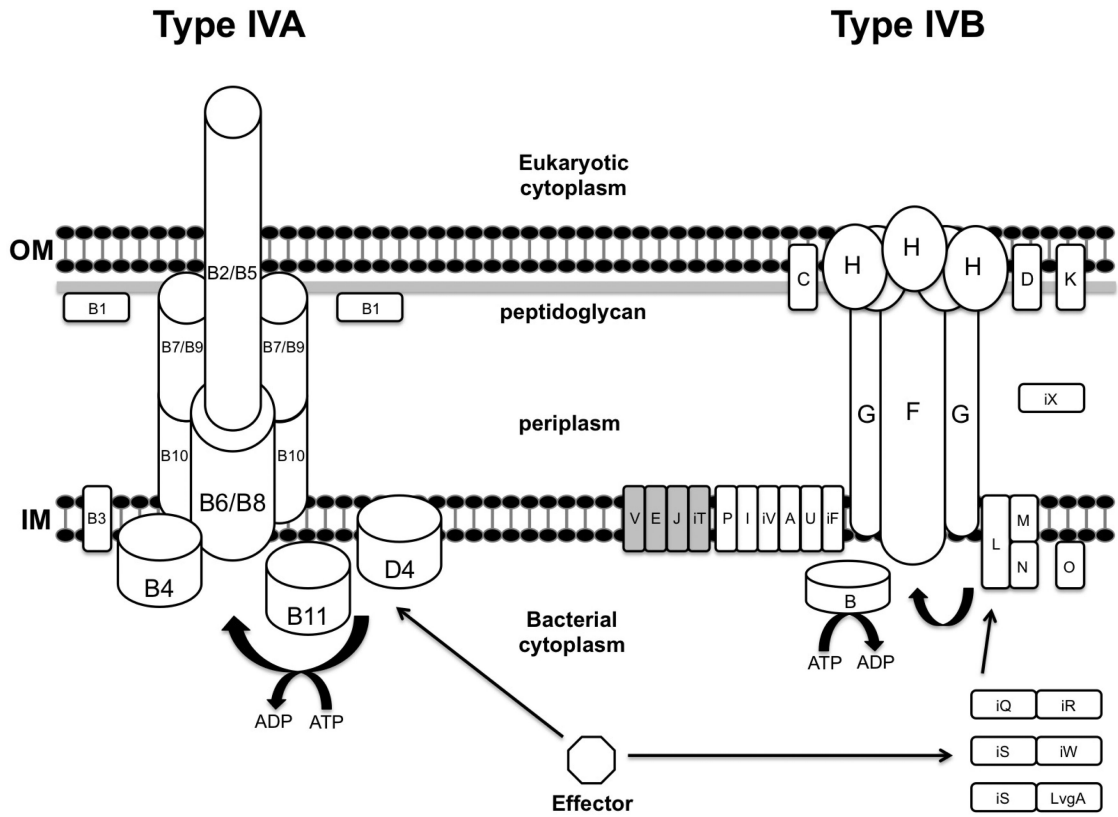
Figure 2. T4SS organization
T4SSs are multi-component machines that collectively serve to transport effector proteins from the bacterial cytoplasm to the eukaryotic cytosol, bypassing a periplasmic intermediate. This is accomplished by VirB/D4 proteins in the T4ASS and Dot/Icm proteins in the T4BSS. Each system forms a central channel through which effectors transit. In the bacterial cytoplasm, ATPases (VirB4, VirB11, VirD4, and DotB) provide the energy necessary for translocation, while chaperone proteins deliver effectors for entry into the central pore. DotV, DotE, DotJ, and IcmT (light gray) have not been localized experimentally but are predicted to be inner membrane proteins. The known or predicted function of individual T4SS components is described in the text.
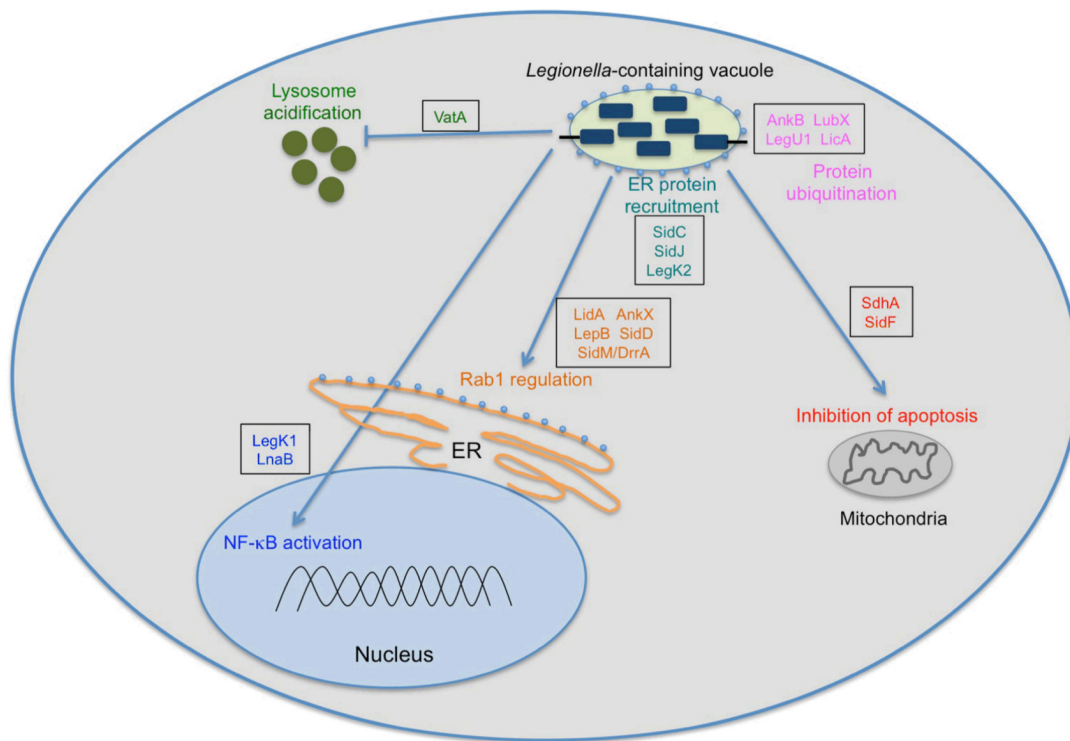
Figure 3. Diversity of *Legionella* effector activities
Legionella produces over 300 Dot/Icm T4SS substrates that control numerous events during intracellular growth. Many effectors are involved in regulating formation and modification of the *Legionella*-containing vacuole, while others interact with host cell proteins involved in transcription and cell survival. *Legionella* provides an example of the potential versatility of a bacterial T4SS. Effectors are shown in boxed regions and are colored similar to the general process they regulate.

Table 1

T4SS effectors with known activity

Effector	Characteristics	Interacting Host Protein(s)	Function	Ref.
Brucella				
RicA		Rab2	Recruitment of Rab2 to pathogen vacuole	[83]
Anaplasma				
AnkA	Ankyrin repeats, EPIYA motif	SHP-1, Abi-1	Binds host DNA	[78,85-87]
Ats-1		Bax	Inhibits apoptosis	[89]
Bartonella				
BepA	FIC domain	Unknown	Inhibits apoptosis	[91,93]
BepC/F		Unknown	Cofilin-dependent uptake	[11]
BepG		Unknown	Invasome-mediated uptake	[12]
Legionella				
RalF	Sec7 homology	ARF-1	Activates ARF	[94]
YlfA		Unknown	Inhibits yeast growth	[95]
SidC/J/LegK2		Unknown	Recruit ER proteins	[97-99]
LidA		Rab1	Disrupts secretory transport	[101,106]
SidM/DrrA		Rab1	GEF/GDI	[106-109]
AnkX	Ankyrin repeats, FIC domain	Rab1	Phosphocholine transferase	[110]
LepB		Rab1	GAP	[109]
SidD		Rab1	deAMPylase	[111]
SidK		VatA	Inhibits lysosome acidification	[112]
VipA	Coiled coil domain	Unknown	Alters Golgi transport	[113]
VipD		Unknown	Alters MVB formation	[113]
LepA/B	Coiled coil domain	Unknown	Escape from protozoa	[116]
SdhA		Unknown	Inhibits apoptosis	[118-119]
SidF		BNIP3, Bcl-rambo	Inhibits apoptosis	[120]
LegK1	Ser/Thr kinase	IκBα	Phosphorylates IκBα	[124]
LnaB	Coiled coil domain	Unknown	Activates NF-κB	[125]
AnkB	Ankyrin repeats, F-box	Parvin B	Recruits ubiquitinated proteins	[126-130]
LegU1/LicA	F-box, choline kinase (LicA)	BAT3 (LegU1)	Form ubiquitination complexes	[131]
LubX	U-box	Clk1, SidH	Ubiquitinates Clk1, mediates SidH degradation	[79,132]
SidI		eEF1a, eEF1Bγ	Induces host stress response	[133]
Coxiella				
AnkG	Ankyrin repeats	p32 (gC1qR)	Inhibits apoptosis	[137]