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MULTI-AUTHOR REVIEW

Salmonella effector proteins and host-cell responses

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Abstract Acute gastroenteritis caused by *Salmonella enterica* serovar typhimurium is a significant public health problem. This pathogen has very sophisticated molecular machinery encoded by the two pathogenicity islands, namely *Salmonella* Pathogenicity Island 1 and 2 (SPI-1 and SPI-2). Remarkably, both SPI-1 and SPI-2 are very tightly regulated in terms of timing of expression and spatial localization of the encoded effectors during the infection process within the host cell. This regulation is governed at several levels, including transcription and translation, and by post-translational modifications. In the context of a finely tuned regulatory system, we will highlight how these effector proteins co-opt host signaling pathways that control the ability of the organism to infect and survive within the host, as well as elicit host pro-inflammatory responses.

Keywords *Salmonella* · Type three secretion · Invasion · Inflammation

Introduction

Salmonella enterica serovar typhimurium (*S. typhimurium*) is a facultative intracellular pathogen that causes a variety of diseases in humans and animals, ranging from gastroenteritis to systemic infection. The disease is primarily spread by the contamination of water and food items with fecal matter from infected hosts and is often self-limiting, but can cause prolonged complications [33]. The ability of *S.* typhimurium to cause disease in humans is related to its horizontal acquisition of virulence genes termed pathogenicity islands (SPI). In *S.* typhimurium SPI-1 and SPI-2 contain genes for two type-three secretion systems (T3SS). TTSSs are specific to Gram (-) bacteria and likely evolved from the flagella basal body. Each TTSS contains a motor, a needle complex, and a translocon through which secreted effectors are delivered into host cells [74].

The secreted effectors co-opt host-cell signal transduction cascades that provide various pivotal functions to the organism, including promoting bacterial entry, controlling inflammatory responses, and regulating bacterial survival within the cell (Table 1). The SPI-1 T3SS (T3SS1) is primarily associated with invasion [27], while the SPI-2 T3SS (T3SS2) seems to principally promote the intracellular survival of at least *S.* Typhimurium, as it has been suggested that survival of *S.* Typhi in macrophages does not require SPI-2 effectors [25]. Despite these differences in T3SS1 and TTSS2, some effectors from both systems are actually required at the same time [8, 47], suggesting the coordination involved in regulating SPI-1 and SPI-2 is more complex than previously thought. The regulation of the timing of synthesis and secretion of these effectors is achieved through several tiers of checkpoints, such as transcription and translation, post-translational modifications, and spatial sequestration-mediated control. In this review, we have assembled information to specifically emphasize how the timing of the delivery and the spatio-localization of *Salmonella* effectors contribute to a successful infection at the expense of the host.

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Table 1 *Salmonella* effector proteins and their interaction with host cell

Effector	Location	Cellular function	Targets	TTSS apparatus
AvrA	SPI-1	Controls *Salmonella*-induced inflammation, inhibition of the NF-kB pathway (16, 81)	IKBa, B catenin	1
SipA	SPI-1	Promotes actin polymerization and plays a key role in bacterial entry. It is activated and cleaved by caspase-3 (32, 73)	F-actin caspase-3	1
SipB	SPI-1	SPI-1 translocon component	Cholesterol	1
SipC	SPI-1	SPI-1 translocon component, induces actin bundling to promote invasion (13, 61)	F-actin	
SipD	SPI-1	SPI-1 translocon component		1
SptP	SPI-1	Reverses the inflammatory changes caused by earlier effectors. Down regulates IL-8 (26, 32)	Cdc42, Rac1	1
SopA		HECT3 ubiquitin ligase (32)		1
SopB	SPI-5	Inositol phosphate phosphatase that promotes bacterial entry. Promotes SCV biogenesis (32, 82)	Inositol phospates	1
SopD		Promotes macropinocytosis and fluid secretion (4, 72)		1 and 2
SopE	Bacteriophage	Initiates the process of invasion by promoting the activation of Rho GTPases (35)	Cdc42, Rac1	2
SopE2	Bacteriophage	Initiates the process of invasion by promoting the activation of Rho GTPases (35)	Cdc42	2
PibB2	Bacteriophage	PipB2 works as a linker for the microtubule motor kinesin-1 (35)	Kinesin-1	2
SifA		Sif formation and membrane integrity, mimics activated small GTPases (1, 7)	SKIP	2
SopD2		Sif formation		2
SpiC	SPI-2	Regulates Sif formation and helps regulate TTSS2 secretion	Hook 3	2
SseB		Promotes pore formation through which proteins reach the host-cell cytoplasm (62)		2
SseC		Promotes pore formation through which proteins reach the host-cell cytoplasm (62)		2
SseD		Promotes pore formation through which proteins reach the host-cell cytoplasm (62)		2
SseJ	SPI-2	Promotes perinuclear localization of the SCV. Works with SifA in maintaining the integrity of the SCV membrane (62)	Cholesterol	2
SseF	SPI-2	Promotes perinuclear localization of the SCV (69)		2
SseG	SPI-2	Sif formation (71)		2
SseI		Migration of actin filaments around the SCV (58)	F-actin	2
SspH1	Bacteriophage	Inhibition of NF-kB gene expression downregulates IL-8 (34)	PKN1	2
SpvB	spv locus	Sif downregulation (49)		
SSeL	SPI-2	Displays deubiquitinating activity. Helps downregulate inflammatory responses to *Salmonella* infection	IKBa	2
SlrP		Bears E3 ubiquitin ligase activity (6)		2

Interplay of *Salmonella* effectors: invasion and infection

Transcriptional and translational regulation during invasion

Inside the host, after entering the lumen of the small intestine, *S. Typhimurium* traverses the intestinal mucus layer and immediately senses the microenvironment (i.e., pH, oxygen tension, osmolarity) of the intestinal epithelial cells. At this point, the first level of control over the timing of the expression of secreted effectors is achieved at the transcriptional level. *Salmonella* operate an elaborate transcriptional machinery controlled by a master regulator encoded on SPI-1 called HilA [3]. The expression of HilA is directed by a multi-component feed forward regulatory loop consisting of HilD, HilC, and RtsA, which are AraC-like regulators that can independently activate HilA expression [22]. HilD can be repressed by HilE, and HilC and RstA further amplify the inducing signal. Another protein, FliZ, was identified to be a major positive regulator

of this system. Through genetic and biochemical analyses, FliZ was found to post-translationally control HilD, and as a result, to positively regulate HilA expression [14]. This control mechanism is independent of other flagellar components, and is not mediated through HilE or through FliZ-mediated RpoS regulation. Moreover, mutants lacking FliZ expression were significantly attenuated in their ability to colonize mouse intestines, but could still cause systemic infection [14, 41, 51]. Therefore, a lack of either SPI-1 activation or of FliZ can attenuate intestinal colonization.

A recent study has also revealed a role for DNA adenine methylation (DAM) in post-transcriptional control of SPI-1 gene expression [53]. In this study, DAM deficient mutants (*dam*-) of *Salmonella* were attenuated in mouse models and presented several virulence-associated defects. In addition, impaired interaction of the *dam* mutants with the intestinal epithelium correlated with reduced secretion of SPI-1 effectors. It is interesting to note that several regulators of T3SS1, such as HilA, HilC, HilD, and InvF, were found in significantly lower levels in the *dam* mutant.

Salmonella relies on T3SS2 to survive and replicate intracellularly

The virulence genes located in SPI-2 are controlled by the regulatory circuits of three different two-component regulatory systems: SpiR/SsrB, EnvZ/OmpR, and PhoQ/PhoP (reviewed by [24]). SsrB is the response regulator, and membrane resident SpiR is the predicted integral cognate sensor. SsrB protein binds to the promoters of all SPI-2 functional gene clusters and is essential for expression of the structural, regulatory, and effector components of the SPI-2 locus [79]. HilD has been identified as a major regulator controlling the crosstalk between the SPI-1 and SPI-2 regulatory networks [12]. HilD binds directly to the regulatory regions of the *ssrAB* operon (the coding regions of *ssrA* and *ssrB*) and counteracts the repression exerted by the negative regulator, H-NS, or *ompR* (a factor required for the activation of SPI-2 genes).

Setting the platform for invasion and infection

Secreted effectors

In the intestinal lumen several environmental cues (i.e., osmolarity, oxygen tension, pH) trigger the upregulation of *Salmonella* T3SS1. Therefore, upon colonization, it is likely that *Salmonella* begins to secrete effector proteins into the intestinal milieu. This concept is supported by studies showing 80–90% of secreted *Salmonella* effector proteins were found either associated with non-adherent bacteria or in the infection media, whereas only about 10% were actually translocated into the host cell [15]. The observation that *Salmonella* secretion and translocation appear to be uncoupled led to the speculation that *Salmonella* effector proteins functionally interact with the host cell both extracellularly and intracellularly.

An example of effector-extracellular epithelial interactions is illustrated by our recent work investigating the molecular action of the *Salmonella* effector invasion protein A (SipA). SipA promotes gastroenteritis by harboring two functional motifs that individually trigger mechanisms of inflammation or bacterial entry [78]. To facilitate infection, SipA activates the host enzyme, caspase-3, within 4 h of infection. This enzyme is required for SipA cleavage at a specific recognition motif that divides the protein into its two functional domains and activates SipA in a manner necessary for pathogenesis. What is striking about this observation is this processing of SipA was found to occur on the outer surface of the intestinal epithelium, suggesting SipA needs to be cleaved before interacting with the apical surface ([73]; Fig. 1). Whether other effector proteins are processed extracellularly in a similar manner remains to be determined.

In recent years, technologies to identify secreted effectors have been developed founded on a sequence-based method called Effective T3. This methodology uses parameters such as length, position, and composition of the protein of interest to predict the probability of it being a secreted effector. For *Salmonella*, the Effective T3-based strategy predicted that close to 3% of the total proteome was secreted, and this portion has been called the secretome of the pathogen [2]. Another study investigating secreted *Salmonella* effectors used sequence information based on several parameters, including the taxonomy of the protein sequence, to determine the probability of the protein being secreted [71]. This study identified 400 *Salmonella*-secreted proteins, many of which were outside the SPI-1 and SPI-2 loci.

Epithelial translocation of early T3SS1 effectors

Early upon contact with enterocytes, *S. typhimurium* engages the translocation of secreted effectors in a highly timed and coordinated manner. The *Salmonella* effectors SopE, SopE2, and SopB initiate the process of invasion (Fig. 1). Together, these proteins promote the activation of host-cell Rho GTPases, which subsequently turn on signal transduction pathways, priming not only the entry of the organism, but also setting in motion the NF-$_{K}$B- dependent inflammatory cascade [35, 76]. Entry of the organism into host cells requires intimate interaction with the host cytoskeleton, which is partly mediated by SipC, a membrane-anchored protein and a component of the bacterial translocon. SipC contains two membrane-spanning regions

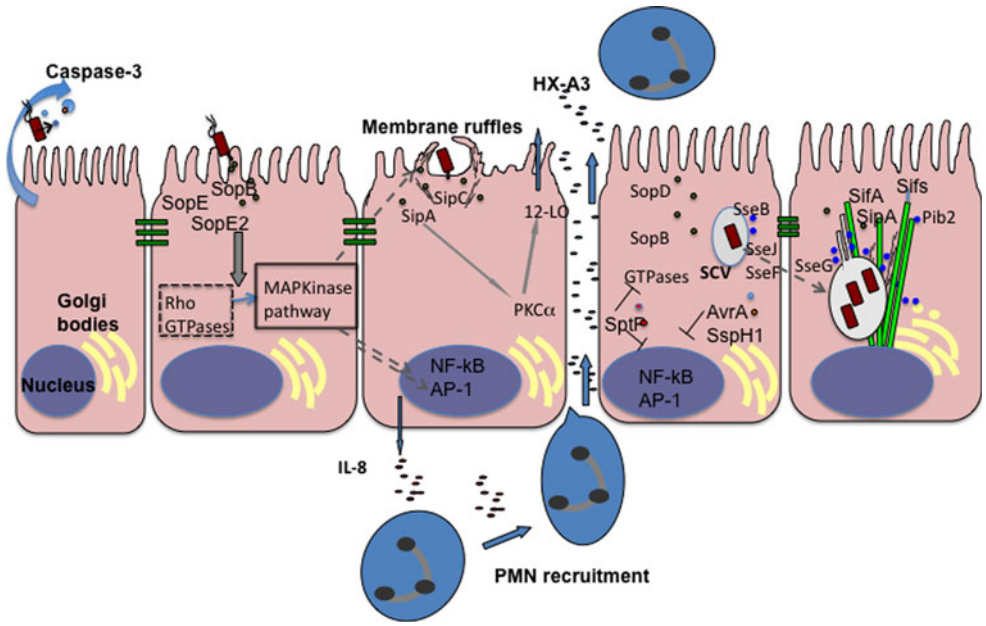

Fig. 1 Spatial dynamics of *Salmonella* effectors during infection. Secretion of effectors and their action begin prior to bacteria entry. SipA secretion leads to activation of caspase-3, which may act as the starting point of the inflammatory pathways induced by the bacteria. Upon contact with epithelial cells, the bacteria translocate early effectors through the type three secretion system encoded by *Salmonella* pathogenicity island-1 (SPI-1, green circles). This leads to the induction of pathways that activate the transcription factors AP-1 and NF-κB. These processes trigger major cytoskeletal rearrangements, resulting in bacterial entry into the epithelial cells along with the basolateral release of interleukin 8 (IL-8), which is a crucial step in polymorpho mononeuclear leukocyte (PMNs) recruitment. SipA and other proinflammatory effectors induce the PKCα driven pathway of eiconasoid biosynthesis, which culminates in the apical release of hepoxilin-A_3 (HXA_3). HXA_3 forms a gradient along the paracellular space, which guides PMNs to the apical surface of the epithelium, a process that also damages the epithelial layer. Endocytosed bacteria remain in a spacious vacuole called the *Salmonella*-containing vacuole (SCV). At this point, several effectors, such as SptP and SspH1, function to re-establish homeostasis by inhibiting proinflammatory mechanisms. Bacteria within the SCV secrete effectors through another type III secretion system encoded by *Salmonella* pathogenicity island-2 (SPI-2, blue circles). These effectors promote the stability of the SCV and the survival of bacteria within the SCV. Also seen in the schematic is the persistence of some SPI-1 effectors even after the formation of SCV

with N and C termini facing the host-cell cytoplasm. This topological assembly of SipC is key to the actin nucleation and the translocation processes. Remarkably both of these processes are primarily dependent on the C-terminus of SipC [61]. Chang and coworkers [13] demonstrated that the effector translocation function and actin-nucleating function are dissociable.

SipA is unique since it plays multiple roles in bacterial entry and inflammation [48, 82]. As described above, this effector is important even prior to bacterial entry into the epithelial cells. During the entry process, SipA has been shown to promote actin filament polymerization by reducing the threshold monomer concentration required for polymerization. In addition, SipA interacts with the assembled filaments and prevents depolymerization [83]. Structural studies investigating SipA-actin binding have revealed that SipA acts as a 'molecular staple' using its two extended arm domains to tether actin monomers that are in close proximity [30]. The net effect of SipA and SipC activity is the bundling of actin filaments, which leads to the formation of membrane ruffles, a key step in *Salmonella* entry into the non-phagocytic epithelial cells [57, 82]. Remarkably, SipA is not necessary for bacterial entry, probably because of functional redundancy of the effectors [48]. However, very early during infection a SipA mutant displays a reduced entry rate [82]. From cytosolic, temporal, and localization points of view, SipA demonstrates a high degree of specialization.

SopB, which bears homology to mammalian inositol phosphate 4 phosphatase, is also implicated in bacterial entry into the host cell [84]. SopB has phosphoinositide phosphatase activity which, by dephosphorylation of phosphatidylinositol-4,5-bisphosphate at the plasma membrane, promotes the invasion process [63]. SopB also contributes to the activation of a RhoG exchange factor SGEF (Src homology 3 domain-containing guanine nucleotide exchange factor), a guanosine nucleotide exchange factor for RhoG, which results in actin rearrangements contributing to the *Salmonella* entry process [66]. Later on in the infection process, SopB activity also promotes SCV biogenesis (described below and in Fig. 1).

The actions of SipC, SipA, and SopB, particularly their effects on actin, lead to major cytoskeletal rearrangements, and thus formation of membrane ruffling. After

actin rearrangement-driven membrane ruffling, the invasion process relies on the formation of phagosomes to contain the internalized bacteria. This process, assisted by SopB and SopD, requires pinching off of these large endocytic vacuoles, called macropinosomes. Remarkably, SopD has been reported to be translocated by both T3SS1 and TTSS2 [72]. SopD shares a strong homology to SopD2, and in concert with SopB it induces fluid secretion and inflammation in a bovine-ligated ileal loop model of infection [40]. Initially, Bokowski et al. reported localization of SopD at the sites of bacterial invasion in a SopB phosphatase activity-dependent manner. Furthermore, SopD plays a role in membrane fusion and macropinosome formation during infection [4]. In another study using stable isotope labeling of amino acids in cell culture (SILAC), and quantitative mass-spectrometric based methods, the intracellular target of SopD was confirmed to be small G protein Cdc42 [69]. In the same study intracellular localization of SopB was reported to be at the plasma membrane.

Late SPI1 effectors ensure damage control

Once *Salmonella* gets inside the host cells, the surface architecture of the cells returns to normal (Fig. 1). Therefore, shortly after endocytosis, *Salmonella* must engage a second set of secreted effectors that ensures repair and homeostasis of the invaded cell (Table 1). SptP, a protein that exhibits opposing activities to SopE and SopE2, relieves the invasion-induced damage by reversing the changes to the host-cell membrane as early as within 3 h of infection [26, 29]. How virulence proteins with opposing abilities precisely perform and fulfill their task is a pivotal question that remains to be addressed. However, a plausible explanation is through very strict regulation of the timing of the expression, translocation, and turn-over of these effectors in the host cell. Detailed studies by Kubori et al. [45] have shed light onto this possibility as they demonstrated that soon after infection, SopE undergoes degradation by the ubiquitin-proteasomal complex, whereas SptP survives longer before getting degraded by the same complex. Another study focused on the temporal dynamics of SptP and SopE2 with fluorescent tagging. Using FIASH/tetracysteine labeling, Van Engelenburg and Palmer demonstrated that these two effectors display very different secretion kinetics and rates of degradation [77].

Salmonella expresses several other proteins that reverse the changes caused by earlier effectors. For instance, SptP and SspH1 participate in downregulating IL-8, and Hagara and Miller found that SspH1 localizes to the nucleus and inhibits NF-kB-dependent gene expression. This observation has lead to the compelling hypothesis that suppressing pro-inflammatory responses is critical to intracellular survival and *Salmonella* pathogenesis [34].

Survival inside the host

Formation of *Salmonella* containing vacuole

Once *Salmonella* makes its way into the host epithelial cell and its effectors have reversed the physiological changes to the cell following entry, it must adapt to the new environment. Thus, shortly after endocytosis the bacteria trigger mechanisms that promote sustenance and multiplication inside the host cell (Fig. 1; Table 1). *Salmonella* relies on the T3SS2 to survive and replicate within the colonic epithelial cells and in macrophages. Intracellular survival and replication are required for *Salmonella* to disseminate and cause systemic infection. Within macrophages and intestinal epithelial cells, *Salmonella* resides inside a small vacuole called the *Salmonella*-containing vacuole (SCV) that persists anywhere from a few hours to days (reviewed by [5]). During SCV biogenesis, several Rabs (Rab5A/B/C) from the host are acquired and retained in the vacuole. This includes several proteins that are absent in a model phagosome; however, some proteins like Rab8, Rab13, Rab23, Rab35 that are usually present in model phagosomes are absent in SCVs. Rab7, which is a component of late phagosomes, is acquired very early by SCV.

Following the formation of the SVC, the microenvironment within the vacuole undergoes drastic changes that eventually trigger activation of T3SS2 (discussed below). At least 20 *Salmonella* effector proteins are known to be translocated by TTSS2 across the phagosomal membrane into the eukaryotic cell cytoplasm. Specific roles and functional details for all the effectors are not yet well understood, and so far no specific effector has been identified as the key factor responsible for altered vesicular trafficking. However, SopB, though a SPI-1 protein, persists at least 12 h post-infection and contributes to the maintenance of the SCV as well as to long-term systemic infection in mice [32, 47]. For example, SopB transcripts were detected at least 8 h post-infection in macrophages [20]. SopB serves to selectively remove M6PR-positive membrane from the SCV, and such pathways of recycling could be important in deciding the fate of the vacuole [11]. During SCV maintenance, SopB alters the charge on the SCV membrane, thereby displacing several typical components of the vacuolar membrane and ultimately preventing it from fusing with lysosomes [4]. Therefore, by the virtue of its lipid-modifying property, SopB could determine several

outcomes during infection, such as signal transduction during early infection and SCV maturation during later infection.

Activation of TTSS2

The expression and assembly of T3SS2 is induced as a direct result of changes in phagosomal conditions (Fig. 1). Those conditions include an acidic medium, with a pH that fluctuates between 5 and 5.5, decreased concentration of divalent cations (Ca^{2+} and Mg^{2+}) and phosphates [18], and the presence of antimicrobial peptides. Under these conditions the expression of T3SS2 reaches its peak within 2 to 4 h post-infection. The expression of SPI-2-encoded genes is regulated by three two-component systems, SpiR/Ssrb, EmvZ/OmpR and PhoQ/PhoP (reviewed by Fass and Grossman [24]). Currently, the functions of T3SS2 remain incompletely defined. However, its importance in *Salmonella* virulence and intracellular survival is well understood, as T3SS2 mutants fail to grow within animal models [37]. A reasonable hypothesis for the role of TTSS2 is that it allows intracellular replication in epithelial and phagocytic cells. This would permit the infection of distant organs during the late stage infection by altering host vesicular trafficking, and by the redirection of essential molecules like lipids and amino acids to the SCV.

Protein delivery across the SCV

The translocation of TTSS2 effector proteins across the SCV requires the formation of a translocon complex that promotes the formation of a pore through which proteins reach the host-cell cytoplasm (Fig. 1; Table 1). It has been reported that the secreted proteins SseB, SseC, and SseD associate in an SCV surface-located macromolecular structure [62]. In the absence of SseB, substrate proteins SseC and SseD are still secreted but do not reach the cell surface. In contrast, SseC and SseD are not required for the secretion and surface location of SseB. The translocated effectors most important during intracellular survival seem to be SifA, SseJ, SseF, SseG, SopD2, and PipB2, as strains mutated for these effectors exhibit virulence defects in mouse infection models [43, 75].

Perinuclear localization of the SCV

Following the activation of T3SS2, the SCV migrates to the perinuclear region in close proximity to the Golgi network, where it undergoes a crucial phase of development (Fig. 1). It has been recently shown that *Salmonella* targets the Golgi apparatus to acquire nutrients and membrane constituents in order to preserve the integrity of replication within the SCV [70]. This specific positioning requires the translocated effectors SifA, SseG, and SseF [7, 19]. SseF and SseG mutants exhibit a defect in Golgi localization and replication, as well as attenuated virulence in mice [70]. Disrupting localization to the Golgi apparatus inhibits bacterial replication [46]. Consequently, interaction of SCV with an intact exocytic pathway appears to be an important aspect of bacterial virulence.

Formation of *Salmonella* induced filaments

The main outcome during SCV maturation is the formation of *Salmonella*-induced filaments (Sifs). As depicted in Fig. 1, Sifs are long filamentous membrane structures necessary for the proper positioning of the SCV in close proximity to the Golgi apparatus and near the perinuclear region of the host. Sif tubules extend from the surface of the SCV and appear to be derived from late endocytic compartments; they contain LAMP-1, vacuolar adenosine triphosphatase (vATPase), lysobisphosphatidic acid, and cathepsin [21].

Sif formation requires the localization of T3SS2 effectors SifA and PipB2 to the SVC. PipB2 functions as a linker for the plus-end-directed microtubule motor kinesin-1 and mediates its recruitment [36]. SifA binds to a eukaryotic protein named SifA kinesin-interacting protein (SKIP) that also binds to the plus-ended-directed microtubule network and regulates the level of kinesin-1 on the SCV [7]. SifA was recently identified as a member of the WxxxE family of bacterial TTSS effectors that subvert host-cell functions by mimicking activated small GTPases or their pathways [1]. Members of this family include IpgB1, IpfB2, and Map (produced by *E. coli* and *Shigella)*. These proteins induce actin cytoskeleton rearrangements similar to that induced by activated GTPases Cdc42, Rac1, and RhoA [1]. Effectors with the WxxxE motif do not have known structural similarities to GTPases, and their activity is unaltered by GTPase inhibitors [1]. Sif formation also requires the presence of an intact microtubule network [10, 31], and the bidirectional nature of the tubule movement suggests the involvement of distinct minus-end-directed and plus-ended-directed motors.

There are some discrepancies in the field regarding the interaction of SifA with other proteins for the induction of tubular filaments. Nonetheless, [65] showed that Sif formation appears to be induced in a cooperative manner by SifA and SseJ, which interact in a protein complex with SKIP and RhoA family GTPase to promote phagosomal tubulation. Additionally, SipA was shown to persist very late after the formation of SCVs; in both macrophages and non-phagocytic cells, molecular crosstalk between SipA and SifA was observed [8].

The appearance of Sifs coincides with the initiation of bacterial replication several hours after invasion of the host

cell [21]. The physiological function of Sifs is incompletely understood, although *Salmonella* mutants that cannot make Sifs exhibit attenuated virulence in mouse models of *Salmonella* infection and reduced replication rate within cultured macrophages. These data suggest that Sif formation plays a key role in *Salmonella* pathogenesis. [75]. Thus, it is inferred that the main role of Sifs is to increase the size of the SCV to better accommodate bacterial replication during systemic infection.

Actin polymerization around the SCV

Several hours after *Salmonella* entry into either epithelial cells or macrophages, a collection of F-actin filaments is observed surrounding the SCV. In fact, *S.* Typhimurium-infected cells treated with an inhibitor of actin polymerization result in decreased bacterial replication, suggesting that actin manipulation is an important factor for bacterial replication [17].

Interestingly, the polymerized actin that encases the SCV depends on the T3SS2. Although the effector proteins responsible for the movement of actin filaments to surround the SCV have not been identified, two effectors, SspH2 and SseI, have been shown to co-localize with the polymerized actin cytoskeleton [58]. Both SspH2 and SseI interact through homologous N-terminal domains with the host actin-binding protein, filamin. However, neither SspH2 or SseI is essential for the formation of F-actin around the SCV since double mutants still show normal SCV-associated actin polymerization [58]. In addition, direct evidence that actin polymerization plays an important role in intracellular survival is lacking.

Some *Salmonella* strains express the SpvB protein, an intracellular toxin encoded by the *spv* locus that has been shown to induce vacuole associated actin depolymerization [49], presumably by ADP-ribosylating actin. Similarly, *Salmonella* strains that lack the SPI-2 genes *ssaV* and *ssaJ* are unable to depolymerize actin in human macrophages [9]. These results imply that SpvB requires SPI-2 to gain access to the host-cell cytoplasm. Recent data also suggest that the T3SS2 is required for SpvB-mediated actin depolymerization [9].

Effector spatial regulation

Spatial sequestering of effectors

Several SPI-1 and SPI-2 effectors show distinct localization patterns during infection (Table 1). Engelenberg and coworkers have developed a fluorescent tag-based method to study the translocation of effectors in real time [77]. These authors labeled three SPI-2 effectors, SteA, SteC, and Pib2, to validate this method. PipB2 displayed highly dynamic behavior of tubules emanating from the *Salmonella*-containing vacuole labeled with both endo- and exocytic markers. SteA was preferentially localized in Golgi bodies. This segregation suggests that targeting and localization of effectors may be important for promoting *Salmonella* pathogenesis.

Using a small intereference RNA (siRNA)-based screen, Mota et al. reported the contribution of SCAMPs (secretory carrier membrane proteins) in the maintenance of the SCV in the Golgi region of Hela cells. This indicates a role for SCAMPs in vacuolar membrane dynamics. Moreover, SCAMP3 was shown to be involved in manipulating specific post-Golgi trafficking that might allow *Salmonella* to acquire nutrients and membrane lipids, or to control host immune responses [59]. In both macrophages and non-phagocytic cells, SipA was necessary for bacterial multiplication within the SCV. Remarkably, SipA was involved in the modulation of SCV morphology and perinuclear distribution by acting cooperatively with the SPI-2 effector, SifA [8]. These findings define an unexpected additional function for SipA after *Salmonella* entry and reveal crosstalk between effectors secreted by the two distinct TTSSs (Fig. 1).

Salmonella-mediated post-translational modifications

Post-translational modifications are now recognized as a very important strategy employed by the pathogen to govern spatial and temporal regulation of their effectors. Molecular mimicry of some key components of host-cell post-translational modification pathways is one way by which *Salmonella* and some other pathogens achieve this end.

SopA, a SPI-1 effector involved in causing inflammation, is one example of a pathogen-associated protein mimicking a host protein. Exhibiting a unique means of host-pathogen interaction, SopA undergoes post-transcriptional and post-translocational ubiquitination by host modification pathways. SopA itself acts as a HECT3 ubiquitin ligase. In what could add another level of complexity, SopA has been shown by our laboratory to possess a caspase-3 cleavage site, and it is cleaved by caspase-3 post-translationally, a process that is necessary for SopA-mediated inflammation. In addition, SseL, another secreted effector, displays deubiquitinating activity in vitro. By acting as a deubiquitinating enzyme, this molecule inhibits the degradation of IkBα, a central regulator of the NF-kB pathway. In addition, the SPI-2 effector, SlrP, has recently been reported to bear E3 ubiquitin ligase activity [6]. This bacterial enzyme was found to mediate ubiquitination of host thioredoxin. Stable expression of SlrP in Hela cells resulted in a significant decrease of thioredoxin activity, which in turn increased cell death.

Ubiquitination-dependent recruitment and control of the biological activity of SopB are perhaps the best examples of how post-translational modification may be manipulated by pathogens to ensure infection and intracellular survival [44]. SopB has been documented to persist even after it has been ubiquitinated, and so how this effector escapes the degradation machinery has been a long-standing question. Mutation of the SopB residue that is conjugated by ubiquitin results in the loss of SopB activity but does not affect its turnover. In this case, ubiquitination is affecting a process that is distinct from mediating protein stability.

One of the rapidly emerging areas in host-pathogen interaction studies is how the pathogen uses various forms of post-translational modification systems of the host towards its own survival. How *Listeria monocytogenes* manipulates host sumoylation to establish infection, described in a report by Ribet and colleagues, is another illustration of this example [68].

Host response

Neutrophil recruitment is established

Cdc42 activation promoted by SopE, SopE2, and SopB triggers several mitogen-activated protein kinase (MAPK) pathways. Specifically, ERK, Jnk, and p38 pathways are induced, the net result of which is terminal activation of major regulators, such as AP-1 and NF-kB [39, 66]. The activation of these regulators is key to stimulating pathways of inflammation as they establish the basolateral secretion of IL-8, which is a requirement for PMN migration [56]. Concomitant to this, SipA mediates arachidonic acid metabolism in epithelial cells, which leads to the apical release of HXA_3 (hepoxilin A_3). Once released HXA_3 forms a gradient along epithelial intercellular junctions, a step that is crucial in recruitment of the PMNs at the apical epithelial surface [60].

Damage control: The multifunctional protein, AvrA, is anti-inflammatory

AvrA is a multifunctional protein that affects several pathways during *Salmonella* infection [16, 81]. This effector deubiquitinates IkBα, and in doing so, blocks its degradation and inhibits NF-kB activation. AvrA also deubiquitinates β-catenin and further blocks the NF-kB pathway [81].

During later stages of infection, AvrA is associated with interferon gamma responses [52]. Additionally, [50] reported the ability of AvrA to restore the tight junctions of the epithelia so as to dampen inflammatory pathways. Therefore, cells colonized with AvrA-deficient bacterial cells exhibited decreased cell permeability, disruption of tight junctions, and increased inflammatory responses [50]. In contrast, AvrA-expressing bacteria led to increased permeability and restoration of tight junctions. An intriguing result from their study was that AvrA expression itself is controlled by a complex mechanism; CsrA/CsrB appears to regulate AvrA in a post-transcriptional manner [42]. Wu and coworkers reported that AvrA-expressing *Salmonella* infection led to significant accumulation of acetylated p53 in comparison to infection with an AvrA-deficient strain. Moreover, HCT116p53-/- cells had decreased inflammatory responses relative to the control parental cell line [80]. Further, a recent study investigating gene expression profiling in the mouse colon following *Salmonella* infection sheds light on several potential functions of AvrA. Upregulations of pathways such as mTOR, NF-kB, growth factor, oxidative phosphorylation, and MAPK signaling were early effects of AvrA. AvrA also possesses a potential caspase-3 cleavage site [73]; the significance of this motif remains to be determined.

Host innate immunity is important to control *Salmonella* infection:

Following passage across the intestinal epithelium *Salmonella* are taken up by macrophages. Like in epithelial cells the bacteria are able to survive in macrophages by forming an SCV, whereby *Salmonella* has mechanisms to secrete effectors into the cytoplasm [28]. Following phagocytosis of *Salmonella,* macrophages become immunologically activated by cytokines and exhibit a profound increase in their microbicidal, degradative, and secretory functions.

Recognition of *Salmonella* by the innate immune system is mediated through pattern recognition receptors that bind to conserved molecules from microorganisms, most notably Toll-like receptor (TLR) family members (reviewed by Prost LR et al. [67]). TLRs activate transcriptional responses to extracellular and vacuolar pathogen-associated molecular patterns. Stimulation of these receptors results in the release of inflammatory signals, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), from the macrophages. This process likely involves detection of a number of bacterial ligands called pathogen-associated molecular patterns (PAMPs) by the TLR family, and in particular by the lipopolysaccharide (LPS) receptor TLR-4/MD-2. Not surprisingly, mice deficient in TLR-4 are more susceptible to infection with *S.* Typhimurium [64]. Other components of innate immune responses, such as interferon-γ, IL-12, and TNF-α, have been shown to be important to control *S. typhimurium* infection in the mouse or humans. These cytokines are required for macrophage activation and efficient killing of *Salmonella* in systemic

infections [23, 38, 55]. Macrophages also respond to *Salmonella* through caspase-1 induction and secretion of the proinflammatory cytokine IL-1β through Ipaf (ICE protease-activating factor), a member of the NLR family [54]. These mechanisms of immune activation are unique to macrophages, as compared to intestinal epithelial cells, mainly owing to differences in TLR expression and the concomitant pathways triggered by these two cell types.

Conclusion

Salmonella infection is a major health problem in the developing and developed world. Therefore, understanding the details of the molecular mechanisms employed by this pathogen to gain access into the host and to induce inflammation is extremely important, and perhaps fundamental for the design of novel vaccine strategies or therapeutics. This review focused on the TTSS effects of *Salmonella* because the ability of this pathogen to invade the non-phagocytic cells of the intestine is key to its pathogenesis. During the infection process, one of the most remarkable features of this organism is its capacity to temporally and spatially regulate the dynamics of its virulence factors. Understanding how *Salmonella* controls this elaborate regulatory machinery is not only fascinating but may also reveal novel insights regarding its pathogenicity.

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