REVIEW

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Master manipulators: an update on *Legionella pneumophila* Icm/Dot translocated substrates and their host targets

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ABSTRACT: Macrophages are the front line of immune defense against invading microbes. Microbes, however, have evolved numerous and diverse mechanisms to thwart these host immune defenses and thrive intracellularly. *Legionella pneumophila*, a Gram-negative pathogen of amoebal and mammalian phagocytes, is one such microbe. In humans, it causes a potentially fatal pneumonia referred to as Legionnaires' disease. Armed with the Icm/Dot type IV secretion system, which is required for virulence, and approximately 300 translocated proteins, *Legionella* is able to enter host cells, direct the biogenesis of its own vacuolar compartment, and establish a replicative niche, where it grows to high levels before lysing the host cell. Efforts to understand the pathogenesis of this bacterium have focused on characterizing the molecular activities of its many effectors. In this article, we highlight recent strides that have been made in understanding how *Legionella* effectors mediate host-pathogen interactions.

Legionella: an intracellular pathogen

Legionella pneumophila is a Gram-negative bacterium that survives and replicates within host cells. In the environment, *Legionella* is found in warm, fresh-water aquatic habitats, where it is a natural pathogen of unicellular protozoan hosts [1]. This microbe was initially identified as a human pathogen in 1976, when attendees of the American Legion Convention in Philadelphia fell ill with symptoms that included high fever and pneumonia. This outbreak resulted in 29 deaths [2,3]. *Legionella*, present in the hotel's air conditioning system, was determined to be the etiological agent of this illness, dubbed Legionnaires' disease. Patients were infected through inhalation of contaminated aerosols.

Inside the lungs, *Legionella* is phagocytosed by alveolar macrophages, which are permissive for *Legionella* growth. The *Legionella*-containing vacuole (LCV) does not passively mature along the endocytic pathway towards the degradative lysosome, but is instead transformed into a replication-promoting compartment that is reminiscent of the endoplasmic reticulum (ER). Ultrastructural studies of infected phagocytes show initial association of the LCV with smooth vesicles and mitochondria, occurring as early as 15 min post phagocytosis [4]. By 8 h postinfection (hpi), the LCV is studded with ribosomes, markers of the ER. ER-specific membrane and luminal proteins also localize to the LCV during infection, including calnexin and BIP, respectively [5,6]. Rab1 and Sec22b, proteins involved in the trafficking of ER-derived vesicles to target membranes, are found on intact LCVs isolated from 1 and 14 h-infected macrophages [5]. This suggests that the LCV adopts an identity that is independent from the endocytic pathway [7]. Taken together with data that shows the LCV localizes to the perinuclear space proximal to the ER, it is clear that *Legionella* evades intracellular degradation by establishing an ER-associated replicative compartment [4,8].

KEYWORDS

• autophagy • intracellular pathogen • *Legionella pneumophila* • macrophages • protein translocation

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LCV biogenesis and intracellular replication requires the activity of the Icm/Dot type IV secretion system, which delivers approximately 300 effector proteins into host cells [9–11]. These proteins manipulate host biological processes in order to promote bacterial growth. Accordingly, mutants lacking a functional Icm/Dot secretion apparatus are avirulent, failing to evade fusion with the lysosome [12–14]. The localization of lysosomal markers LAMP1 and Rab7 to the vacuole of the avirulent *dotA* mutant within 5 min of phagocytosis indicates that wild-type *Legionella* must act quickly to counteract host defenses [15]. *Legionella* does this by engaging the type IV secretion system and initiating protein translocation upon contact with host cells.

The essential role that the Icm/Dot secretion system plays in *Legionella* pathogenesis suggests that determining the function of its numerous translocated proteins is the key to understanding *Legionella* virulence strategies. Consequently, identifying and characterizing Icm/Dot translocated substrates (IDTS) has been the focus of intense investigation over the past two decades. A significant obstacle to this endeavor is the fact that individual deletion of a vast majority of IDTS has no effect on the intracellular colonization of host cells by *Legionella*. This is likely due to redundancy among effectors within the IDTS repertoire. Despite this challenge, significant progress has been made in determining the function of a number of IDTS, indicating that *Legionella* utilizes the Icm/Dot secretion system to manipulate a vast number of host cell processes, including vesicle trafficking, translation, transcription and survival [16–18]. In this article, we highlight recent analysis of the IDTS repertoire, its composition and seeming redundancy, as well as the characterization of IDTS with newly identified functions that expand the range of cell biological functions of *Legionella* effectors.

Functional redundancy: problem solved?

While approximately 300 translocated substrates of the Icm/Dot system have been identified, significant questions remain regarding their individual contributions to intracellular survival and replication. Of the IDTS, only SdhA and DimB, when deleted, exhibit severe defects in intracellular replication [19,20]. Functional redundancy is invoked to explain the shockingly low number of translocated substrates that are essential for intracellular colonization. This strategy

ensures that very few individual proteins are absolutely required for intracellular growth [16]. Experimental validation of this hypothesis has been observed for a small number of proteins that impact a narrow range of the known cell biological processes that are manipulated during *Legionella* infection [19,21,22].

To identify *Legionella* genes that are essential for growth *in vitro* under nutrient rich conditions, O'Connor *et al.* performed a transposon site hybridization screen [23]. A total of 588 essential genes were identified. Subsequent mapping indicated that seven distinct genomic loci, comprising 27.1% of the coding capacity of the organism, contained no genes required for growth *in vitro* (Figure 1A). Strains containing single individual deletions of five clusters exhibited wild-type growth in broth culture. This was also true for a pentuple mutant, in which these five gene clusters were deleted simultaneously. Amazingly, this pentuple mutant, lacking 31% of the known IDTS, was not markedly defective for intracellular growth in permissive murine macrophages (Figure 1B). These translocated substrates were dispensable for infection, likely due to functional redundancy. This wholesale redundancy is only evident upon infection of mammalian cells, the so-called accidental host for *Legionella* [24]. The authors found that the pentuple mutant was differentially compromised for growth in distinct amoebal species, the natural hosts of *Legionella* in aquatic environments. This was also true for *Legionella* strains containing different combinations of cluster deletions (Figure 1C). Given the modular nature of these genomic clusters, the increased variability in their gene composition and synteny, and their significantly lower GC content, the authors hypothesize that these clusters may have been horizontally acquired by *Legionella* over time. They also hypothesize that these clusters are all maintained due to the selective pressure to survive in an aquatic environment that contains numerous, diverse amoebal hosts with different intracellular environments and challenges. Thus, it appears that this large cadre of effectors is maintained in order to equip *Legionella* with the ability to parasitize distinct and diverse intracellular niches.

Functional redundancy may be a general *Legionella* strategy for maintaining a broad host range of infection. In addition to the Icm/Dot type IV secretion system, *Legionella* utilizes a type II secretion system, the *Legionella* secretion

Figure 1. Functional redundancy and host range expansion. (A) Linear schematic of the *Legionella* genome that depicts the seven genomic loci that contain no essential genes. The total number of genes and the number of Icm/Dot translocated substrates within each cluster is noted for the wild-type *Legionella* strain and for the pentuple strain, which contains simultaneous deletions in five of the identified clusters. Clusters 2, 4, 6 and 7 could not be removed completely. The number of genes that remain after genetic manipulation are indicated. (B) The wild-type *Legionella* strain is able to replicate in amoeba and primary murine bone marrow-derived macrophages from A/J mice. The pentuple strain, on the other hand, is not able to replicate in amoeba, indicating that one or more of the nonessential genes is specifically required for replication in amoeba. (C) A schematic of distinct amoebal species – *Dictyostelium discoideum* (Dd), *Hartmannella vermiformis* (Hv) and *Acanthamoeba castellanii* (Ac) – within the aquatic environment of a fresh water pond is shown. *Legionella* mutants that contain deletions of individual clusters exhibit distinct intracellular growth phenotypes in different amoebal species, suggesting that the presence/absence of these clusters define the amoeba host range.

pathway (Lsp), to deliver proteins into its extracellular space. Approximately 27 proteins secreted via this pathway have been identified [25]. Mutants lacking the type II secretion apparatus are defective for growth in amoeba, macrophages and mice, indicating that these secreted proteins play an important role in pathogenesis [26]. Recent work by Tyson *et al.* sought to determine if individual Lsp substrates were required for replication in multiple host cells, including human U937 macrophages and the amoebal strains *Hartmannella vermiformis*, *Acanthamoeba*

castellanii and *Naegleria lovaniensis* [26]. Of the 17 mutant strains tested, 13 grew similarly to wild-type *Legionella* in all strains tested. Of the remaining four, NttA (hypothetical protein) was impaired for growth in *A. castellanii* only, while PlaC (phospholipase), ProA (metalloprotease) and SrnA (T2 RNase) were impaired for growth in both *H. vermiformis* and *N. lovaniensis*. Thus, similar to the translocated proteins of the Icm/Dot type IV secretion system, the proteins of the Lsp appear to serve redundant functions that confer host-specific virulence in

Legionella. Whether there is functional interplay between these two systems and the proteins they introduce into the host environment remains to be seen. Why *Legionella* employs two distinct mechanisms of extracellular protein delivery is also a mystery. The answers to these two questions can offer great insights into *Legionella* virulence strategies.

The high degree of functional redundancy encountered among the *Legionella*-translocated substrates has greatly limited the ability of researchers to interrogate the molecular nature of *Legionella*-host interaction using classical forward genetic mutation analysis. Additionally, multiple host pathways contribute to the intracellular replication of *Legionella*, particularly the early secretory system that contributes membrane for LCV biogenesis from multiple input pathways. This was demonstrated by showing that disruption of intracellular growth required simultaneous interference by multiple double-stranded RNAs [22]. Recent work has extended this multigene-targeting strategy to simultaneous perturbations in both *Legionella* and the host in order to identify translocated substrates that are important for intracellular growth [27]. Insertional mutagenesis and depletion (iMAD) involves challenging double-stranded RNA-depleted host cells that are disrupted in one intracellular pathway with transposon insertion mutants of *Legionella*. The rationale behind this strategy is that mutations removing translocated substrates that target one pathway will result in defective intracellular growth in host cells that are depleted for a second, redundant pathway. Standard genetic approaches were unable to identify roles for many of these proteins in intracellular growth because manipulation of multiple pathways by the organism masks defects resulting from loss of a single pathway. O'Connor *et al.* used this approach to screen for *Legionella* genes required for growth in five distinct *Drosophila* host cell backgrounds that were each depleted for a single protein involved in the early secretory system [27]. This analysis showed that 55 Icm/Dot substrate genes were required for growth in at least one knockdown host background. This includes 44 genes that had not previously been shown to contribute to intracellular replication.

Hierarchical clustering of the mutants identified by iMAD, which was performed based on their patterns of intracellular growth in the *Drosophila* knockdown strains, identified

14 distinct functional groups. Proteins within a single functional group were predicted to target the same host pathway. To test this hypothesis, the authors generated *Legionella* mutants that contained different combinations of double mutants within and between the various functional groups. Consistent with this hypothesis, only *Legionella* strains that contain simultaneous deletions in distinct functional groups exhibit an intracellular growth defect in *Drosophila* cells. This phenotype was also observed during infection of amoeba and macrophages, natural hosts of *Legionella*. This type of analysis has great predictive, hypothesis-generating power. Proteins with known functions that are present within a functional group can be used to predict the phenotype of uncharacterized effectors within the same group. *L. pneumophila* mutants defective for SdhA are unable to maintain vacuole integrity, resulting in decay of the vacuolar membrane during infection [28]. The authors predicted that a $\Delta wipB\Delta lidA$ mutant would be similarly defective in maintaining vacuolar integrity based on the clustering of WipB with SdhA in the same functional group, and demonstrated that this was indeed the case [27].

Functional redundancy is an experimental barrier that continues to plague *Legionella* research. Recent work, however, has greatly broadened our understanding of the role that this phenomenon plays in the biology of *Legionella*, allowing the bacteria to maintain a broad host range across amoebal species [26,27]. Genetic tools, such as iMAD, offer novel approaches to surmounting the experimental barrier imposed by functional redundancy; however, analyses to date have focused on host trafficking pathways and vacuolar biogenesis. Many of the uncharacterized effectors are likely functioning during the later stages of infection, and defining the host biological pathways that are targeted and how redundancy among effectors work within those contexts is the next challenge for investigators.

Build your own vacuole: co-opting host vesicular trafficking

Intracellular organelles have unique protein compositions. Consequently, the vesicular trafficking pathways that mediate the delivery of these macromolecules are very specific and highly regulated. Distinct small GTPase proteins serve as markers and activators of fusion with cargo vesicles that originate from particular compartments

[29]. Rab1, a member of the Rab GTPase family is localized to the Golgi apparatus where it drives fusion specifically with vesicles that originate from the ER. Arf1 is a small GTPase that promotes vesicle formation on the Golgi membrane, vesicles that traffic back to the ER. During infection, Rab1 and Arf1 localize to the LCV in an Icm/Dot-dependent manner, suggesting that IDTS are playing a role in their recruitment [5,30]. The translocated proteins responsible for this recruitment have been identified as RalF, an Arf1 guanine exchange factors (GEFs) and DrrA (also known as SidM), a Rab1 GEF [31–33]. Sar1, a small GTPase that regulates vesicle formation on the ER membrane, does not localize to the LCV. Its activity, however, is vital to *Legionella*

pathogenesis, as expression of a dominant negative Sar1 variant, SarH79G, severely inhibits the ability of *Legionella* to replicate intracellularly [34].

The activity of GTPases, from their localization to target membranes, to their activation, deactivation and subsequent removal from the membrane, is tightly regulated [29]. The cycling of the Rab1 GTPase on the LCV membrane is modulated by multiple *Legionella* IDTS (**Figure 2**). The GEF activity of DrrA/SidM is necessary to release the GDP dissociation inhibitor from the Rab1-GDP. This enables the insertion of an active Rab1-GTP into membranes, where it promotes the fusion of ER-derived vesicles with the LCV [35,36]. The final step in the Rab GTPase cycle involves Rab inactivation by GTP hydrolysis and

Figure 2. *Legionella* Icm/Dot translocated substrates manipulate host vesicle trafficking pathways. *Legionella* evades fusion with endosomal compartments and generates an intracellular compartment that is reminiscent of the ER. The acquisition of ER components is mediated by IDTS that hijack host vesicle trafficking pathways to promote fusion between ER-derived vesicles and the LCV. *Legionella*-driven processes are highlighted using red arrows, while host-driven processes are depicted with black arrows. These translocated substrates include the guanine exchange factors RalF and DrrA/SidM, which recruit Arf1 and Rab1 to the LCV membrane. Several translocated effectors modulate the activity of Rab1. DrrA AMPylates (~) Rab1 and works with the t-SNARE syntaxin 3 and the v-SNARE Sec22b to promote fusion between ER vesicles and the LCV. The effector LidA is thought to act as a tether that stabilizes the interaction between activated Rab1 and the LCV. SidD is a deAMPylase. AnkX modifies Rab1 by adding a PC (#) moiety, which is reversed by the activity of Lem3. LepB is a Rab1 GTPase-activating protein that inactivates Rab1 and triggers its removal from the LCV membrane. Although their functions are unknown, Rab6 and Rab8 localize to the LCV. *Legionella* also manipulates the retrograde vesicle trafficking pathway. The IDTS RidL inhibits the activity of the SNX that are critical for retromer assemble and retrograde trafficking. ER: Endoplasmic reticulum; GDI: GDP dissociation inhibitor; IDTS: Icm/Dot translocated substrate; LCV: *Legionella*-containing vacuole; PC: Phosphocholine; SNX: Sorting nexin.

GTPase removal from the membrane. This activity is mediated by the IDTS LepB, a Rab1-specific GTPase-activating protein (GAP) [37].

Recent work has highlighted additional *Legionella*-mediated modifications to Rab1. Rab1 can be AMPylated by DrrA/SidM [38]. This activity was discovered after the N-terminal domain of DrrA/SidM was crystalized and found to be structurally similar to glutamine synthetase adenylyl transferase. The full-length DrrA/SidM possesses AMP-transferring activity, which results in the AMPylation of Rab1 on Tyr77. With this modification, activated Rab1 is refractory to GAP-mediated GTP hydrolysis and is constitutively active. This posed the following conundrum: Rab1 localizes to the LCV only during the first 4 h of infection [37], after which its diminished levels are coincident with increased LepB levels. This can only occur if Rab1-GTP is hydrolyzed and removed, which is not possible if Rab1 remains AMPylated. Therefore, Rab1 AMPylation must be reversible. The deAMPylase, SidD, was identified by two groups [39,40]. SidD exhibited *in vitro* deAMPylase activity towards Rab1 and is required for the removal of Rab1 from the LCV during the early stages of infection.

Another recently discovered Rab1 post-translational modification is phosphocholination or 'PCylation' [41,42]. This activity was identified using liquid chromatography–tandem mass spectrometry analysis on *Legionella*-infected cells that ectopically expressed the Rab1 protein. Rab1 PCylation required AnkX, a translocated protein that disrupts vesicle traffic and induces cytotoxicity when introduced into host cells [43]. This modification was made on Ser76, directly adjacent to the AMPylated Tyr77, and is thought to inhibit Rab1-targeted GEF and GAP activities. A dephosphocholinase effector, Lem3, was also identified after the observation that PC-Rab1 lost its phosphocholine (PC) moiety after incubation with *Legionella* cell lysate [42]. DePCylation was subsequently observed in an *in vitro* assay with purified PC-glutathione S-transferase Rab1 and Lem3.

LidA is a translocated substrate of the Icm/Dot secretion system that localizes to the cytosolic face of the LCV, where it associates with Rab1 [8,32]. In the absence of LidA, there is a kinetic delay in the recruitment of Rab1 to the LCV. LidA has no GAP or GEF activity, but is able to associate with Rab1 in both the GDP and GTP-bound states [32]. Additionally,

AMPylation and PCylation do not interfere with the LidA/Rab1 interaction. However, beyond these observations, very little is known about the mechanism of LidA function. Recent *in vitro* analysis of purified LidA sought to characterize its activity biochemically [44]. Neunuebel *et al.* found that LidA has positive effects on Rab1 activation, preventing spontaneous nucleotide release from Rab1, as well as GAP-mediated inactivation [44]. This is, however, confounded by the fact that they also observed that purified LidA inhibits Rab1 interaction with downstream Rab effectors. Purified LidA interferes with the ability of *Legionella* proteins to promote AMPylation, deAMPylation, phosphocholination and dephosphocholination. The authors propose a model in which LidA serves as a tether for Rab1 that promotes its activation. Given that DrrA/SidM activity is required for Rab1 recruitment, they suggest that LidA interacts with Rab1 after it is AMPylated, locking the protein in this state.

Biochemical analysis indicates that LidA also binds Rab6 and Rab8; however, very little is known about the role these small GTPases play during infection [32]. Proteomic analysis of purified *Dictyostelium* LCVs indicate the presence of Rab8 [45]. Rab6-specific antibodies fail to show localization to 30-min-old LCVs in infected mouse macrophages [30]. Despite the lack of evidence for an association of Rab6 with the LCV, Chen and Machner recently demonstrated that Rab6 is required for efficient intracellular replication of *Legionella* [46]. Approximately 20% of vacuoles are replication-incompetent in the presence of a dominant-negative Rab6. Analysis of the interaction of LidA with the Rab6 isoform Rab6A', using purified recombinant proteins, indicates that LidA preferentially binds GTP-associated Rab6A' and does so at a 1:2 stoichiometry. Subsequent analysis showed that LidA inhibited the GAP activity of purified Rab6A'. Thus, Rab6 is required for efficient *Legionella* infection and LidA modulates its activity.

While the factors involved in the recruitment of ER-derived vesicles to the *Legionella* vacuole have been identified, the factors that drive the actual membrane fusion events are unknown. Typically, fusion is driven by interactions between SNARE proteins present on the fusing vesicle (v-SNAREs) and on the target compartment (t-SNAREs). Arasaki *et al.* showed that the fusion events that transform the *Legionella* vacuole involve the v-SNARE Sec22b on the

ER-derived vesicles, and the plasma membrane t-SNARE complex syntaxin on the nascent vacuole [47]. More recent work has demonstrated that the *Legionella* effector DrrA/SidM interacts with plasma membrane-derived Syntaxin3 present on the vacuole in a Rab1-mediated reaction involving these normally noncognate SNAREs [48]. This interaction is sufficient to drive membrane fusion and content exchange of cargo in a Sec22b v-SNARE-dependent reaction.

The extensive analysis of LCV biogenesis has elucidated molecular mechanisms used by *Legionella* to manipulate anterograde trafficking pathways in the host. Recent work now shows that *Legionella* also manipulates retrograde trafficking (Figure 2) [49]. Retrograde trafficking describes the transport of cellular contents from the plasma membrane and endosomes to compartments associated with the early secretory apparatus. This process plays a critical role in recycling cargo receptors and contributes to the maintenance of organelle identity and function [50]. Cargo selection of specific membrane proteins is mediated by the multisubunit retromer complex, which is composed of a heterotrimeric cargo recognition subcomplex and a membrane-deforming sorting nexin subcomplex [51]. Finsel *et al.* have identified a *Legionella*-translocated protein, RidL, which interacts with and interferes with the activity of the host retromer during infection [49]. Immunoprecipitation/mass spectrometry analysis of RAW246.7 macrophage and *Dictyostelium discoideum* lysates indicate that purified RidL interacts with the host retromer, pulling down components of the cargo recognition complex (Vps35, Vps26 and Vps29). A direct interaction between purified glutathione S-transferase-RidL and Vps29 was also observed. RidL was characterized as an Icm/Dot-translocated substrate that promotes intracellular replication. The $\Delta ridL$ mutant exhibited a modest growth defect in murine macrophages and amoeba. RidL localizes to the membrane of the LCV during infection. Consistent with this observation, GFP fusion proteins of VPS29 and Vps35, components of the retromer, localizes to the LCV in these host cells. This localization was Icm/Dot dependent, but RidL independent. Interestingly, siRNA knockdown of retromer components results in an approximately twofold increase in *Legionella* intracellular replication, indicating that retromer activity negatively impacts *Legionella* growth and suggests that RidL is negatively

regulating retromer activity. The authors find that RidL competes with SNX1 for binding to the LCV, consequently inhibiting retrograde traffic. Additionally, ectopic RidL expression is sufficient to inhibit the retrograde traffic of host cells. Taken together, these data indicate that retrograde traffic is detrimental to the intracellular growth of *Legionella* growth and that the translocated effector RidL promotes replication by inhibiting this pathway. Interestingly, McDonough *et al.* have shown that the retromer plays an opposite role in the pathogenesis of *Coxiella burnetii*, where it is thought to promote replication [52]. During infection, the biogenesis of the *C. burnetii* replicative vacuole, an acidified lysosome-derived compartment, and intracellular replication requires a functional *C. burnetii* Icm/Dot type IV secretion system. In this case, depletion of the retromer cargo recognition components VPS35 and VPS29, as well as sorting nexins SNX2, SNX3, SNX5 and SNX6, inhibited the translocation of *C. burnetii* Icm/Dot effectors, as well as intracellular replication. This defect in translocation could explain the diminished intracellular replication, as the Icm/Dot effectors are essential for intracellular growth. The authors suggest a model in which the retromer could be responsible for bringing to or excluding from the vacuole key replication promoting factors. Conversely, it is possible that retrograde transport could alter the environment within the LCV in a manner that is detrimental to *Legionella*.

Build your own vacuole: hijacking host lipids

Membrane lipid composition also shapes the identity and function of subcellular compartments. Phosphoinositides (PI) are lipids that are present on the cytosolic surface of organelles. They serve as anchors for proteins that play roles in vesicle trafficking and signaling events. *Legionella* has devised mechanisms to co-opt host lipid metabolic pathways to promote LCV biogenesis and intracellular growth [53].

Phosphatidylinositol 4-phosphate, PI(4)P, is a PI that is enriched on the membranes of the Golgi apparatus. *Legionella* is able to co-opt host lipid metabolic pathways to facilitate the enrichment of PI(4)P on the surface of the LCV, an ER-like replication compartment [54]. A functional Icm/Dot type IV secretion system is required for this phospholipid enrichment, suggesting that *Legionella* proteins may be able

to manipulate host phospholipid pools, as well as interact with them. Initial studies focused on identifying *Legionella* proteins that bound to these PIs, presumably anchoring them to their site of action. SidC, its paralog SdcA, and DrrA/SidM were among the first PI(4)P-binding IDTS identified [54,55]. These proteins have all been shown to contribute to the interaction of the LCV with the ER [32,56]. LidA, an IDTS that interacts with DrrA/SidM to modulate Rab1 activity, also binds to PI(4)P, although it has a greater preference for PI(3)P *in vitro* [55]. Since PI(3)P is enriched on endosome membranes and plays a role in promoting interactions with endosomes and lysosomes, LidA binding to the LCV is thought to be mediated through interactions with PI(4)P. A subset of effectors have been identified that have a preference for binding to PI(3)P. This includes the IDTS SetA, a glucosyltransferase, and the secreted protein LpnE, which interacts with host PI(5)P phosphatase (OCRL1) [57–59].

Recent work has focused on identifying and characterizing the *Legionella* proteins that alter the host lipid landscape, endeavoring to answer the question of how the nascent LCV with high levels of PI(3,4)P₂ and PI(3,4,5)P₃ is converted to an ER-like LCV that is enriched for PI(4)P [54]. PI(3,4)P₂ and PI(3,4,5)P₃ promote interactions with endosomes and lysosomes, while PI(4)P promotes interactions with the ER [53]. Hsu *et al.* took a bioinformatic approach, scanning the *Legionella* genome for putative PI phosphatases that contained a conserved CX₅R motif [60]. SidF was one of 29 IDTS that contained this motif, and the authors used the malachite green phosphatase assay to show that purified SidF is a PI(3)P with specificity for PI(3,4)P₂ and PI(3,4,5)P₃, generating PI(4)P and PI(4,5)P₂, respectively. This activity is dependent on the catalytic cysteine in the CX₅R motif. SidF contains two transmembrane domains and localizes to the LCV during infection of primary murine macrophages, suggesting that the site of its activity during infection is the LCV membrane. While the overall sequence and structure of SidF did not resemble that of known phosphatases, the crystal structure of the N-terminal catalytic domain shows that the catalytic core resembles that of other PI phosphatases and is ideally shaped to accommodate PI(3,4)P₂ in the active site. The observed hydrolysis of PI(3,4,5)P₃ also occurs, but is less efficient than hydrolysis of the 5-hydroxy lipid. These results establish that SidF

is a PI phosphatase that could alter the PI profile of the LCV, increasing PI(4)P levels and promoting association with ER vesicles. Accordingly, deletion of SidF results in a 30% decrease in SidC localization to the LCV, consistent with SidF playing a role in PI(4)P enrichment on the LCV. SidF is the first *Legionella* protein that has been shown to directly modify host PIs.

Genetic screens have recently identified two *Legionella* IDTS that manipulate host phospholipid distribution, LecE and LpdA (via diacylglycerol [DAG]) [61,62]. Both proteins were shown to localize to the LCV during *Legionella* infection of U937 human-derived macrophages. LpdA, a phospholipase D domain-containing protein, is thought to promote conversion of phosphatidylcholine to phosphatidic acid (PA). Dephosphorylation of PA generates diacylglycerol (DAG), a reaction that is mediated by the Pah1 phosphatase in *Saccharomyces cerevisiae*. The IDTS LecE increases Pah1 activity in yeast cells, resulting in an increase in DAG production. Ectopic expression of LpdA and LecE in Cos7 cells containing GFP PA and DAG biosensors, respectively, showed that the effectors dramatically altered the quantity and distribution of these lipids in a manner that was dependent on key catalytic residues. Present at the LCV membrane, the activity of LpdA and LecE could contribute to the enrichment of PI(4)P on the LCV, converting phosphatidylcholine to DAG [61,62]. In this model, higher DAG levels on the LCV would recruit PKC and PKD, which interact with membranes through DAG-binding domains. PKC could then activate PKD, resulting in recruitment of PI4KIIIB to the membrane, consequently increasing levels of PI(4)P.

Hijacking the host transcriptome

The regulated processes of transcription and translation are excellent targets for engineering an intracellular environment hospitable to pathogen survival. It has been shown that *Legionella* targets the host proteome by inhibiting protein synthesis (Figure 3) [63]. Inhibition is mediated by several IDTS, including glycosyltransferases Lgt1, Lgt2 and Lgt3, which target eEF1A and SidI, which targets eEF1A and eEF1By [64–66]. SidL has also been implicated in protein synthesis inhibition, but its target remains unknown [67].

Recent work indicates that *Legionella* may also target the host transcriptome. Two groups independently characterized homologs of a translocated substrate possessing a eukaryotic

Figure 3. *Legionella* lcm/Dot translocated substrates manipulate host transcription and translation. The *Legionella* effectors Lgt1, Lgt2, Lgt3, Sidl and SidL inhibit host protein translation. Two groups separately characterized a SET domain-containing lcm/Dot translocated substrate that localizes to the nucleus during infection and modifies histones. These groups reach different conclusions about the activity and function of this SET domain protein. Model 1: RomA was characterized in *Legionella pneumophila* strain Paris by Rolando *et al.* [68]. In this model, RomA is a methyltransferase that represses transcription by methylating histone H3K14, preventing H3K14 acetylation, which is a marker of active transcription. Model 2: LegAS4 is the RomA homolog in *L. pneumophila* strain Philadelphia. Li *et al.* propose a model in which LegAS4 methylates histone H3K4 associated with rDNA [69]. This methylation is thought to promote rDNA transcription.
rDNA: Ribosomal DNA.

SET domain, RomA in *L. pneumophila* strain Paris and LegAS4 in *L. pneumophila* strain Philadelphia [68,69]. SET domains are found in histone lysine methyltransferase, suggesting a role for this protein in host cell histone modification during infection [70,71]. Both groups demonstrated that the purified protein methylates H3 histones and that conserved catalytic residues in the SET domain are required for this activity. They also showed that the effector localizes to the nucleus during infection, consistent with its predicted role in histone modification. The authors, however, offer different models for how histone methylation manipulates host transcription during infection (Figure 3). Rolando *et al.* identified a novel histone H3K14 methylation pattern mediated by RomA and predict that it

plays a role in repressing transcription of a variety of targeted genes [68]. Li *et al.*, on the other hand, predicted that LegAS4 methylates histone H3K4 and H3K9 and propose that this methylation plays a role in activating ribosomal DNA (rDNA) transcription [69].

To determine the effect of RomA methylation on histones, Rolando *et al.* performed mass spectrometry analysis on *in vitro*-methylated histone H3, which they determined to be the preferred substrate of RomA [68]. This approach identified a novel mammalian histone H3K14 methylation pattern and the authors showed that this RomA-dependent H3K14 methylation occurs in the context of a *Legionella* infection. Immunofluorescence analysis of infected THP-1 cells probed for H3K14me2 showed

positive nuclear staining of cells infected with wild-type *Legionella* and not the $\Delta romA$ strain. They could also detect the presence of RomA-dependent H3K14me2 in THP-1 infected cell lysates as early as 4 hpi, increasing at 8 hpi. The activity of this protein is required for efficient infection of amoebal and human cells, indicating that this histone modification may play an important role in virulence. The previous observation that H3K14 acetylation was a marker for transcriptional activation led to the hypothesis that methylation might serve as a marker to inhibit acetylation and promote transcriptional repression. The pattern of H3K14me2/3 colocalization with 4',6-diamidino-2-phenylindole-bound heterochromatic regions is reminiscent of the H3K9me3 marks for transcriptional repression, providing further evidence in support of this hypothesis. Chromatin-precipitation/sequencing analysis of THP1 macrophage-like cells infected with either wild-type *Legionella* or the $\Delta romA$ mutant was used to determine which genes were enriched for H3K14-methylated histones at their transcriptional start sites. This approach identified 3470 unique genes, 2968 of which were annotated using DAVID (Database of Annotation, Visualization, and Integrated Discovery) software [72]. These genes were placed in functional categories that included cell death, kinase activity and gene expression. The authors highlighted genes in one of the most enriched annotation clusters: genes involved in the regulation of immune responses. This cluster included cytokines (IL-6 and TNF- α), chemokines (CXCL1 and CXCL2) and the receptors Nalp3 and TLR5, suggesting that these genes are targets of bacterial repression during infection. This result is, however, at odds with previously published work showing that immune genes such as IL-6 and TNF- α are highly induced during infection with virulent *Legionella* strains [20,73]. While the authors have identified a novel mechanism of activity for a previously uncharacterized effector, the biological consequence of its function during infection needs to be examined further.

Li *et al.* determined the LegAS4 site of methylation on H3 by probing their *in vitro* methylation reaction mixtures with antibodies to known H3 lysine methylation patterns, including H3K4, H3K9, H3K27 and H3K36 [69]. The presence of LegAS4 induced the most dramatic increase in H3K4 dimethylation (H3K4me2). The nucleolar localization of transfected GFP-LegAS4 in

HeLa cells led the authors to hypothesize that LegAS4 might be playing a role in regulating rDNA transcription. Chromatin immunoprecipitation of LegAS4, followed by quantitative real-time PCR to determine if there was a specific association with rDNA indicated that LegAS4 bound rDNA in the intergenic space and the promoter region. LegAS4 expression in cells also correlated with increased H3K4me2 methylation at the rDNA promoter, as well as increased pre-ribosomal RNA synthesis. These phenotypes are all dependent on the catalytic histone methyltransferase activity of LegAS4, indicating that this effector may be playing a role in modulating rDNA transcription. Increased rDNA expression was observed during *Legionella* infection of *Acanthamoeba castellanii*, murine bone marrow-derived macrophages and human U937 monocytes. This increase in rDNA expression was dependent on the Icm/Dot secretion system, but independent of LegAS4 expression, as a $\Delta legAS4$ mutant still induced rDNA transcription. The authors cite functional redundancy to explain this lack of phenotype with the deletion strain. Taken together, these data suggest that LegAS4 positively regulates rDNA expression during infection.

These two groups take different approaches to determine the function of this SET domain IDTS and each identifies a distinct site of methylation on histone H3. Rolando *et al.*, identifying H3K14, found that mutating the lysine of H3K4 to alanine on histone octamers resulted in a decrease in RomA-dependent methylation *in vitro* [68]. However, they could not detect methylated H3K4 if they tried to probe RomA-treated octamers with antibodies to di- or tri-methylated H3K4, thus concluding that the effect of RomA on H3K4 methylation is indirect. On the other hand, Li *et al.* would not detect the H3K14 methylation because they probed only for known methylation patterns [69]. What is clear from both works is that this effector localizes to the nucleus and modifies histones. What remains unsolved is the biological consequence and significance of this activity.

Autophagy

Autophagy is a process used by eukaryotic cells to degrade cytosolic components [74]. These cytosolic proteins and organelles are encapsulated and sequestered in a *de novo* synthesized phagosome that matures and fuses with degradative lysosomes. Under normal conditions,

autophagy plays important roles in cellular development and homeostasis. During periods of starvation, however, this 'self-eating' mechanism is upregulated, resulting in an increase in lysosome-generated macromolecules, nutrients the cell can utilize to promote its own survival. Additional stresses can also induce autophagy, including microbial infection. In this antimicrobial capacity, autophagy can promote lysosomal delivery of microbes that evade the endosomal trafficking route. As such, autophagy has been implicated in the host response to *Listeria monocytogenes*, *Shigella flexneri* and *Coxiella burnetii* [75]. Consequently, autophagy is a barrier to intracellular colonization that pathogens must overcome. This can be carried out in a number

of different ways, either by evasion or disruption. Recent papers indicate that *Legionella* is targeted by the host autophagy during infection and that it utilizes an Icm/Dot substrate to thwart this host antimicrobial response (Figure 4) [76,77].

Autophagy is induced during infection of nonpermissive host cells upon *Legionella* infection. These nonpermissive host cells restrict the intracellular growth of *Legionella* and ultimately clear the pathogen [78]. Proteins required for initiation of autophagosome formation, ATG7, and autophagosome maturation, ATG8, localize to the LCV during infection of these cells, suggesting that autophagy may play an antimicrobial role on the host response to *Legionella* [79]. Evidence in support of this hypothesis is

Figure 4. Model of autophagy in the context of *Legionella* infection. *Legionella pneumophila* is a vacuolar pathogen. During infection, the LCV acquires ubiquitinated proteins. In this model, ubiquitination selectively targets the LCV for autophagy. Cytosolic LC3 is lipidated, conjugated to PE. LC3-PE inserts into the autophagosome membrane where it promotes the growth and maturation of the autophagosome. (A) In nonpermissive cells, this nascent LC3-PE studded phagophore is recruited to the LCV where it binds the p62 adaptor protein that is selectively bound to ubiquitin on the surface of the LCV. The autophagosome matures and surrounds the LCV. In the final step of maturation in nonpermissive host cells, the autophagosome fuses with the lysosome and the luminal contents are degraded. (B) The fate of *Legionella* is different in permissive host cells. Although LC3 is lipidated, the *Legionella* Icm/Dot translocated substrate RavZ irreversibly removes PE from LC3, generating LC3* that cannot be reconstituted with PE. The release of LC3* from the nascent phagophore halts autophagosome formation. As a result, *Legionella* replicates intracellularly until the host cell succumbs to the infection. LCV: *Legionella*-containing vacuole; PE: Phosphatidylethanolamine.

provided by siRNA knockdown experiments in which the depletion of the critical autophagy factor ATG5 results in an increase in intracellular replication of *Legionella* during infection [76,80]. The manner in which this pathway is triggered, however, remains a mystery. Clues to resolving this issue may come from recent papers that indicate that the p62 protein localizes to the LCV in nonpermissive macrophages. p62 is an adaptor protein that links ubiquitinated substrates with the autophagic machinery via ATG8/LC3 [76,81]. Dorer *et al.* previously showed that the LCV stained positively for ubiquitin on its surface [22]. Experiments performed upon infection of host cells with the *L. pneumophila* strain 130b show that the F-box-containing IDTS AnkB promotes recruitment of ubiquitinated proteins to the LCV [82]. Ubiquitinated proteins subsequently serve as a nutrient source for intracellular *Legionella*, as they are targeted to the proteasome for degradation into constituent amino acids [83]. This colocalization of LC3, p62 and ubiquitin suggests that ubiquitination of the LCV may also trigger autophagic uptake. Khweek *et al.* isolated a *Legionella* mutant that was able to replicate within restrictive C57Bl/6 macrophages [76]. The fact that this mutant is less capable of recruiting ubiquitin to the LCV surface, and that this correlates with reduced p62 localization supports this hypothesis. Further connections between the LCV, ubiquitin and autophagy come from evidence that Cdc48/p97 is essential for intracellular replication of *Legionella* [22]. siRNA depletion restricts intracellular replication in permissive host cells. The reduced intracellular growth correlates with an accumulation of ubiquitinated proteins on the vacuolar surface. The canonical role for mammalian Cdc48/p97 is to extract ubiquitinated proteins from membranes and deliver them to the proteasome. Recent work indicates that p97 plays a critical role in modulating autophagy [84]. Cells with lowered p97 function accumulate LC3, while p62-positive autophagosomes do not mature fully to fuse with lysosomes. The increasing number of links between ubiquitination, autophagy and the LCV suggest that these pathways might interact to regulate *Legionella* pathogenesis.

Legionella replicates in murine A/J macrophage, despite the presence of autophagy markers on the surface of the LCV [79]. This suggests that *Legionella* is able to halt the maturation of the autophagosome. In recent work, a *Legionella* Icm/Dot effector was identified that

can irreversibly inactivate a key autophagy factor required for maturation [77]. This work was based on the observation that wild-type *Legionella* is able to inhibit the lipidation of ATG8/LC3, dependent on a functional type IV secretion system. Deletion strains of *Legionella* were screened in search of mutants that lost the ability to inhibit LC3 lipidation [23,85]. Subsequent analysis of the Icm/Dot substrates that are missing in one strain unable to interfere with LC3 processing indicated that RavZ, a putative cysteine protease, was solely responsible for this inhibition. Host ATG4 is a cysteine protease that cleaves pro-LC3, exposing a C-terminal glycine residue that is required to lipidate LC3 and localize it to membranes [86]. Based on the putative cysteine protease activity, the authors examined the ability of RavZ to cleave LC3 in an *in vitro* conjugation system. They found that RavZ was indeed a deconjugating enzyme that was able to reverse the conjugation of phosphatidyl ethanolamine to LC3. However, unlike ATG4, cleavage by RavZ removes that C-terminal glycine, leaving RavZ-cleaved LC3 refractory to re-conjugation by the host autophagy system and ultimately unable to promote autophagosome maturation. It is worth noting that the Δ ravZ deletion mutant does not have an intracellular growth defect and does not become autophagocytosed, leaving the question of how the ubiquitinated replication vacuole is able to bypass autophagy in the absence of RavZ activity unanswered.

Vacuole maintenance & host cell death

Premature host cell death during infection impairs pathogenicity because it eliminates the protective, nutrient-rich intracellular niche. Pathogens must therefore devise strategies to prolong the lifespan of the host cell until they are prepared to trigger host death and transmit to another host. *Legionella* employs multiple strategies to promote this host survival and they all require substrates of the Icm/Dot secretion system. First, infection induces that activation of antiapoptotic genes downstream of NF- κ B [20,87–88]. Second, ‘pro-death’ host genes BNIP3 and Bcl-rambo are targeted and inactivated by the multidomain effector SidF. Finally, the effector SdhA prevents cells from undergoing a dramatic and inflammatory cell death by preventing death-inducing microbial products from entering the host cell cytosol [19,28].

SdhA is one of two translocated effectors that have severe intracellular growth defects when

deleted individually. Infection with this mutant is toxic to macrophages derived from permissive mouse strains [19]. These macrophages display signs of nuclear degradation, mitochondrial disruption, membrane permeability and caspase activation, indicating that SdhA protects host macrophages.

Recent work by two groups has endeavored to provide insights into how SdhA exerts its protective effect. A suppressor screen identified *Legionella* genes that are important for triggering cell death in the absence of SdhA [28]. This included PlaA, a phospholipase that cleaves lysophosphatidic acid. The deletion of *plaA* in the Δ *sdhA* deletion background resulted in attenuation of the Δ *sdhA* deletion phenotypes. Attenuation required phospholipase activity. PlaA is homologous to the *Salmonella* protein SseJ, which is important for maintaining the integrity of the *Salmonella*-containing vacuole during infection. To determine if the Δ *sdhA* mutant is defective in maintaining the integrity of its LCV, the authors examined infected cells and vacuoles isolated from infected cells. Consistent with a similar role in *Legionella*, Δ *sdhA* vacuoles stained positively for galectin-3, a marker of disrupted vacuoles. Additionally, vacuoles isolated from Δ *sdhA*-infected U937 macrophages were permeable to antibodies used in staining, but wild-type-infected macrophage vacuoles were not.

Mammalian cells possess immune surveillance systems to detect cytosolic perturbations, including contamination with microbial products (DNA, flagellin, secretion systems) [89,90]. Detection of these products by cytosolic Nod-like receptors triggers the assembly of multiprotein complexes, inflammasomes, which activate the caspases that promote cell death. Caspase-1 was initially implicated in the cell death that occurred downstream of Δ *sdhA*-mediated vacuolar disruption [28]. Paradoxically, the host cell cytotoxicity was independent of the canonical AIM2-ASC inflammasome that is thought to activate caspase-1 [28,90]. Recent work by Aachoui *et al.* resolves this discrepancy [90]. Key to this resolution was the finding that the *Casp1*^{-/-} mice were actually *Casp1*^{-/-}*11*^{-/-} double knockouts. Bone-marrow derived macrophages from *Casp1*^{-/-}*11*^{-/-} double knockout mice exhibited no cytotoxicity when infected with the Δ *sdhA* mutant, compared with macrophages from wild-type mice, which succumbed to the infection. Retroviral complementation with

caspase-11 alone, but not caspase-1, restores the cytotoxicity phenotype. Caspase-11-dependent cytotoxicity, or pyroptosis, was observed for other bacterial pathogens that entered the cytosol, including *Salmonella typhimurium* Δ *sifA* mutants, which access the cytosol through a disrupted vacuolar membrane, and *Burkholderia* species that naturally invade the cytosol. Taken together, these results indicate that SdhA plays a critical role in maintaining the membrane integrity of the LCV, thus allowing *Legionella* to evade detection by cytosolic surveillance systems.

Future perspective

L. pneumophila emerged as a human health threat approximately 40 years ago. Since then, great strides have been made in understanding how this bacterial pathogen interacts with host cells. The Icm/Dot type IV secretion system and its many translocated effectors are central players in this interaction and the work that has been done to elucidate their molecular function has greatly enhanced our understanding of *Legionella* pathogenesis. We have learned that this bacterial pathogen is able to manipulate a wide variety of host cell processes including vesicle trafficking (anterograde and retrograde), protein synthesis, autophagy and cell death. Studies of *Legionella*-host cell interactions have also informed our understanding of host cell biology. For example, the activity of the effector protein RomA uncovered a novel histone modification that could modulate the host transcriptome [68].

Future work in this field must continue to expand our knowledge of the translocated substrates of the Icm/Dot secretion system. However, this work must not be performed in isolation. The functional redundancy among the effectors and the host cell pathways they manipulate suggest that systems approaches, such as iMAD, that interrogate both host and microbe simultaneously, will be critical for advancing our analysis of host-pathogen interactions [23,27].

Recent work provides a better understanding of how and why *Legionella* has amassed its arsenal of effectors. Armed with the knowledge that the IDTS repertoire defines the host range of *Legionella*, studying the interaction between *Legionella* and its natural host, amoeba, can help us gain insights into how environmental hosts drive the evolution of pathogenicity in bacterial species.

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EXECUTIVE SUMMARY

Legionella pneumophila: an intracellular pathogen

- *Legionella pneumophila*, a Gram-negative intracellular bacterium, possesses approximately 300 Icm/Dot translocated substrates. The role that many of these effectors play during infection is largely unknown, although the biochemical activities of a number of these proteins have been identified.
- A molecular analysis of the translocated substrates will provide insights into *Legionella* pathogenesis and the evolution of microbial intracellular survival strategies.

Functional redundancy: problem solved?

- In total, 31% of the Icm/Dot-translocated substrates (IDTS) are dispensable for replication in murine macrophages. Distinct subsets of these effectors define the host range of *Legionella* among environmental amoebal species.
- The development of genetic strategies for simultaneous disruption of host and bacterial genes can uncover phenotypes for effectors that have redundant cellular functions.

Build your own vacuole: co-opting host vesicular trafficking

- *Legionella* hijacks host vesicle trafficking machinery in order to build the replicative *Legionella*-containing vacuole (LCV).
- Several Icm/Dot substrates play important roles in modulating the activity of Rab1, the small GTPase that drives fusion between endoplasmic reticulum (ER)-derived vesicles and target membranes. The substrate activities are diverse and include guanine exchange, GTP hydrolysis, AMPylation and phosphocholination.
- The *Legionella*-translocated effector RidL interacts with the host retromer complex and disrupts retrograde trafficking of vesicles to the Golgi apparatus.

Build your own vacuole: hijacking host lipids

- The *Legionella* LCV is enriched for the phosphatidylinositol 4-phosphate (PI(4)P). This phosphoinositide is typically located on compartments that interact with vesicles from the ER.
- SidF, an IDTS, is the first *Legionella* protein demonstrated to directly interact with and modify phospholipids. It has PI(3)P phosphatase activity and localizes to the LCV during infection. *In vitro* assays and the crystal structure show that it has substrate specificity for PI(3,4)P₂ and PI(3,4,5)P₃.
- LecE and LpdA are *Legionella* IDTS that localize to the LCV, where they are thought to promote an increase in diacylglycerol levels. High diacylglycerol levels could promote and increase in PI(4)P levels, thus promoting association with the ER.

Hijacking the host transcriptome

- The identification and characterization of a *Legionella* effector that methylates histones hints at microbial mechanism for modulating host transcription, although the consequences of this modification remain uncertain.

Autophagy

- Localization of ubiquitin, autophagy-promoting factors, and adaptors that aid in the selection of ubiquitinated cargo for autophagy to the LCV strongly suggests that ubiquitination might be the stimulus that initiates this cellular degradative pathway.
- RavZ was identified as a *Legionella* effector that could irreversibly halt autophagosome maturation, although its removal did not inhibit intracellular replication. This suggests that there are other pathways that might protect the bacteria from degradation in the lysosome.

Vacuole maintenance & host cell death

- Maintaining vacuolar integrity is critical for promoting intracellular colonization during microbial infection. Dedicated virulence factors prevent vacuolar lysis.

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