Review Article

Impact of *Salmonella enterica* Type III Secretion System Effectors on the Eukaryotic Host Cell

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Type III secretion systems are molecular machines used by many Gram-negative bacterial pathogens to inject proteins, known as effectors, directly into eukaryotic host cells. These proteins manipulate host signal transduction pathways and cellular processes to the pathogen's advantage. *Salmonella enterica* possesses two virulence-related type III secretion systems that deliver more than forty effectors. This paper reviews our current knowledge about the functions, biochemical activities, host targets, and impact on host cells of these effectors. First, the concerted action of effectors at the cellular level in relevant aspects of the interaction between *Salmonella* and its hosts is analyzed. Then, particular issues that will drive research in the field in the near future are discussed. Finally, detailed information about each individual effector is provided.

1. Introduction: Type III Secretion Systems and *Salmonella*

Gram-negative bacteria have evolved several machineries, known as secretion systems, for transport of substrates across their cell membranes in response to various environmental cues. Secretion of proteins is often essential for pathogenicity, biofilm formation, modulation of the eukaryote host, and nutrient acquisition. There are at least seven different secretion systems classified as type I (T1SS) to type VI (T6SS) and the CU (chaperone-usher) system. Two main mechanisms for transport operate in these secretion systems: the proteins can be exported directly from the cytoplasm out of the cell by a one-step process or by a two-step process where the protein is first exported through the inner membrane to the periplasm and then moved across the outer membrane. The T2SS, T5SS, and the CU transport substrates from the periplasm across the outer membrane. They are classified as two-step translocation pathways as they rely on the general secretory pathway, Sec, or the Tat pathway for the first step of transfer across the inner membrane. T1SS, T3SS, T4SS, and T6SS are one-step transport systems that carry out simultaneous translocation of substrates

across both membranes without periplasmic intermediates [1].

Many Gram-negative bacterial pathogens of animals or plants, including members of the genera *Salmonella*, *Shigella*, *Yersinia*, *Escherichia*, and *Pseudomonas*, rely on T3SSs to inject proteins directly into the eukaryotic host cells. Substrates of T3SSs, known as effectors, are transported via a flagellum-like injectisome from the cytoplasm of the bacterial cell across the inner and outer membrane to the cytoplasm of a eukaryotic host cell. These complex nanomachines consist of at least 20 different subunits and are composed of a basal body residing in the inner membrane, periplasm and outer membrane of the bacterial cell, a needle, located in the extracellular space, and a channel-like translocator that inserts into the host plasma membrane. Effectors are targeted to the T3SS by a signal that is usually located within the N-terminal 20 to 30 amino acids. These regions are structurally disordered and their amino acid sequences are not universally conserved, but they often share specific amino acid compositions or patterns. In addition to the N-terminal T3S signal, translocation depends, at least in some cases, on a second protein region, located within the N-terminal 50 to 100 amino acids of effector proteins, that

provides the binding site for a T3S chaperone. Chaperones presumably promote the recognition of secreted proteins by components of the T3SS. Additional functions of the chaperones are preventing the premature degradation of T3SS substrates and contributing to impose a hierarchy on the translocation of effector proteins [2]. The diversity of functions of T3SS effectors as well as the lack of conserved sequences has hampered the comprehensive identification of candidate effectors. Collectively, these virulent factors contribute, in some cases redundantly, to remodel host cellular functions, subvert immunity, establish a survival niche, and promote pathogen proliferation. Completing the repertoire of effectors for different pathogens, as well as understanding the molecular functions, and identifying the host targets for every effector are subjects of intense research in the field of cellular microbiology. This recent discipline is at the interface between microbiology and cell biology and attempts to use pathogens as tools to tackle questions in cell biology and to employ cell biology methods to understand the pathogenicity of microbes [3].

Salmonella are predominantly pathogenic Gram-negative, rod-shaped, motile bacteria belonging to the family Enterobacteriaceae that probably diverged from a common ancestor with *Escherichia coli* about 100 million years ago. The genus *Salmonella* includes the species *S. bongori* and *S. enterica*. *S. enterica* is further divided into several subspecies and more than 2500 serovars. Serovars causing disease in humans and other warm-blooded animals mostly belong to *S. enterica* subspecies *enterica*, whereas *S. bongori* and the other subspecies of *S. enterica* are usually associated with cold-blooded animals. *Salmonella* can cause food poisoning, gastrointestinal inflammation, typhoid fever, and bacteremia, depending on the serovar and the host. For instance, *S. enterica* serovar Typhi causes typhoid fever in humans, whereas *S. enterica* serovar Typhimurium usually causes self-limited gastroenteritis in humans and a systemic typhoid-like disease in mice. *S. enterica* serovar Typhi is also an example of strict adaptation to a specific host, human, whereas *S. enterica* serovar Typhimurium has a wide range of hosts, including rodents, cattle, and mammals. Systemic infection usually starts with the ingestion of contaminated food or water. After crossing the intestinal mucosa, *Salmonella* infects macrophages and spreads via the blood to the liver and spleen where it multiplies intracellularly. The typical intracellular niche of *Salmonella* is a modified phagolysosome known as *Salmonella*-containing vacuole (SCV), but the mode of entry and the strategy to survive inside the target cells varies according to the type of cell and depends on the temporal expression of particular genes by *Salmonella*. Target cells include M cells, gut epithelial cells, macrophages, neutrophils, monocytes, dendritic cells, granulocytes, B cells, and T cells [4].

In addition to the flagella system, which is also considered a T3SS, *S. enterica* encodes two virulence-related T3SS, T3SS1 and T3SS2 (Figure 1), on *Salmonella* pathogenicity island 1 (SPI1) and *Salmonella* pathogenicity island 2 (SPI2), respectively [5–7]. Some T3SS substrates are encoded on SPI1 and SPI2, but many are encoded outside these pathogenicity islands, usually within DNA segments that

exhibit features consistent with their horizontal acquisition. T3SS1 becomes active upon contact with epithelial cells in the intestine of the infected host and translocates effectors across the host cell plasma membrane. Some of these effectors are involved in induction of localized membrane ruffling and bacterial invasion. T3SS1 effectors also trigger activation of mitogen-activated protein kinase (MAPK) pathways, production of proinflammatory cytokines, recruitment of polymorphonuclear leukocytes (PMNs), and induction of acute intestinal inflammation. T3SS2 is found in all subspecies of *S. enterica* (but not in *S. bongori*). This system is expressed intracellularly in response to acidic pH and nutrient limitation found in the lumen of the SCV. It translocates effectors across the membrane of the SCV. These effectors are required for the modulation of the intracellular environment [8].

This paper summarizes our current knowledge about the functions of *Salmonella* T3SS effectors. First, because effectors often act cooperatively, sometimes redundantly or in opposite ways, it is important to consider their concerted actions on relevant steps of the infection process at the cellular level. The paper continues with an analysis of the issues that will drive future research in the field of *Salmonella* T3SS effectors. Finally, as a sort of glossary, detailed and up-to-date information is given about each particular effector with special emphasis in biochemical activities, host partners, and targeted cellular pathways and processes. This information is summarized in Table 1.

2. Cellular Processes and Pathways Manipulated by *Salmonella* Effectors

2.1. Actin Cytoskeleton and Invasion. Central to the pathogenesis of *Salmonella* is its ability to invade intestinal cells: M-cells, epithelial cells, and dendritic cells [9–11]. The best-characterized *Salmonella* invasion mechanism requires T3SS1 that initiates a process known as the “trigger” mechanism. It has been recently shown that *Salmonella* can also invade cells by a “zipper” entry process, typical of other pathogens like *Listeria* [12], through the Rck outer membrane protein, which induces a local accumulation of actin, leading to a discrete membrane alteration [13]. Another outer membrane protein, PagN, and the pore-forming hemolysin HlyE also contribute to invasion by unknown mechanisms [14–16]. In addition, recent data indicate that other unknown entry routes may be used depending on the serotype, the host, and the cell-type considered [17, 18]. However, given its relative importance and the scope of this review, the focus here will be on the T3SS1-dependent entry mechanism.

T3SS1-dependent invasion is a process with several steps [19], some of them common to other invasion mechanisms, that includes (i) movement in the gut lumen by passive diffusion and active motility and chemotaxis; (ii) transient interactions with the mucosal surface; (iii) reversible binding to target cells via adhesins; (iv) irreversible T3SS1-mediated docking; (v) translocation of bacterial effectors through T3SS1; (vi) manipulation of the host cells by effectors

FIGURE 1: Schematic representation of *Salmonella* T3SS1 and T3SS2. HM: host plasma membrane; VM: vacuolar membrane; OM: outer membrane; IM: inner membrane. T3SS1 components and effectors are in blue. T3SS2 components and effectors are in red. Effectors translocated through both systems are in purple.

leading to rapid appearance of membrane ruffles; (vi) host cell invasion with formation of spacious vacuoles. Figure 2 summarizes the role of T3SS1 effectors in this process. At least six effectors induce remodeling of actin cytoskeleton: SipA, SipC, SopB, SopE, SopE2, and SptP. SipA and SipC directly bind to actin, cooperate to promote the formation of actin filaments at the site of bacterial adhesion, and prevent filament disassembly by host factors [20]. SipA modulates the actin-bundling activity of T-plastin [21] and inhibits the filament-depolymerizing factor ADF/cofilin and the filament capping and severin protein gelsolin [22]. SipC possesses distinct domains that are able to nucleate actin (central amino acid region) and to promote actin bundling (C-terminal region) [23, 24]. SopE, SopE2, and SopB do not directly interact with actin but mediate the activation of small GTPases of the Rho family that are required for the formation of highly branched actin networks. As explained in the last section of this paper, SopE and SopE2 mimic mammalian guanine nucleotide exchange factors (GEFs) to activate the GTPases Rac1 and Cdc42 by catalyzing exchange

of GDP for GTP [25, 26], whereas SopB indirectly activates RhoG targeting its GEF, SGEF [27]. After bacteria entry, membrane ruffling is terminated by the GTPase-activating protein (GAP) activity of SptP that inactivates Rac1 and Cdc42, reverting the actin cytoskeleton to its basal state after bacteria entry [28].

One important factor contributing to actin cytoskeleton reorganization and T3SS1-mediated *Salmonella* entry is tyrosine phosphorylation. Two host nonreceptor tyrosine kinases have been shown to be involved in invasion: Abl (Abelson tyrosine kinase) and FAK (focal adhesion kinase). Abl-mediated tyrosine phosphorylation of its substrates CrkII, an adaptor protein, and Abi1, a component of the WAVE2 complex, is enhanced during host cell invasion, and inhibition of CrkII phosphorylation impairs bacterial entry [29]. The physical interaction between FAK and the scaffolding protein p130Cas is necessary for *Salmonella* internalization. Although phosphorylation of p130Cas is induced during *Salmonella* invasion, this is not required for bacterial internalization, but p130Cas appears to be necessary

TABLE 1: List of known *Salmonella* type III secretion system effectors.

Effector	Localization	T3SS	Activity	Cellular targets	Pathway/cellular process
AvrA	SPI1	1	Acetyltransferase Deubiquitinase	β -catenin, ERK2, I κ B α , MKK4, MKK7, p53	Antiapoptotic, anti-inflammatory
CigR	SPI3	2			
GogB	Gifsy-1	2		FBXO22	Anti-inflammatory
GtgA	Gifsy-2	2			
GtgE	Gifsy-2	1/2	Protease		Prevents recruitment of Rab29
PipB	SPI5	2			
PipB2		1/2		KLC (kinesin light chain)	SIF extension
SifA		2	GEF	Rab7, RhoA, SKIP	SIF, SIFT, SCV formation
SifB		2	GEF		
SipA/SspA	SPI1	1		Caspase-3, F-actin, T-plastin	Actin assembly, disruption of tight junctions, PMN migration, SCV positioning
SipB	SPI1	1		Caspase-1	Pyroptosis
SipC/SspC	SPI1	1		Cytokeratin 8, cytokeratin 18, Exo70, F-actin, syntaxin 6	Actin nucleation, SCV maturation
SipD/SspD	SPI1	1			
SirP		1/2	E3 ubiquitin ligase	ERdj3, Trx	Apoptosis
SopA		1	E3 ubiquitin ligase	Caspase-3, HsRMA1, Ubch7	Invasion, escape from SCV, PMN migration
SopB/SigD	SPI5	1	Phosphoinositide phosphatase/GDI	Cdc24, Cdc42	Invasion, nuclear responses, SCV maturation, fluid secretion
SopD		1/2			Invasion, inflammation, fluid secretion
SopD2		2			Inhibition of LNT formation
SopE	SopE ϕ	1	GEF	Cdc42, Rab5, Rac1	Actin remodeling, inflammation
SopE2	Phage remnant	1	GEF	Cdc42, Rac1	Actin remodeling, inflammation
SpiC/SsaB	SPI2	2		Hook3, TassC	Inhibition of fusion of SCV with lysosomes
SptP	SPI1	1	GAP	Cdc42, Rac1, VCP, vimentin	Reversion of actin reorganization, inhibition of ERK activation
SpvB	Plasmid	2	Tyrosine phosphatase Actin ribosyltransferase	G-actin	Actin depolymerization, delayed cytotoxicity
SpvC	Plasmid	1/2	Phosphothreonine lyase	ERK2	Inactivation of MAPK
SpvD	Plasmid	1/2			
Srff	Iol island	2			
SseB	SPI2	2			
SseC	SPI2	2			
SseD	SPI2	2			
SseF	SPI2	2			
SseG	SPI2	2			
SseI/SrffH	Gifsy-2	2		Junction plakoglobin, TIP60 Caprin-1, desmoplakin Filamin A, IQGAP1, TRIP6	SIF formation, SCV positioning SIF formation, SCV positioning Modulation of host cell migration
SseJ		2	Acyltransferase	RhoA, RhoC	Dynamics of the SCV

TABLE 1: Continued.

Effector	Localization	T3SS	Activity	Cellular targets	Pathway/cellular process
SseK1		2			
SseK2		2			
SseK3	Phage ST64B	2			
SseL		2	Deubiquitinase	IκBα, OSBP-1, talin, ubiquitin	Autophagy
SspH1	Gifsy-3	1/2	E3 ubiquitin ligase	PKN1	
SspH2	Phage remnant	2	E3 ubiquitin ligase	14-3-3γ, AIP, BAG2, Bub3, Filamin A, Proflin-1, Sgt1, UbcH5-Ub	
SteA		1/2			
SteB		2			
SteC		2	Kinase		Actin meshwork
SteD		2			
SteE	Gifsy-3	1/2

FIGURE 2: T3SS1-dependent invasion of eukaryotic cells. *Salmonella* triggers its own internalization into host cells using a mechanism characterized by the appearance of large membrane ruffles at the bacterial entry site. Bacterial effector proteins SipA, SipC, SopB, SopE, and SopE2, which are injected into host cells through T3SS1, induce this process. Actin remodeling, mediated by host factors represented in yellow, and membrane fusion, mediated by host factors represented in brown, are involved in the mechanism of internalization. The process is reverted by the effector SptP.

for the proper assembly of actin during invasion [30]. The relationship of these kinases with Cdc42 and Rac1 is not clear, but the activation of these GTPases is not impaired in the absence of FAK or Abl, suggesting that they are regulating other components that function either downstream or in parallel with Rac1 and Cdc42. A potential target of Cdc42 is the tyrosine kinase Ack. This kinase is activated upon infection with *Salmonella* in a T3SS1-dependent manner. However, although Ack is involved in *Salmonella*-induced cellular responses mediated by the MAPK ERK, it does not appear to be required for *Salmonella* internalization [31]. Other host factors that are recruited to the actin-rich ruffles (also known as phagocytic cups) induced by *Salmonella* are Shank3 and IQGAP1. Shank3 is a large cytoskeletal scaffold protein. IQGAP1 is another scaffold protein that interacts with actin, Rac1, and Cdc42 and is necessary for efficient activation of Rac1 and Cdc42 by *Salmonella*. It has been suggested that interactions of IQGAP1 with *Salmonella*-activated Rac1 and Cdc42 maintain them in their active form [32, 33]. Another host factor that contributes to bacterial invasion by altering the dynamics of the actin cytoskeleton is the ubiquitin C-terminal hydrolase UCH-L1 [34]. Since SopA, SopB, and SopE are substrates of the ubiquitin proteasome system, it has been suggested that UCH-L1, acting as a deubiquitinating enzyme, could play a role in controlling the half-life of these effectors and/or the activity of small GTPases of the Rho subfamily.

Concomitant with the actin remodeling process, three of these effectors also regulate fusion of membrane from

different sources at the site of entry contributing to the expansion of ruffles. SipC interacts with Exo70, a component of the exocyst complex, which mediates docking and fusion of exocytic vesicles with the plasma membrane [35]. SopE can also activate RalA, a GTPase required for assembly of the exocyst [35]. Finally, SopB stimulates membrane fusion at the site of entry by increasing local levels of PI3P resulting in the recruitment to membrane ruffles of VAMP8, a v-SNARE protein that mediates homotypic fusion of early and late endosomes and regulates exocytosis by forming a SNARE complex with syntaxin 4 and SNAP23 [36].

2.2. Tight Junction Alterations. The epithelial cells lining the small intestine form a barrier that keeps the gut contents in the gut cavity, the lumen. These epithelial cells are bound by tight junctions, specialized cell junctions that function as barriers to the diffusion of some membrane proteins and lipids between apical and basolateral domains of the plasma membrane, and seal neighboring cells together preventing even small molecules from passing into the lumen. Tight junctions are composed of two major transmembrane proteins, claudins and occludins, intracellular peripheral membrane proteins called ZO proteins and several other associated proteins. *Salmonella* is one of the pathogens that have developed specific strategies to alter these structures during infection, resulting in diarrhea and other pathogenic effects [37]. *Salmonella* modifies tight junctions through the action of four T3SS1 effectors (Figure 3): SipA, SopB,

FIGURE 3: Effects of T3SS1 effectors on epithelial tight junctions. T3SS1 effectors SipA, SopB, SopE, and SopE2 contribute to disruption of tight junction by decreasing the amounts of ZO-1 (ZO) and phosphorylated occludin. In contrast, AvrA is a tight junction stabilizer.

SopE, and SopE2 [38]. All of them contribute to the effects of *Salmonella* infections in cell lines: decrease in transepithelial resistance, increase in permeability, decrease in ZO-1 expression, and decrease in the amount of phosphorylated occludin, which is the form that is present in tight junctions. Surprisingly, another T3SS1 effector, AvrA, has been described as a tight junction stabilizer [39].

2.3. Biogenesis of the *Salmonella*-Containing Vacuole. Following internalization, *Salmonella* establishes its intracellular niche in a modified phagosome termed the *Salmonella* containing vacuole (SCV). This is a unique membrane-bound compartment where *Salmonella*, depending on the host cell type, can establish a replication niche [40]. Its biogenesis and maturation are characteristically accompanied by the formation of different types of tubules originating and connected to the SCV [41]. Additional hallmarks of the biogenesis of the SCV are the movement of the vacuole from the plasma membrane to a perinuclear position [42], the recruitment of several members of the Rab family of small GTPases [43], the interaction with endocytic and exocytic pathways, and the involvement of T3SS1 and T3SS2 effectors in the modulation of the process. The SCV biogenesis can be divided arbitrarily into three stages: early (10 min–1 h postinfection), intermediate (1 h–4 h), and late (>4 h) [8, 41] (Figure 4).

The early stage is governed by T3SS1 effectors SopB and SptP. The role of SopB is complex and involves direct, through its phosphoinositide phosphatase activity, as well as indirect manipulation of the host membrane phosphoinositide contents. (i) SopB activates Akt via $PI(3,4)P_2$ and $PI(3,4,5)P_3$ [44]. (ii) Its activity is necessary for reducing

the levels of $PI(4,5)P_2$, necessary for efficient formation of the vacuole [45], and phosphatidylserine. Reducing the levels of these negatively charged lipids results in the exclusion of several Rab from the SCV and may serve to delay the fusion with the lysosome [46]. (iii) SopB recruits the GTPase Rab5 to the SCV membrane, that in turn attracts the Rab5-interacting protein Vps34, a PI3K that generates PI3P in the SCV membrane [47]. The accumulation of this phosphoinositide is necessary for the recruitment of the sorting nexins SNX1 and SNX3, which are important regulators of membrane trafficking [48, 49]. SNX1 shifts from its endosomal localization to the site of bacterial entry within the first minutes of *Salmonella* infection. Then, it forms highly dynamics tubules named spacious vacuole-associated tubules (SVATs) that are accompanied by a reduction in vacuole size and removal of cation-independent mannose-6-phosphate receptor from the nascent SCV. This receptor is a late endosome marker involved in delivering soluble lysosomal enzymes to lysosomes. Its absence from the SCV was originally seen as an evidence of lack of SCV-lysosome fusion [50], but there are reports that support a model in which SCVs can fuse to lysosomes, and then cation-independent mannose-6-phosphate receptor is removed in an SNX1-dependent manner [49, 51, 52]. SNX3 associates to specific tubular structures (SNX3 tubules) that last from 30 min to 2 h after invasion and contribute to the recruitment of Rab7 and LAMP1 to the SCV and to the movement of the vacuole [48]. The function of the effector SptP in the biogenesis of the SCV involves its GAP activity, that downregulates Cdc42 and Rac1 and reverts membrane ruffling [28], and its phosphatase activity, which dephosphorylates VCP [53], although this activity could be related to later events (see what follows).

FIGURE 4: Biogenesis of the *Salmonella*-containing vacuole. After internalization, *Salmonella* establishes an intracellular niche inside a modified phagosome known as *Salmonella*-containing vacuole (SCV). The initial step in SCV biogenesis (0-1 h) is governed by T3SS1 effectors (in blue) SopB and SptP and is characterized by the formation of SVATs and SNX3 tubules. The movement of the SCV to a juxtanuclear position during the intermediate stage of development (1-4 h) requires the participation of the T3SS1 effectors SipA and SopB and the T3SS2 effectors (in red) SifA, SseF, and SseG. Many effectors are involved in the final stage of maturation and in the maintenance of the SCV. Replication is initiated 4-6 h postinvasion and is accompanied by the formation of different kinds of tubules known as SIFs, SISTs, and LNTs. Effectors involved in the formation of these tubules are depicted in red (T3SS2 effectors) and in purple (effectors of both systems). The maturation process is also characterized by the interaction with the host endocytic and secretory pathways.

The intermediate stage in the SCV development is characterized by dynein-mediated movement of the vacuole along microtubules to reach a juxtanuclear position adjacent to the microtubule-organizing center [42]. This process requires the participation of the T3SS1 effectors SipA and SopB, and the T3SS2 effectors SifA, SseF, and SseG (see below for details of the function of these effectors on SCV biogenesis). The actin-based motor nonmuscle myosin II appears to contribute to SCV positioning in a process involving the phosphatase activity of SopB [54].

During the late stage of SCV maturation, a set of T3SS effectors, exhibiting both cooperative and antagonistic activities, are involved in the maintenance of the vacuole at its perinuclear position, as well as in the generation of different types of tubular networks, namely, *Salmonella*-induced filaments (SIFs), *Salmonella*-induced secretory carrier membrane protein 3 (SCAMP3) tubules (SISTs), and LAMP1-negative tubules (LNTs), that are more easily observed in

cultured epithelial cells. SIFs are tubules extending from the SCV that form along a scaffold of microtubules and derive from late endocytic compartments. They have the same composition as that of SCV membranes and contain LAMPs, Rab7, cholesterol, lysobisphosphatidic acid, vATPase, and cathepsin D [55-57]. But, they are also positive for SCAMP3 [58], indicating that these tubules can incorporate membrane from the secretory pathway. Formation of SIFs involves effectors PipB2, SifA, SopD2, SseF, SseG, and SseJ, whereas SpvB appears to have a negative effect on SIF formation (see the last section of this paper for a detailed explanation of the function of these effectors). A model of SIF formation [41] suggests that a putative GEF activity of SifA activates the small GTPase RhoA, which then binds and activates SseJ. As a consequence, the lipid composition of the SCV membrane is changed, promoting the formation of tubules. Both PipB2 and the complex SifA-SKIP bind to kinesin-1, link the SCV and nascent tubules to microtubules,

FIGURE 5: *Salmonella*-induced host cell death. (a) *Salmonella* induces apoptosis in epithelial cells 12–18 h postinfection (p.i.). Effectors AvrA and SopB (secreted through T3SS1), SpvB (secreted through T3SS2), and SlrP (secreted through T3SS1 and T3SS2) have been suggested to be involved in this process through different mechanisms. Ub: ubiquitin. (b) *Salmonella* expressing T3SS1 induces rapid pyroptosis in macrophages. Pyroptosis is a proinflammatory form of programmed cell death that depends on caspase-1 activation. The T3SS1 effector SopB, the T3SS1 rod protein PrgJ, and flagellin secreted through T3SS1 are involved in the induction of rapid pyroptosis. (c) Noninvasive *Salmonella* induces delayed pyroptosis in infected macrophages. T3SS2 effectors, SpvB and SseL, and flagellin are involved in this form of cell death.

and promote the elongation of tubules along microtubules. SopD2 associates to late endosome and can contribute to SIF formation by targeting endocytic vesicles to the SCV and nascent tubules [59]. Finally, SseF and SseG mediate bundling of microtubules near the SCV that can promote fusion of aggregated vesicles into tubules [60]. SISTs also contain SCAMP3 and T3SS2 effectors but lack LAMP1 and other late endocytic markers [58]. The formation of SISTs requires effectors PipB2, SifA, SopD2, SseF, and SseG. The mechanisms to segregate SISTs from SIFs remain unknown. Other tubules that arise at the late stage of SCV maturation are LNTs. These tubules are enriched in bacterial T3SS2 effectors but lack host LAMP1 and SCAMP3. Similarly to SIFs and SISTs, they form along microtubules in a kinesin-1-dependent manner, but they lack all the markers of SIFs and SISTs except vATPase. A report suggests that SifA and SopD2 exert positive and negative roles, respectively, in the formation of LNTs and that PipB2 is involved in their centrifugal extension [59]. The role of the *Salmonella*-induced tubular networks is unclear, although it could be related to the interception of nutrients or membranes from host cell trafficking [58].

During its maturation, the SCV becomes a replicative niche for *Salmonella* in many tissue culture models. However, this is not the case in fibroblasts or dendritic cells. This is consistent with the low replication rate observed *in vivo* in acute or chronic infections and suggests the existence of mechanisms that restrict replication, but not survival, of bacteria inside the SCV [40]. Interestingly, replication in epithelial cells can also occur in the cytosol, and although

it occurs only in less than 20% of infected cells, it accounts for the majority of the net replication [61]. In addition, in epithelial cells, a subset of SCVs is not maintained at a juxtanuclear position but moves towards the cell host periphery [62]. This centrifugal movement depends upon host microtubules and kinesin and the T3SS2 PipB2 and is associated with a decrease in the T3SS1 effectors SipA and SopB [62]. Both escape from the vacuole and centrifugal displacement of the SCV have been associated with the ability of *Salmonella* to achieve cell-to-cell transfer in order to repeat the intracellular cycle [62, 63].

2.4. Cell Death. *Salmonella* induces cell death in host cells using T3SS-dependent and -independent mechanisms [64]. Three processes of T3SS-dependent cell death will be discussed here (Figure 5): epithelial cell apoptosis, rapid T3SS1-dependent macrophage pyroptosis, and delayed T3SS2-dependent macrophage pyroptosis [65].

Salmonella invades intestinal epithelial cells during the enteric phase of infection and triggers death of cultured epithelial cell lines *in vitro* by apoptosis. This process requires bacterial entry and replication, and the phenotypic expression of apoptosis is delayed for 12–18 h after bacterial entry [66]. Apoptosis is a form of programmed cell death that can be initiated by external or internal stimuli. It involves the activation of a distinct subset of initiator caspases that activate executioner caspases, like caspase-3, which cleave cellular substrates to produce the features associated with apoptosis: reduced mitochondrial membrane potential, cell

surface exposure of phosphatidylserine, cytochrome cleavage, nuclear condensation, DNA fragmentation, and maintenance of an intact plasma membrane. Phagocytic cells ingest the apoptotic bodies that result, without accompanying inflammatory response [65]. Both T3SS1 and T3SS2 are required for triggering apoptosis in epithelial cells. Although it has been difficult to assess the role of T3SS1 independent from its function in cell invasion, there are at least some effectors translocated through this system that contribute to modulate the apoptotic process. Also, the T3SS2 effector SpvB is clearly required for apoptosis [67]. SlrP, which can be translocated by T3SS1 and T3SS2, has been recently suggested to contribute to epithelial cell death, likely through its interaction with thioredoxin-1 and with ERdj3 [68, 69]. At least two effectors, AvrA and SopB, can have antiapoptotic roles: AvrA suppresses the JNK MAPK apoptotic pathway [70], whereas SopB activates Akt, a kinase that can exert prosurvival effects [71]. Together, these effectors could be responsible for the delay in apoptosis seen in epithelial cells. This delay could be beneficial for the pathogen in order to establish a stable intracellular niche and avoid adaptive immunity [70]. Apoptosis at the later stages of the intestinal phase of the disease generates apoptotic bodies that are phagocytosed, together with *Salmonella*, by the incoming macrophages. Some of these macrophages could serve as sites for *Salmonella* proliferation in the mucosa and submucosa, whereas others carry bacteria to other tissues, allowing the progress of the systemic disease [64].

In macrophages, *Salmonella* expressing T3SS1 rapidly trigger another form of programmed cell death, called pyroptosis, which is dependent on caspase-1 [72, 73], a central mediator of innate immunity that is not activated in apoptosis. Caspase-1 activation results in production of active IL-1 β and IL-18, rapid cell lysis, and release of proinflammatory intracellular contents [74]. This phenomenon has been observed in macrophages and dendritic cells infected with *Salmonella* grown under conditions that favor expression of T3SS1, and the cytotoxic effect is detected within 1–2 h [75, 76]. Initial studies suggested that the T3SS1 translocase SipB was involved in this process through a direct interaction with caspase-1 [77]. Later studies revealed that caspase-1 activation requires the host inflammasome components ASC and NLRC4 (also known as Ipaf). The inflammasome is a multiprotein signaling platform that can be activated by flagellin and by the T3SS1 rod protein PrgJ [78–80]. Both proteins share a common sequence motif that triggers NLRC4 activation and are injected into the host cell cytosol through T3SS1. Murine NLR proteins NAIP5 and NAIP2 directly recognize flagellin and PrgJ, respectively. The NAIP proteins then physically interact with NLRC4, resulting in activation of the NLRC4 inflammasome and macrophage innate immunity [81]. It has been recently shown that *Salmonella* infection also triggers PKC δ -dependent phosphorylation of NLRC4 at serine-533 and that this is another important step in formation of a fully functional inflammasome [82].

Caspase-1 activation appears to play a protective role in host defense against bacterial infections. Consistent with this, expression of T3SS1 and bacterial flagellin are repressed

during systemic bacterial infection [83, 84]. In fact, when macrophages are infected with *Salmonella* grown under conditions that repress expression of T3SS1, rapid killing does not take place. However, delayed macrophage death becomes apparent 18–24 h postinfection. This form of cell death (i) requires T3SS2 and the effectors SpvB and SseL, (ii) is mediated by caspase-1, and (iii) results in production of IL-1 β , DNA cleavage, cell lysis, and inflammation [85–88]. Interestingly, during this form of delayed macrophage death, *Salmonella* activates two inflammasome receptors, NLRP3 and NLRC4 [89]. Activation of NLRC4 is dependent on T3SS2 and flagellin, but the T3SS2 rod protein SsaI does not contribute to caspase-1 activation. The signal that activates NLRP3 is unknown, but a relevant intermediary host factor is GBP5 (guanylate binding protein 5) [90]. This form of cell death has features of pyroptosis. However, other authors have observed a delayed form of macrophage death similar to the apoptotic process described previously for epithelial cells [91].

2.5. Exploitation of the Ubiquitin System. Ubiquitination is a reversible posttranslational modification that consists of the covalent attachment of ubiquitin onto a target protein. The process consists of a three-step enzymatic cascade involving the activity of an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2), and an ubiquitin ligase (E3), which controls the specificity of the reaction by recruiting the target protein [92]. Ubiquitination is found in all eukaryotic cells and plays important roles in protein degradation, signal transduction, transport of membrane proteins, and host defense. E3 enzymes belong to different families that are mechanistically distinct: the homologous to E6-AP C-terminus (HECT) domain family, the really interesting new gene (RING) domain family, or the U-box domain family. HECT E3s form a thioester intermediate with the ubiquitin before transferring it to the substrate. RING and U-box E3s function as scaffolds to bring E2 and substrate into proximity [93, 94]. Ubiquitination can be reverted by a family of isopeptidases called deubiquitinases that catalyzes the removal of ubiquitin from ubiquitinated proteins [95]. *Salmonella* interacts with the host ubiquitin pathway in several ways: some T3SS effectors are ubiquitination substrates, and several effectors act as E3 ubiquitin ligases, while others mimic deubiquitinases [96, 97] (Figure 6).

Ubiquitination has been shown for the T3SS1 effectors SopA, SopB, SopE, and SptP. Interestingly, SopE and SptP are both polyubiquitinated and marked for proteasome-dependent degradation; however, SopE is more quickly degraded, ensuring the sequential activity of these effectors [98]: first, SopE, acting as a GEF for Cdc42 and Rac1, contributes to *Salmonella*-invasion mediating actin binding polymerization and ruffle formation; then SptP, acting as a GAP on the same small GTPases, switches off actin polymerization. Ubiquitination also regulates the intracellular localization of SopB: it is transported from its initial localization at the plasma membrane to the SCV after monoubiquitination at several lysine residues [99]. Finally, SopA is ubiquitinated by the host E3 ubiquitin ligase

FIGURE 6: Exploitation of the ubiquitin system by *Salmonella*-secreted effectors. SopA, SopB, SopE, and SptP are substrates of the host ubiquitination system. AvrA and SseL possess deubiquitinase activity. SopA, SlrP, SspH1, and SspH2 are E3 ubiquitin ligases. GogB interferes with the E3 ubiquitin ligase activity of the host SCF complex.

HsRMA1. It has been proposed that monoubiquitinated SopA could contribute to *Salmonella* escape from the SCV into the cytosol of epithelial HeLa cells, whereas polyubiquitination of SopA leads to its degradation by the host proteasome pathway [100].

In addition to being a substrate for ubiquitination, SopA is itself an E3 ubiquitin ligase. Although it has little sequence identity to eukaryotic E3s, it is considered as a HECT-like E3 ligase because of its structure and proposed mechanism of action [101]. The substrates for the E3 activity of SopA are unknown, but this activity appears to be involved in *Salmonella*-induced PMN transepithelial migration [102]. Three additional *Salmonella* T3SS effectors, SlrP, SspH1, and SspH2, possess E3 ubiquitin ligase activity, but because they differ from all known E3 ligases in structure and mechanism, they can be considered as a new class of cysteine-dependent E3 ubiquitin ligase, the novel E3 ligase (NEL) family. However, they share some generalized characteristics with the HECT family of ubiquitin ligases, including the presence of a conserved cysteine residue on a loop that

is critical to the ubiquitination reaction, the formation of a stable covalent link with ubiquitin, and the requirement of conformational changes for biochemical activity [103]. Proposed substrates for the activities of SlrP and SspH1 are mammalian thioredoxin and the serine/threonine protein kinase PKN1, respectively [69, 104].

Salmonella has a T3SS1 effector, AvrA, and a T3SS2 effector, SseL, with deubiquitinase activity. Both effectors interfere with nuclear factor κ B (NF- κ B) signaling through their suggested substrates: NF- κ B, I κ B α , and β -catenin, for AvrA, I κ B α for SseL [88, 105–107]. SseL has been shown to be involved in the regulation of autophagy. This is a process that involves degradation of intracellular components via the lysosome and that can be used by eukaryotic cells to control and degrade intracellular bacteria (xenophagy). Some examples of autophagy where *Salmonella* T3SS could have a role have been described. (i) SCV membranes damaged by bacteria entering the cytosol attract galectin-8 [108], and polyubiquitinated proteins accumulate on the bacterial surface [109]. Galectin-8 and ubiquitin are then detected

FIGURE 7: Nuclear responses induced by *Salmonella* effectors. Some of the effects of *Salmonella* T3SS effectors on host signal transduction pathways, leading to induction or inhibition of host immune responses, are depicted.

by autophagy receptors [110–112]. T3SS1 is involved in this process maybe by damaging the SCV membrane with its pore-forming activity. Also, the T3SS1 translocase SipB has been suggested to induce autophagy and cell death in macrophages [113, 114]. (ii) *Salmonella* within vacuoles can induce a cellular response leading to the formation of T3SS2-dependent ubiquitinated aggregates that attract autophagy markers. This process is opposed by the SseL deubiquitinase activity [115].

2.6. Nuclear Responses. T3SS effectors contribute to the transcriptional changes that are observed in *Salmonella*-infected cells (Figure 7). The effects of individual effectors on some signal transduction pathways are mentioned in other sections of this paper. This section will focus on transcriptomic studies.

Microarray analyses performed on host RNA responses have exploited a variety of *in vivo* and *in vitro* models and have allowed the study of the impact of pathogens on host cells on a global scale [116]. In one of the first studies of this type, differential host cell gene expression was examined in an *in vitro* model of *S. enterica* serovar Typhimurium infection using the RAW264.7 murine macrophage cell line [117]. This study detected significant changes in the expression of numerous genes encoding chemokines, cell surface receptors, signaling molecules, and transcriptional activators at 4h postinfection. Genes encoding inducible nitric oxide synthase (iNOS), MIP-1 α , MIP-1 β , MIP-2 α , IL-1 β , TNF α receptor, CD40, I κ B α , I κ B β , TGF β 1, TGF β 2,

caspase-1, Fas, TDAG51, TRAIL, LIF, Egr-1, NF-E2, IRF-1, and *c-rel* were among the upregulated genes, whereas expression of Ski, B-myb, Fli-1, c-Fes, cyclin D1, and cdk4 was downregulated. Another study that investigated the response of U-937 human monocytes to *S. enterica* serovar Typhimurium infection and the role of PhoP in this response detected upregulation of IL-8, MIP-1 α , MIP-1 β , IL-23p19, and I κ B α [118]. Genes upregulated in the human intestinal epithelial cell line HT-29 infected with *S. enterica* serovar Dublin for 3, 8, or 20 h, included several cytokines (G-CSF, Inhibin β A, EBI3, MIP-2 α , IL-8), kinases (TKT, Eck, HEK), transcription factors (IRF-1), and HLA class I [119]. Other interesting examples of global *in vitro* and *in vivo* studies are the gene expression profiling in chicken heterophils infected with *S. enterica* serovar Enteritidis [120], the analysis of transcriptional responses to *S. enterica* serovar Choleraesuis infections in pig mesenteric lymph nodes [121], the analysis of the gene expression response of the rat small intestine following oral infection with *S. enterica* serovar Enteritidis [122], and the transcriptional profiles from *S. enterica* serovar Typhi-infected children [123]. However, the role of the T3SSs on host transcriptional responses was not investigated in these studies.

Recently, the impact of T3SS1 and particular effectors on the global transcriptional response of cultured human Henle-407 epithelial cells infected with *S. enterica* serovar Typhimurium was analyzed [124]. The genes up-regulated by wild-type *Salmonella* infection included genes whose products are proinflammatory such as several chemokines and cytokines and their receptors (MIP-2 α , MIP-2 β , IL-8, IL-1 α ,

IL-11, IL-1R1, COX-2, TNFRSF10D, IL-4R, TNFRSF12A) and genes encoding transcription factors that amplify the immune response (Fos, FosB, FosL1, Jun, JunB, EGR1, EGR3, ATF3, STAT3). *Salmonella* infection also stimulated the expression of genes whose products limit the immune response (tristetraprolin, suppressors of cytokine signaling SOCS2 and SOCS3, I κ B ζ and several members of the DUSP family of tyrosine phosphatases). The transcriptional profiles of epithelial cells infected with an *invA* mutant strain, that lacks a functional T3SS1, or with a “effectorless” mutant strain, lacking genes encoding T3SS1 effectors AvrA, SlrP, SopA, SopB, SopD, SopE, SopE2, SptP, and SspH1, were similar to that of uninfected cells, demonstrating that the transcriptional reprogramming triggered by the wild-type strain depended on one or more T3SS1 effectors. Additional experiments showed that SopB, SopE, and SopE2 were responsible for the stimulation of innate immune responses in epithelial cells in a manner that is independent of the canonical innate immune receptors and conserved bacterial products or pathogen-associated molecular patterns. These effectors redundantly mediate the stimulation of Rho-family GTPases leading to the activation of MAPK (ERK, p38, and JNK) and NF- κ B signaling pathways [124]. In a recent *in vivo* study using a bovine-ligated ileal loop model, the transcriptional profiles of bovine host Peyer’s patches inoculated with wild-type or a *sipA sopA sopB sopD sopE sopE2* mutant of *S. enterica* serovar Typhimurium were compared at seven time points, from 15 min to 12 h [125]. The main difference was that the wild-type infection induced a biphasic host response with increased gene expression activity at 1 h and 12 h postinfection, whereas infection with the mutant strain induced a progressive increase in gene expression over time. In addition, both strains showed significantly different patterns of host response at early time points of infection within phosphatidylinositol, CCR3, Wnt, and TGF- β signaling pathways and in the regulation of actin cytoskeleton.

Microarrays have also been used to analyze the role of AvrA in host transcriptional responses *in vitro* and *in vivo* [126–128]. Comparison of the effects of wild-type and *avrA* strains of *S. enterica* serovar Typhimurium on cultured intestinal epithelial cells suggested a specific role of this effector in inhibiting the *Salmonella*-induced activation of the JNK pathway, whereas no interference with NF- κ B activation was observed [126]. In contrast, *in vivo* experiments carried out with RNA from mouse colon mucosa showed that NF- κ B was one of the top-10 signaling pathways targeted by AvrA, although the effects were different at 8 h and 4 days postinfection [127]. Another *in vitro* study suggested a role of AvrA in *Salmonella*-induced p53 acetylation in epithelial cells [128].

The role of T3SS1 and T3SS2 in the transcriptional response of chicken macrophages to *S. enterica* serovar Enteritidis infection have also been investigated using cDNA microarrays [129]. This work suggested a role for the T3SS1 effector SipA and for the T3SS2 effector PipB in suppressing host innate response, since individual mutants lacking one of these effectors elicited higher transcription levels of certain chemokines and RhoA than the wild-type strain.

2.7. Inhibition of Antigen Presentation in Dendritic Cells.

Dendritic cells are phagocytic cells with a pivotal role in the immune response [130]. In addition to playing a critical role in innate immunity, they are probably the most efficient and critical of all antigen-presenting cells [131]. They can sample a diverse array of antigens in peripheral tissues, transport the antigens to local lymph nodes, and present them to T cells as peptides bound to both MHC class I and II products. *S. enterica* serovar Typhimurium is efficiently taken up by dendritic cells, and these cells can serve as an alternative invasion pathway to M cells [132]. Initial studies carried out with an immortalized cell line suggested that *Salmonella* in these cells resides in a compartment that is different from that described for *Salmonella* inside macrophages because it lacks the late endosomal/lysosomal membrane marker LAMP1 [133]. However, in murine bone marrow-derived dendritic cells, *S. enterica* serovar Typhimurium resides in a membrane-bound compartment that has acquired late endosomal markers [134]. In these cells, *Salmonella* represents a static, nondividing population that is able to mount a functional T3SS2. The activity of this system is not required for intracellular survival [135] but is necessary for the correct maturation of the SCV in dendritic cells [134]. *Salmonella* inhibits the capacity of dendritic cells to process and present antigens by the MHC class II pathway and their ability to stimulate T cell proliferation in a T3SS2-dependent manner [136, 137]. T3SS2 effectors PipB2, SifA, SlrP, SopD2, and SspH2 are equally important for the interference with antigen presentation, whereas SseF and SseG contribute to a lesser extent. In contrast, effectors GogB, PipB, SifB, SseI, SseJ, SseK1, SseK2, and SspH1 have no contribution to this phenotype [138]. *Salmonella* interferes with MHC class II antigen presentation by specifically reducing cell surface HLA-DR expression in a process that requires SifA [139] and is mediated by T3SS2-dependent polyubiquitination, leading to removal of mature, peptide loaded, $\alpha\beta$ dimers from the cell surface [140]. The specific effector involved in this posttranslational modification is unknown, since, surprisingly, effectors that are known to interfere in the ubiquitination pathway (SlrP, SopA, SseL, SspH1, or SspH2) were not required for class II downregulation [140].

3. Future Directions

The study of *Salmonella* T3SS effectors has provided significant progress in our understanding of host-pathogen interactions in the last two decades. More than 40 effectors have been identified, and the characterization of the biochemical activities and host targets of some of them have been successfully addressed in the last years. The mechanisms for the establishment of an intracellular niche have been described in detail for some serovars, especially in certain cultured cell models, and *Salmonella* effectors have become an invaluable tool for the study of the cell biology of the host. However, many aspects of the contribution of these proteins to the relationship between *Salmonella* and its hosts at the cellular level and at the organism level remain unknown.

Some of the topics in this field that are likely to be the subject of intense research in the next years are discussed in this section.

3.1. The Effector Repertoire. In spite of the recent availability of fully sequenced genomes, identifying T3SS effectors has not always been an easy task, since they do not share universally conserved features. A variety of different approaches have been used to identify some genes as good candidates to encode effectors. T3SS-dependent translocation has been then tested using different methodologies. Genes in SPI1 or SPI2, where the structural components of T3SSs are encoded, were obvious candidates, but most effectors are encoded outside these islands. Coregulation with SPI1 or SPI2 genes has been a guide in some cases [141]. Sequence similarity was useful for only a few effectors, like the effectors belonging to the *Salmonella* translocated effector (STE) family, which share an N-terminal secretion signal [142]. Because of the lack of conserved sequences, computational prediction of T3SS effectors has been a difficult challenge. Functional redundancy is another hurdle, since mutants lacking only one effector are usually as virulent as the wild type. An interesting functional approach to find new effectors was based on the generation of fusions with the catalytic domain of CyaA from *Bordetella pertussis* [143]. This is a calmodulin-dependent adenylate cyclase, and, since calmodulin is present in the eukaryotic cytosol but not in bacteria, translocation of a CyaA fusion into the host is detected as an increase in cAMP concentrations in *Salmonella*-infected cell cultures [144]. Although useful, this approach is time consuming and has limitations. More recently, the proteomic analysis of culture supernatants has been an efficient way to identify T3SS effectors [145]. Although the combinations of these and other approaches have probably been successful in finding the majority of *Salmonella* effectors, it is likely that other effectors exist, and finding them is an important task in this field. To do that, besides finding new screen methods, it could be useful trying the same screens under different conditions affecting: culture conditions, host cell types, or postinfection times. Also testing different serovars and a variety of strains is important since certain effectors are strain-specific.

An important, related topic is the study of the specificity of an effector for a particular T3SS and of the degree of cross-talk between systems. This is especially relevant in *S. enterica* since these bacteria possess three T3SS: the virulence-related T3SS1 and T3SS2 and the flagellar system. In fact, as mentioned above, flagellin can be secreted through T3SS1 or T3SS2 in the context of macrophage infections and induction of pyroptosis. Conversely, escape of effectors through the flagellar system has also been observed under certain circumstances [146]. Examples of effectors that have been shown to be secreted by T3SS1 and T3SS2 have been given above. They were initially seen as exceptions but the number is increasing. This is not surprising, since effectors from different bacterial species are efficiently secreted ectopically. In principle, translocation *in vivo* could be more restricted depending on the expression patterns of effectors and their cognate T3SSs. There are, however, evidences of

overlapping in the expression of *Salmonella* T3SSs [147] and, therefore, this question should be studied in detail, since translocation through different systems open the possibility of different functions for the same effector. Specificity could also be connected with the presence of chaperones [148] that, in many cases, have not been identified yet. Related to this question is the kinetics of translocation and persistence in the host of each effector. There seems to be a hierarchy of translocation [149]. How is this imposed? How relevant is this for the infection process?

3.2. Biochemical Activities and Host Targets. Defining the biochemical activities and cellular targets [150] of T3SS effectors are the initial steps to understand their function. As seen in Table 1, this information is missing for a significant number of known *Salmonella* effectors. Similarity to proteins with known activities has been useful in some cases, but in many other cases, effectors mimic the activity of eukaryotic proteins without sharing significant sequence similarity [151]. Studying the effects of the expression of individual effectors in host cells, although nonphysiological, is a powerful tool that can help in the discovery of their activities. This can be carried out not only in mammalian cells but also in more simple models, like the yeast, that are more amenable to genetic analysis [152, 153]. Defining host substrates for the catalytic activities of effectors is another relevant subject of research. For instance, there are three known E3 ubiquitin ligases of the NEL family: SlrP, SspH1, and SspH2. SlrP and SspH1 have been shown to ubiquitinate Trx and PKN1, respectively, *in vitro*, but this has not been confirmed *in vivo*, and there are no putative substrates for SspH2. Finding the host binding partners for every effector (wild-type or catalytically dead to stabilize transient interactions) will provide putative substrates. The classical yeast two-hybrid screen [69] or the quantitative proteomics technique based on stable isotope labeling of amino acids in cell culture (SILAC) are examples of efficient methods that are being used to identify interaction partners [154]. Computational methods are also useful to predict host-pathogen protein-protein interactions [155]. Structural studies of effectors, especially in complex with their cognate host partners, are also instrumental in understanding their function and their mechanisms of action [156].

3.3. Global Responses. Details have been given in a previous section about the analysis of global transcriptional responses of the host to *Salmonella* infections and the role of T3SS in these responses. The analysis is far from complete because the role of individual effectors has been studied only in a few cases. DNA microarrays or deep sequencing [157] are being used to compare the effect of wild-type and specific mutant strains of *Salmonella*. These methods could also be used to analyze the transcriptional profiles of cultured cells expressing or not a particular effector. In addition, proteomics will provide the pattern of global responses at the protein level, which should be useful, for instance, to define the substrates for the effectors that interfere with the host ubiquitination pathway.

3.4. Different Lifestyles. The SCV has been historically seen as the primary survival and replication niche for intracellular *Salmonella*. However, the behavior of a strain of *S. enterica* is different depending on the host cell type, and, although the SCV is a replicative niche in cultured epithelial cells and macrophages, this is not the case in fibroblasts or dendritic cells [40]. In addition, *Salmonella* can have a bimodal lifestyle in epithelial cells and replicate in the SCV and in the cytosol, as has been beautifully analyzed recently in human HeLa cells [61]. This issue is still more complex if we take into account the heterogeneity that is observed in tissue culture infection models, where neither all cultured host cells are infected nor all pathogen cells inflict alterations in host physiology [158]. Defining the specific cell types, where *Salmonella* survive and replicate *in vivo*, and understanding the role of T3SS effectors in establishing different intracellular lifestyles and different host responses will be an interesting subject of research in the next years. This effort should be assisted by new technologies that allow real-time and single-cell analysis.

A related topic is the study of the relationships between different serovars of *S. enterica* and their hosts and the different outcomes that can result in terms of pathological manifestations. T3SS effectors play a central role in this context; therefore, defining the effector repertoire for every serovar and every strain and comparing the effects of different serovars on the same host cells are other relevant subjects of research. In contrast with the impressive amount of data accumulated about the role of T3SSs in the pathogenesis of *S. enterica* serovar Typhimurium, limited data is available concerning the role of these systems in *S. enterica* serovars Typhi or Paratyphi, which are responsible for systemic diseases in humans. The *in vivo* study of these serovars has been hampered by their strict adaptation to a particular host, but the development of new models is in progress [159].

3.5. Extracellular Roles and Applications of Effectors. T3SS1 effectors are easily detected in the culture media of *Salmonella* growing under standard laboratory conditions. The physiological relevance of this host-independent secretion remains to be explored. Interestingly, it has been recently shown that *S. enterica* serovar Typhimurium overexpressing SPI1 displays an adherent biofilm that is able to massively recruit heterologous nonbiofilm forming bacteria [160]. Although this phenotype is not observed when SPI1 is expressed from its original genomic location, the authors suggest a number of potential uses for engineered biofilm formation including bioremediation, biofuel cell design, and engineered infections for beneficial purposes. Because of its ability to export protein to the extracellular environment, T3SS1 is, in fact, a potential useful tool when proteins need to be exported for their function or to ease purification. T3SSs from several bacteria have already been used to export recombinant proteins including enzymes, peptides to induce an immune response, and spider silk proteins, but the range of foreign proteins that can be secreted and their limits are currently under study [161]. Effectors are also promising candidates to be carriers for delivery of heterologous vaccine

antigens, especially T3SS2 effectors that are synthesized only when *Salmonella* is inside host cells [162].

3.6. *Salmonella* in Plants. Many reports have linked food poisoning with the consumption of raw vegetables and fruits contaminated with *Salmonella* and other enteric bacteria [163]. Therefore, there is a great interest in understanding the interaction between *Salmonella* and plants. The role of plants in the life cycle of *Salmonella* and the ability of these bacteria to use plants as alternative hosts to human and other animals have been recently reviewed [164, 165]. *Salmonella* usually enters agricultural environments via animal feces. Animals can directly excrete waste onto plants or can contaminate surface water used for irrigation and pesticide or fertilizer diluent. Once it has found its way to plants, *Salmonella* actively attaches to plant tissues, colonizes plant organs, and uses them as viable hosts. In addition, plants inoculated with *Salmonella* have been reported to show reduced vigor [166], or chlorosis and wilting of leaves [167]. However, *Salmonella* is not considered a phytopathogen because Koch's postulates were not completed on any plant.

It is well established that T3SS effectors secreted by plant pathogens like *Pseudomonas syringae* are recognized by host plant cells and induce an immune response that usually result in a hypersensitive cell death response at the infection site [168]. Interestingly, components of *Salmonella* T3SS1 also appear to be recognized by plants, since mutants lacking this system (*spaS* or *sipB* mutants) exhibit increased colonization of alfalfa roots and wheat seedlings [169]. These results contrast with the reduced proliferation observed for mutants *prgH* and *ssaV*, lacking T3SS1 and T3SS2, respectively, in *Arabidopsis thaliana* [170]. These mutants also induced more apparent symptoms in *Arabidopsis* plants, suggesting that, in this case, T3SSs are necessary to prevent the hypersensitive response. In fact, transcriptomic analysis indicated that 649 host genes are induced specifically by the T3SS1 mutant, and many of these genes encode proteins related to responses against pathogens [170]. Consistent with this idea, wild-type bacteria, but not a T3SS1 mutant, were able to suppress the oxidative burst and the increase in extracellular pH after inoculation of a tobacco cell culture [171]. Clearly, the role of *Salmonella* T3SSs and the outcome of the interactions are different depending on plant species and cultivars and on *Salmonella* serovars [166, 172]. It has been recently shown that *Salmonella* effector SseF, but not AvrA, PipB2, SseG, SseJ, SseL, SopB, or SopD2, triggers a hypersensitive-like response in *Nicotiana benthamiana* when expressed in leaves by *Agrobacterium tumefaciens*-mediated infiltration or when translocated into leaves by the T3SS of *Xanthomonas campestris* [173]. However, *Salmonella* was unable to elicit this response in *N. benthamiana*, and more studies are needed to understand the extent of the contribution of individual effectors during endophytic growth.

Many questions remain unresolved about the role of T3SSs in *Salmonella*-plant interactions: what are the signals that induce expression of T3SS1 and T3SS2 in plants? How does *Salmonella* achieve the delivery of effectors across plant

cell walls and plasma membranes? What is the role of individual effectors?

3.7. Final Remarks. The topics discussed in this section are just a few examples of the issues that are likely to be the subject of investigation in the near future in this fertile research field. No doubt, *Salmonella*, and particularly its T3SS effectors, will continue to be an outstanding tool in basic cell biology studies. Examples of advances in the understanding of host cells processes, like innate immunity, programmed cell death, cytoskeleton organization, or membrane trafficking, have been presented in this paper. Many more are expected to arise, as the details of the effector functions are unveiled.

4. An Alphabetical Guide to *Salmonella* T3SS Effectors

4.1. AvrA. AvrA is a T3SS1 effector that shares sequence similarity with YopJ of the animal pathogen *Yersinia pseudotuberculosis* and AvrRxv of the plant pathogen *Xanthomonas campestris* pv. *vesicatoria* [174]. Several signaling pathways have been proposed as targets of AvrA. Initial studies used transient expression of *avrA* in human epithelial HeLa cells and infections with *S. enterica* serovar Typhimurium *pho-24* (or its AvrA⁻ variant). The *pho-24* mutation induces constitutive activation of the PhoQ/PhoP two-component system, and this activation increases expression of *avrA*. These studies suggested that AvrA inhibits the NF- κ B pathway [175]. Inhibition of c-Jun N-terminal kinase (JNK) and NF- κ B signaling pathways were observed in transgenic *Drosophila* and murine models leading to suppression of innate immunity, inflammation, and apoptosis during natural infection [176]. Recently, DNA microarray analysis of mouse colon mucosa infected with wild-type or AvrA⁻ *Salmonella* suggested that several pathways, including mTOR, NF- κ B, platelet-derived growth factors, vascular endothelial growth factor, oxidative phosphorylation, and MAPK signaling, are specifically regulated by AvrA *in vivo* [127]. Using the streptomycin pretreatment mouse model of enteric salmonellosis, it was shown that AvrA modulates survival of infected macrophages likely via JNK suppression and prevents macrophage death and rapid bacterial dissemination. AvrA suppression of cell death in infected macrophages may allow for establishment of a stable intracellular niche typical of intracellular pathogens [70]. In addition, bacteria expressing *avrA* decrease the intestinal permeability in comparison with AvrA-deficient bacteria through an increase in the tight junction proteins ZO-1 (Zonula occludens-1), occludin, and claudin-1. It is suggested that this effect of AvrA is mediated by a decrease in the expression of inflammatory cytokine interleukin-6 (IL-6) [39].

Two biochemical activities have been proposed for AvrA that can explain some of its effects in the host: deubiquitinase activity and acetyltransferase activity. Suggested targets for the deubiquitinase activity of AvrA are two inhibitors of NF- κ B, I κ B α , and β -catenin [106, 107]. In addition, several members of the Wnt family have been shown to

be upregulated by AvrA [177], and since Wnt2 is regulated by ubiquitination, AvrA may stabilize Wnt2 by removing the ubiquitin from Wnt2 [178]. This, in turn, stabilizes β -catenin, a downstream target of Wnt. Proposed targets for the acetyltransferase activity of AvrA are MAPK kinase 4 (MKK4) in a *Drosophila* model, leading to inhibition of JNK [176] and p53. p53 acetylation is increased by AvrA *in vitro* and in an *in vivo* mouse model. Activation of the p53 pathway through acetylation would lead to cell cycle arrest and would block apoptosis and inflammation [128]. Both YopJ and AvrA require the eukaryotic host cell factor inositol hexakisphosphate for activation of their acetyltransferase activity [179].

In a recent study, *pho-24* strains with wild-type or mutated *avrA* were used in a model of chronic *Salmonella* infection in mice. In this model, *Salmonella* persistently promotes small intestinal and colonic epithelial proliferation *in vivo* over 10–27 weeks [180]. This study also showed that, under these conditions, *Salmonella* strains expressing AvrA enhance expression of Akt, potentiate phosphorylation and acetylation of β -catenin, induce β -catenin nuclear translocation, and increase transcription of target genes, which could be the explanation for increased proliferation. In addition, AvrA inhibits the *Salmonella*-induced activation of the JNK pathway through its physical interaction with MKK7: the catalytic mutant of AvrA (C172S) interacted with MKK7 (but not with MKK3, MKK4, or MKK6) in the yeast two-hybrid system. Interestingly, AvrA is phosphorylated at conserved residues by a T3SS-effector-activated ERK pathway. This phosphorylation could have a negative effect on AvrA activity [126].

4.2. GogB. GogB is encoded within the bacteriophage Gifsy-1, which is present in most *S. enterica* serovar Typhimurium strains [181]. GogB is a secreted substrate of T3SS1 and T3SS2, but it is translocated specifically through T3SS2 to the host cytoplasm during infection [182]. Very recently, F-box only protein 22 (FBXO22) was identified as the host cell target of GogB. This interaction targets GogB to the Skp, Cullin, F-box containing (SCF) ubiquitin ligase complex to dampen the host inflammatory response by inhibiting I κ B α degradation and NF κ B activation. Therefore, GogB can be seen as an anti-inflammatory effector that manipulates the host ubiquitination system to prevent host inflammatory responses following colonization in order to limit tissue damage and bacterial burden during chronic infection [183].

4.3. PipB. The gene encoding this effector, *pipB* (for pathogenicity island-encoded protein B), is located in SPI5 and contributes to enteropathogenesis in a calf model of infection [184]. This gene is highly induced inside macrophages and epithelial cells [185]. PipB is expressed under SPI2-inducing conditions and is translocated through T3SS2 to the SCV and SIFs [186]. These are stable filamentous lysosomal glycoprotein-containing structures connected to the SCV that are formed in epithelial cells four to six hours after invasion with *Salmonella* [187]. PipB associates with host membranes and is enriched in detergent-resistant

microdomains, also known as lipid rafts [188]. Interestingly, PipB is implicated in intestinal tract colonization of chicks by *S. enterica* serovar Typhimurium [189] and contributes to invasion and survival of *S. enterica* serovar Enteritidis in chicken oviduct epithelial cells [190]. In addition, PipB plays a role in repressing avian β -defensins genes [191] and in stimulating inducible nitric oxide synthase in these cells [192].

4.4. PipB2. PipB2 was described as a *Salmonella* T3SS2 effector [193] with sequence similarity to PipB [186]. PipB2 is synthesized under SPI2-inducing growth conditions and upon infection of macrophages [194], where it localizes to the SCV and to SIFs 12 h postinfection. PipB2 reorganizes late endosome/lysosome compartments in mammalian cells resulting in the centrifugal extension of SIFs away from the SCV along microtubules. This activity is a consequence of its kinesin-1 binding activity [195]. Since SifA, another T3SS2 effector, downregulates kinesin-1 recruitment, PipB2 and SifA demonstrate antagonistic activities [195]. PipB2 promotes outward movement of the SCV when myosin II activity is inhibited [54]. The characteristic positioning of SCV to juxtanuclear regions suggests that the kinesin-inhibitory action of SifA may be dominant over the effects of PipB2 at 8 to 14 h postinfection. However, at later stages of epithelial cell infection, there is an outward displacement of a significant proportion of SCVs that is dependent upon host microtubules, kinesin and PipB2, and that is involved in cell-to-cell spread of *Salmonella* during infection [62]. Recently, it has been shown that the level of PipB2 is similar under SPI1- and SPI2-inducing conditions and that, in addition to T3SS2, this effector can use T3SS1 to be translocated into several mammalian cell types [196].

4.5. SifA. *Salmonella*-induced filament gene A (*SifA*) was described as a gene necessary for the formation of SIFs [197]. SifA was initially associated with T3SS on the basis of similarity to the N-termini of effectors SspH1, SspH2, and SlrP and of its specific synthesis under SPI2-inducing conditions in an SsrB-dependent manner. Although several lines of evidence indicated that it was a T3SS2 effector, its translocation could not be demonstrated using the CyaA fusion protocol [142, 198, 199]. However, secretion through T3SS2 of a SifA-M45 fusion was demonstrated *in vitro* [200].

In addition to its role in SIF formation, SifA is required for the development of SISTs. Both SIFs and SISTs are SCAMP3-positive tubules, but while SISTs are devoid of late endocytic markers, SIFs are positive for both late endocytic markers and SCAMP3. *Salmonella* recruits membrane from a *trans*-Golgi network-derived SCAMP3-containing pathway to induce the formation of both types of tubules [58]. SifA is also necessary for the maintenance of the SCV [198]. Because of their inability to maintain the SCV, *sifA* mutants have a defect in replication in macrophages and fibroblasts [198, 199, 201], but they replicate more efficiently in epithelial cells, and this enhanced replication occurs in the host cytosol [202]. Formation of SCVs and SIFs requires the host factor

Rab7 [203], but, unlike SCVs, SIFs are devoid of Rab7-interacting lysosomal protein (RILP) and dynein, and *in vitro* experiments suggested that SifA contributes to uncoupling Rab7 from RILP by interacting with Rab7 [204]. Transfection experiments suggested that SifA directs membrane fusion events involving late endosomes [205]. Data obtained in eukaryotic cell cultures infected with *Salmonella* established that SifA is required for the recruitment of lysosomal glycoproteins LAMP1 and LAMP2 to the SCV [206, 207]. An additional role for SifA in interfering with MHC class II antigen presentation has also been suggested [139].

Targeting of SifA to membranes requires a C-terminal, CAAX motif (where AA represents two aliphatic residues) that is commonly found in small GTPases [208]. The cysteine residue within this motif is modified by host prenylation machinery [209]. SifA interacts with the host protein SKIP (SifA and kinesin-interacting protein) forming a functional complex that mediates the role of SifA in virulence [210, 211]. SKIP interacts both with kinesin-1 and SifA via its N-terminal RUN and C-terminal PH domains, respectively. Mammalian proteins SKIP and RhoA form a protein complex with *Salmonella* effectors SifA and SseJ that promotes host membrane tubulation [212]. In addition, the SifA-SKIP complex plays an essential role in the formation and/or the anterograde movement of PipB2/kinesin-1-positive vesicles that are likely to derive from SCV [213].

The biochemical activity of SifA is not completely understood. SifA was identified as a member of the WxxxE family of bacterial T3SS effectors that stimulate small G protein signaling events. Members of this family were initially suggested to act in some cases by mimicking activated small GTPases [214], but more recent data suggest that they function as GEFs [215]. In fact, the structure of SifA in complex with the PH domain of SKIP revealed that SifA has two distinct domains: the N-terminus binds to SKIP, and the C-terminus has a fold similar to SopE, a *Salmonella* effector with GEF activity toward Rho GTPases [212]. In addition, SifA can bind to RhoA in human HeLa cells [212], and an interaction of SifA with a Rho GTPase has also been found using the model organism *Saccharomyces cerevisiae* [216]. However, the ability of SifA to catalyze nucleotide exchange on RhoA has not been demonstrated [217]. Interestingly, SKIP also interacts, through its PH domain, with the late endosome GTPase Rab9. This interaction is important to maintain peripheral LAMP1 distribution in cells and is inhibited by SifA, suggesting that bacterial G protein mimicry may result in G protein antagonism [218].

4.6. SifB. This effector was identified through the similarity of its N-terminal amino acid sequence with the translocation signal present in other effectors [142]. Similarly to SifA, SifB is a member of the WxxxE family [214], which after translocation through T3SS2 is targeted to the SCV and SIFs in association with LAMP1 [219].

4.7. SipA. *Salmonella* invasion protein A (SipA) is a protein encoded on SPI1 [220] that is delivered into the host cell through T3SS1 [21]. This effector has a role in invasion of

epithelial cells by modulating actin assembly through its C-terminal actin-binding domain, stabilizing F-actin filaments, and increasing the bundling activity of the host protein T-plastin [21, 221–223]. SipA contributes, together with other T3SS1 effectors, to the disruption of tight junctions, protein complexes intimately linked to the actin cytoskeleton that are located at the interface between epithelial cells [38]. The N-terminal 425 amino acids of SipA trigger PMN transepithelial migration *in vitro* [224–226]. This effect is mediated by the activation of ezrin, via a PKC- α -dependent pathway, that leads to the apical surface expression of multidrug resistance-associated protein 2 (MRP2), which in turn facilitates the apical release of the PMN chemoattractant HXA₃ [227]. SipA also contributes to intestinal inflammation *in vivo* [228, 229]. The proinflammatory effects of SipA require the activity of host pattern recognition receptors NOD1 and NOD2 that leads to NF- κ B activation in a process that does not require its actin-binding domain [230]. A role for SipA in promoting the phosphorylation of Jun and p38 MAPKs coupled to activation of IL-8 expression has also been suggested [231].

Interestingly, in addition to its role during invasion, SipA continues to act long after invasion [232, 233]. From the cytosolic face of the SCV, SipA, through its N-terminal domain, promotes intracellular replication and redistribution of late endosomes and cooperates with SifA in the modulation of SCV morphology and perinuclear positioning. These results emphasize the complex relationships that exist between effectors of different T3SS and provide evidence of secretion through T3SS1 from inside the SCV [232].

4.8. SipB, SipC, and SipD Translocases. SipB, SipC, and SipD [220, 234, 235] are a group of proteins known as translocases that are themselves secreted through T3SS1 and mediate the passage of effectors through the target host cell membrane [236]. SipD is present in the bacterial cell surface, probably localized at the tip of the needle complex, prior to the contact with the host cell. SipB and SipC become surface exposed upon contact of *S. enterica* serovar Typhimurium with epithelial cells [237]. Presumably, these translocases form a pore structure called the translocon through which the effectors are translocated and that mediates intimate association of *Salmonella* with mammalian cells [237].

Because SipD is present in the bacterial surface before invasion, it can act as a sensor for environmental molecules. In fact, SipD interacts with bile salts [238–240], which are present in the intestines and decrease *Salmonella* invasiveness [241].

SipB is involved in the induction of a form of macrophage cell death that is caspase-1 dependent [77] and is known as pyroptosis [72]. This translocase was shown to directly interact and activate caspase-1 [77], which in turn mediates activation of IL1- β in murine macrophages [77] and IL-18 in human dendritic cells and human alveolar macrophages [242, 243].

In addition to its general role in the translocation of T3SS1 effectors, SipC possesses an actin nucleation activity and cooperates with SipA in the production of F-actin foci at sites of bacteria-host cells contact [20, 83, 244]. Topological

analysis suggests that SipC is inserted in the host cell membranes with its N-terminal domain (amino acids 1–120) and its C-terminal domain (amino acids 200–409) in the host cytosol [244]. Residues 201–220 of SipC are necessary for its actin nucleation activity, whereas the C-terminal 88 amino acids are important for its translocase function [23]. Initial studies ascribed the F-actin bundling activity of SipC to its N-terminal domain [244], but a more recent report suggests that this activity is directed by residues 221–260 and 381–409 in the C-terminal region [24]. The C-terminal region is also involved in the interaction with cytokeratin 8 [245, 246]. Besides its role in invasion of epithelial cells, a recent work suggests that in macrophages SipC is localized in the surface of the phagosome, and through its C-terminal domain, it interacts with the SNARE motif of syntaxin 6 and thereby recruits LAMP1 from Golgi-derived vesicles [247]. Other translocases or T3SS1 effectors have no role in this process. These findings correlate with a previous report suggesting that SipC is involved in docking and fusion of exocytic vesicles by directly interacting with Exo70, a component of the exocyst complex [35].

4.9. SlrP. *Salmonella* leucine-rich repeat protein (SlrP) was identified by signature-tagged mutagenesis as *S. enterica* serovar Typhimurium host range factor [248]: an *slrP*-null mutant is as virulent as the wild-type in calves, but it is 6-fold attenuated for mouse virulence after oral infection. The predicted protein has a complete sequence of 765 amino acid residues that can be divided in three domains [249]. The N-terminal domain is necessary for secretion through both T3SS1 and T3SS2 and is similar to the N-termini of other effectors [142]. The central domain contains several copies of a leucine-rich repeat signature, a protein motif frequently involved in protein-protein interactions [250]. Finally, the C-terminal domain is conserved in the effectors SspH1, and SspH2, from *S. enterica*, and the IpaH family of *Shigella flexneri* effectors. IpaH9.8, SspH1, and SspH2 possess E3 ubiquitin ligase activities and define a new class of ubiquitin ligases [103, 251–253]. SlrP is also an E3 ubiquitin ligase that interacts with mammalian thioredoxin-1 (Trx) and, at least *in vitro*, can use this host protein as a substrate [69]. Transient transfections of epithelial cells showed that although the localization of SlrP is mainly cytosolic, a part of this protein is translocated into the endoplasmic reticulum where it can interact with the chaperone ERdj3 [68]. HeLa cells stably transfected with *slrP* are more prone to cell death, and the current working model suggests that SlrP promotes apoptosis in host cells by interfering with the functions of its targets Trx and ERdj3 [68, 69].

4.10. SopA. *Salmonella* outer protein A (SopA) is a T3SS1 effector that contributes to enteric disease [254]. HA-tagged SopA is targeted to the mitochondria of host cells [255]. A role of SopA in invasion of polarized T84 cells [256] and in escape to the cytosol [100] has been described. SopA is a HECT-like E3 ubiquitin ligase whose activity is involved in *Salmonella*-induced PMN transepithelial migration but not in invasion in cultured HeLa cells or in escape of

the SCV [102]. It preferentially uses E2s UbcH5a, UbcH5c, and UbcH7 for its ubiquitination reaction. Although the E2-interacting surface of SopA has little similarity to those of eukaryotic E3s, structural analysis reveals that SopA binds to human UbcH7 on the same region as eukaryotic HECT E3s [101, 257]. Thus, *Salmonella* SopA illustrates a functional mimicry of the mammalian HECT E3 ubiquitin ligase by a Gram-negative bacterial pathogen. Interestingly, it was previously shown that SopA interacts with the host E3 ubiquitin ligase HsRMA1 and is ubiquitinated and degraded by the HsRMA1-mediated ubiquitination pathway [100].

4.11. SopB. SopB (also known as SigD [258]) is a T3SS1 secreted *Salmonella* effector protein [259] that is encoded within SPI5 [184]. SopB is a phosphoinositide phosphatase [260] bearing, in its C-terminal region, motifs of mammalian inositol 4-phosphatases and a putative synaptojanin (inositol 5-phosphatase-) like domain [260, 261]. This activity mediates most of the multiple roles that this effector plays at different levels. (i) Invasion: SopB mediates actin cytoskeleton rearrangements to promote bacterial invasion [262] through activation of RhoG. It activates RhoG by indirectly stimulating SGEF (SH3-containing guanine nucleotide exchange factor) [27]. SopB also hydrolyzes $PI(4,5)P_2$ at the plasma membrane. Hydrolysis of this phospholipid promotes host membrane fission, a process that facilitates bacterial entry [45, 263]. (ii) Nuclear responses: by controlling phosphoinositide signaling during invasion, it can activate the prosurvival serine-threonine kinase Akt (protein kinase B) and protect epithelial cells from apoptosis [44, 264]. In addition, SopB can indirectly activate Rho-family GTPases (Cdc42 and RhoG) leading to activation of MAPK and NF- κ B signaling [124]. It has been suggested that, through its action on Cdc42 and Akt, SopB is responsible for a large fraction of host signaling and phosphorylation events that are induced by *Salmonella* infection [265]. (iii) SCV maturation: SopB recruits Rab5 to the SCV, which in turn recruits Vps34, a PI3-kinase, leading to PI3P accumulation on the SCV [47]. Rab5 is required for the formation of sorting nexin 3 (SNX3) tubules, and depletion of SNX3 partially inhibits Rab7 and LAMP1 recruitment to the SCV and decreases SIF formation. These results suggest that through the modulation of phosphoinositides by SopB at the membrane of the vacuole, *Salmonella* is able to modify the localization of the host protein SNX3 and induce a network of tubules to promote SCV maturation [48]. SopB, by reducing the levels of $PI(4,5)P_2$ and phosphatidylserine in the nascent SCV, reduces the negative membrane surface charge in this compartment. This affects SCV targeting of host cell proteins involved in membrane trafficking and inhibits SCV-lysosome fusion [46]. (iv) Diarrhea: the activity of SopB is required to induce fluid secretion in infected calf intestine loops [260]. This can occur by attenuation of signaling pathways that normally limit chloride secretion [266].

Interestingly, the N-terminal region of SopB may exert a function on the actin cytoskeleton independent of the catalytic domain [267]. Both, a catalytically inactive SopB and

a truncated SopB devoid of the phosphatase domain are able to complex with yeast Cdc42 and to inhibit Cdc42-dependent pathways when expressed in the eukaryotic cell model *S. cerevisiae* [153]. SopB also interacts with mammalian Cdc42 [268] through its N-terminal region independently of its catalytic activity and regardless of the activation state of the GTPase [269]. Structural and biochemical analyses have recently shown that SopB structurally and functionally mimics a host guanine nucleotide dissociation inhibitor (GDI) by contacting key residues in the regulatory switch regions of Cdc42 and slowing Cdc42 nucleotide exchange [156]. The interaction with Cdc42 is important for retention of SopB on the SCV and for bacterial growth [269]. Another independent factor involved in shifting the localization of SopB from the ruffling plasma membrane to endosomal compartments that will mature into SCV is ubiquitination on lysine residues that are present in the N-terminal region of SopB [99, 268, 270, 271].

4.12. SopD and SopD2. SopD and SopD2 are related proteins belonging, like SifA or SlrP, to the STE family, which shares a conserved N-terminal sequence that serves as a secretion signal for both T3SS1 and T3SS2 [142]. The similarity between SopD and SopD2 spans the entire length of both proteins and includes a putative coiled-coil domain in the C-terminal region [272]. SopD2 is a T3SS2 effector, and, although SopD was initially described as a T3SS1 effector [273], its pattern of expression suggests that it may also be translocated into host cells by the T3SS2 [272]. SopD contributes to promote the inflammatory responses and fluid secretion in *Salmonella*-infected intestines [273]. Together with SipA, SopA, SopB, and SopE2 is a major virulence factor responsible for diarrhea during *S. enterica* serovar Typhimurium infection of calves [229] that contributes to invasion of epithelial cells [256]. SopD acts cooperatively with SopB in the induction of membrane fission and macropinosome formation during *Salmonella* invasion [274]. It also contributes to virulence during systemic infection of mice and to optimal replication in macrophages, suggesting that SopD contributes not only to early but also to late stages of disease [275]. Both SopD and SopD2 bind with membranes but using different mechanisms. The binding of SopD is ATP-dependent, requires the full-length protein, has broad distribution on endosomes, and occurs at the invasion site of *Salmonella*. Conversely, SopD2 binding with membranes is ATP-independent, requires only the N-terminus of the protein, and occurs only in late endosomes [272, 274]. As mentioned previously, SifA is necessary for the stability of the SCV and for intracellular replication in macrophages and fibroblasts. Interestingly, deletion of *sopD2* in a *sifA* mutant stabilizes the SCV and restores intramacrophage replication and virulence. This deletion also induces the formation of tubules different from SIFs and SISTs that are called LNTs, indicating that SopD2 inhibits the formation of LNTs. SopD2 also acts as an inhibitor of vesicle transport from the vacuole [59]. These results suggest a role for SopD2 as an antagonist of SifA in terms of vacuolar membrane dynamics.

4.13. *SopE* and *SopE2*. *Salmonella* outer protein E (SopE) was initially characterized as a T3SS1 effector involved in invasion of epithelial cells [276]. This effector localizes to the host plasma membrane [277] and contributes to invasion by stimulating membrane ruffling acting as GEF on Rho GTPases Cdc42 and Rac1 [25, 278]. Both Rho GTPases initiate actin polymerization via nucleation promoting factors that activate the Arp2/3 complex. The best characterized nucleation promoting factors are N-WASP (neural Wiskott-Aldrich syndrome protein) and WAVE (WASP family verprolin homolog). Recruitment and activation of Cdc42 and Rac1 at the membrane by SopE lead to recruitment of N-WASP and the WAVE regulatory complex (WRC). SopE triggers activation of N-WASP and cooperates with Arf1 (recruited and activated by the host GEF ARNO) in the activation of WRC [279].

SopE activates several innate immune signaling pathways [124] and is of major importance for eliciting intestinal inflammation [229] through activation of caspase-1 in enterocytes [280]. Caspase-1 activation results in cleavage and secretion of IL-1 β and IL-18, which are important for the inflammatory response. The *sopE* gene is encoded within a prophage, SopE ϕ , which is not present in all strains of *Salmonella* [281, 282]. Based on the association of SopE ϕ with an epidemic *S. enterica* serovar Typhimurium clone, it was speculated that acquisition of the SopE protein by phage-mediated horizontal gene transfer might have increased the fitness of the resulting strain in the host population [283]. Recently, it was shown that SopE enhances the production of host-derived nitrate, an energetically highly valuable electron acceptor, in a mouse colitis model. Nitrate enhances the growth of *Salmonella* in the gut lumen through anaerobic nitrate respiration [284].

In contrast to *sopE*, *sopE2* appears to be broadly distributed in *Salmonella*. This gene encodes a protein, SopE2, that is 69% identical to SopE and also acts as an efficient GEF for Cdc42 [26, 285] but in contrast with SopE, not for Rac1, suggesting that these two similar effectors can activate different sets of Rho GTPases signaling cascades [286]. Like SopE, SopE2 has been implicated in the pathogenesis of diarrhea and enteritis associated with *Salmonella* infection in calves [229] and in streptomycin-pretreated mice [228]. Consistent with a role in diarrhea and inflammation are the reports indicating that SopE2 is involved in upregulation of macrophage iNOS independently of effects on invasion [287]: that this effector cooperates with flagellin in triggering increased epithelial IL-8 production [288], and that SopE2, together with SopE, SipA, and SopB disrupt tight junction structure and function [38].

4.14. *SpiC*. SpiC (also known as SsaB [289]) is a SPI2-encoded protein with effector and translocator functions. Initially, it was shown to be exported into host cells through T3SS2 and was proposed to interfere with intracellular trafficking to inhibit fusion of SCV with lysosomes [290]. Two host targets have been proposed for SpiC: TassC [291] and Hook3 [292]. TassC is also known as Nipsnap4 or Nps4. It was reported to be a protein that associates with membranes

and partly localizes in lipid rafts [293], although more recently it was shown to be a mitochondrial protein [294]. Hook3 is a member of the microtubule binding Hook family of coiled-coil proteins that forms part of a protein complex that has been involved in vesicle trafficking and/or fusion [295] and in the traffic of pericentriolar satellites [296] and could be a mediator of the proposed role of SpiC as a T3SS2 effector. In addition to its role as an effector, SpiC was found to be necessary for the secretion of SseB, SseC, and SseD *in vitro* [297, 298]. These three proteins are components of the T3SS2 translocon. SpiC interacts with SsaM [299], and both proteins are part of the SsaM/SpiC/SsaL regulatory complex that functions within the bacterial cell to mediate the switch from translocon protein secretion to effector translocation [300]. The complex is dissociated and degraded in response to cytoplasmic pH sensed through the translocon pore, triggering effector delivery [300].

4.15. *SptP*. The T3SS1 effector *Salmonella* protein tyrosine phosphatase (SptP) [301, 302] is a modular protein consisting of two discrete domains. The N-terminal domain functionally mimics a GAP for Cdc42 and Rac1, mediating the reversion of the changes in the actin cytoskeleton that were triggered by effectors like SopE and SopE2 that act as GEF on the same Rho GTPases [28]. The C-terminal half of SptP shares sequence similarity with the prokaryotic tyrosine phosphatase YopH from *Yersinia*, as well as with eukaryotic tyrosine phosphatases, and possesses potent tyrosine phosphatase activity [301]. This activity is involved in reversing the activation of the MAPK ERK that results from *Salmonella* infection [31]. SptP inhibits activation of ERK by interfering with activation of Raf1 in a process that involves both SptP activities [303]. Probably as a consequence of downregulation of ERK, SptP contributes, together with SspH1, to downmodulate IL-8 production after invasion of intestinal epithelial cells [304]. The tyrosine-phosphorylated form of the filament protein vimentin was proposed as a potential binding partner and substrate for SptP, but the significance of this interaction remains unclear. More recently, valosin-containing protein (VCP), a host AAA+ ATPase that performs motor-like function to facilitate protein complex disassembly in diverse cellular processes [305], has been identified as an interacting partner of SptP and a substrate for its phosphatase activity, both *in vitro* and during infection of HeLa cells [53]. Dephosphorylation of VCP leads to an increase in binding of this protein to syntaxin 5, promoting fusion events within the cells that are likely important for the maturation and maintenance of the SCV. In summary, SptP has a role in downregulating membrane ruffling within 1 h of pathogen internalization, but it persists for at least 8 h to be also involved in later events related to SCV maintenance and intracellular bacterial proliferation [53].

4.16. *SpvB* and *SpvC*. Several *Salmonella* serovars belonging to subspecies I harbor plasmids essential for virulence [306]. Only a particular region of the virulence plasmid, *Salmonella*

plasmid virulence (*spv*), is necessary for the virulent phenotype, and this locus is found in the chromosome in certain *S. enterica* lineages [307]. This region contains the operon *spvABCD* (*spvABC* in the chromosomal locus), which is positively regulated by the product of *spvR*. Genes *spvR*, *spvB*, and *spvC* are required for virulence [91].

SpvB has two distinct domains separated by seven proline residues. The C-terminal domain possesses ADP-ribosyltransferase activity that covalently modifies G-actin monomers at arginine-177 and prevents their polymerization into F-actin filaments [308–311]. SpvB inhibits the formation of vacuole-associated actin polymerizations (VAPs) in HeLa cells [312] and negatively regulates the formation of SIFs [313]. The ADP-ribosylation activity of SpvB is also required for a delayed form of cell death that is observed in host cells 18–24 h after infection with noninvasive bacteria or T3SS1 mutants [67, 85, 314, 315]. One report showed that SpvB could be secreted into a culture medium mimicking the intracellular iron concentrations of eukaryotic cells in a T3SS-independent manner [316]. However, several components of the T3SS2, have been shown to be essential for SpvB-induced actin depolymerization and delayed cytotoxicity, strongly supporting the idea that SpvB is translocated into the host cell through T3SS2 [314, 317].

SpvC can be secreted to the culture medium by either T3SS1 or T3SS2, but its translocation into the cytosol of macrophages was shown to be dependent on T3SS2 [318]. Recently, it was demonstrated that SpvC is translocated into the host cell cytosol through T3SS1 when bacteria are grown under SPI1-inducing conditions [319]. This effector has phosphothreoninylase activity [318, 320]. This enzyme uses a novel catalytic mechanism, called eliminolysis [321], that irreversibly removes the phosphate group from a phosphorylated threonine via β -elimination [322–325]. SpvC specifically removes phosphate from threonine in the conserved activation loop motif TXY of ERK and inactivates this MAPK, and it is also active towards p38 and JNK *in vitro* [318, 320]. Inactivation of MAPK is the proposed reason for the effect of SpvC in modulating host immune response by reducing inflammatory cytokines during the early stages of infection [319].

4.17. *SrfJ*. The gene *SsrB* regulated factor J (*SrfJ*) was identified in a screen whose aim was finding genes regulated by *SsrB*, the main positive regulator of SPI2, located outside this island [141]. Because of its pattern of expression, *SrfJ* was immediately suggested as a putative T3SS2 effector. However, secretion of *SrfJ* through this system was shown only very recently [326]. Interestingly, *SrfJ* is similar to human lysosomal glucosylceramidase, an enzyme involved in regulating metabolism of the sphingolipid ceramide [327]. Intriguingly, in addition to being positively regulated by *SsrB*, *SrfJ* is negatively regulated by *IolR*, the regulator of genes involved in *myo*inositol utilization in *Salmonella* [326, 328].

4.18. *SseB*, *SseC*, and *SseD* Translocases. Genes *sseB*, *sseC*, and *sseD* are located in SPI2 and their products display weak similarity to EspA, EspD, and EspB, which are secreted

by the T3SS of the locus of enterocyte effacement of enteropathogenic *E. coli* [289]. *SseB*, *SseC*, and *SseD* are secreted to the bacterial surface through T3SS2 after exposition of bacteria to acidic pH and function as the translocon for T3SS2 effector proteins [329–331].

4.19. *SseF* and *SseG*. The genes encoding these effectors, *sseF* and *sseG*, are located in SPI2 [289]. *SseF* and *SseG* are secreted *in vitro* [200] and translocated into the host cell through T3SS2 [332]. Both effectors associate with SCV membranes and are necessary for the formation of SIFs [332–334]. *SseF* and *SseG* colocalize with microtubules in infected HeLa cells and are responsible for the induction of massive bundling of microtubules at late time points after infection. These bundles serve as a scaffold for SIF formation [335]. In addition to its role in SIF formation, *SseG* and *SseF* physically interact with each other and have a role in the targeting of the SCV to the vicinity of the Golgi network in infected epithelial cells [60, 336, 337]. *SseG* is targeted specifically to the *trans*-Golgi network through a predicted transmembrane domain located in the middle of the protein. An intact Golgi network is required for intracellular replication of *Salmonella*. These results suggest that, besides the selective interactions with the endocytic pathway that are necessary for the maturation of the SCV, interactions with the secretory pathway are also required for intracellular replication [337]. In fact, *Salmonella* can induce a general redistribution of compartments involved in exocytic transport in order to redirect exocytic cargo vesicles to the SCV, and this process is dependent on *SifA*, *SseF*, and *SseG* [338]. Two models have been suggested to explain the role of the *SseF*-*SseG* complex and *SifA* in the spatial distribution of the SCV [339]. The first model is based on the balance between the activity of the microtubule motors kinesin and dynein: as mentioned previously, *SifA* controls the positioning of the SCV by modulating kinesin activity through SKIP; *SseF*-*SseG* would control positioning of bacterial vacuoles by modulating activity of dynein on the SCV [336, 340]. The second model suggests that tethering between *SseF*-*SseG* and Golgi-related molecules determines SCV positioning [42, 337], although no Golgi protein has been found to interact with *SseF* or *SseG*. It is also possible that both kinds of mechanisms, motor balance and tethering, contribute to SCV positioning [339]. Intriguingly, TIP60, an acetyltransferase that catalyzes histone acetylation, has been described as a host interacting partner of *SseF* using the yeast two-hybrid system [341]. TIP60 was upregulated upon *Salmonella* infection, its acetylation activity was increased in the presence of *SseF*, and, interestingly, experimental downregulation of TIP60 in macrophages led to decreased intracellular proliferation of *Salmonella*. However, the interaction has not been confirmed by other methods, and since TIP60 is not directly involved in vesicular trafficking, the functional significance of this interaction is not understood.

4.20. *SseI*/*SrfH*. *Salmonella* secreted effector I (*SseI*), also known as *SsrB* regulated factor H (*SrfH*) was identified as

a member of the STE family on the basis of similarity to the SspH1/SspH2/SlrP N-termini [142] and as a gene located outside SPI2 that was regulated by SsrB, the main positive regulator of SPI2 and T3SS2 [141]. The gene *sseI* lies within the Gifsy-2 prophage. This gene is expressed under SPI2-inducing conditions, and its product is translocated into macrophages in a T3SS2-dependent manner [142]. SseI has been shown to bind to the mammalian actin cross-linking protein filamin and to colocalize with the polymerizing actin cytoskeleton [312]. SseI could play at least two different roles. (i) It has been suggested that, through interaction with TRIP6, a member of the zyxin family of adaptor proteins that regulate motility and adherence, SseI can stimulate migration [342] or regulate cell adherence [343] of phagocytes in order to cause early escape of *S. enterica* out of the gastrointestinal tract and into the blood stream. (ii) SseI blocks migration of macrophages and dendritic cells by a mechanism that involves interaction of SseI with the host factor IQGAP1, a regulator of the cytoskeleton and cell migration [343]. This second role could be critical during later stages of infection and could explain the attenuation of the *sseI* mutant in long-term, but not short-term, systemic infections [207, 343]. The discrepancy between these two roles (stimulation or inhibition of cell migration) has been recently resolved: a naturally occurring allele of *sseI/srfH* promotes the migration of infected phagocytes into the bloodstream, while another naturally occurring allele that differs by only a single nucleotide polymorphism does not [344]. Upon infection of polarized epithelial cells, SseI is targeted to the plasma membrane in a manner dependent on S-palmitoylation of a conserved cysteine residue within its N-terminal domain. This posttranslational modification is necessary for its function in modulating host cell migration [345].

4.21. SseJ. SseJ possesses the N-terminal conserved domain typical of the STE family. It is expressed under SPI2-inducing conditions and translocated through T3SS2 [142]. The C-terminal domain has homology to members of the GDSL motif lipase family, and, in fact, SseJ exhibits phospholipase A1, deacylase, and glycerophospholipid:cholesterol acyltransferase activity. This activity results in the esterification of cholesterol [346–348]. Cholesterol is important in endocytic trafficking events and accumulates in the SCV. It has been suggested that, through cholesterol modification, SseJ is controlling the dynamics of the SCV in cooperation with SifA. SseJ is recruited to the cytosolic face of the SCV where it binds specifically to the GTP-bound form of the RhoA GTPase, and this interaction activates the lipase activity of this effector [349]. SseJ was also able to bind to GTP-bound RhoC (and maybe also to RhoB [350]), but not to other GTPases like Cdc42, H-Ras, or Rac1, and only RhoA acted as a potent activator of the lipase activity of SseJ [349]. Recent structural studies demonstrated that SseJ binds to RhoA through a large surface that includes residues of the switch region II. This is one of the regions in RhoA that undergoes conformational changes upon binding to either GTP or GDP. This region is involved in the interaction of

RhoA-GTP with eukaryotic proteins, like protein kinase N 1 (PKN1), Rock and Rhotekin, which mediate the regulatory effect of the GTPase in several cellular processes. Thus, SseJ competes with eukaryotic effectors for binding to the same surface on RhoA [350].

4.22. SseK1, SseK2, and SseK3. SseK1 and SseK2 were identified because of their similarity to NleB, a known type III secreted protein from *Citrobacter rodentium* [351]. Another effector of the same family, SseK3, is encoded on the ST64B prophage, which is present in a subset of strains and serovars of *S. enterica* [352]. Translocation of these effectors through T3SS2 was demonstrated using the CyaA method, and they have a minor role in virulence, since an *sseK1 sseK2* double mutant and an *sseK1 sseK2 sseK3* triple mutant are slightly attenuated for virulence in mice [351, 352]. Their specific functions in the host cells have not been elucidated yet.

4.23. SseL. *sseL* was described as a member of the SsrB regulon whose product is translocated through T3SS2 [88, 353] onto the vacuolar surface in infected host cells. SseL targets the host cell ubiquitin pathway acting as a deubiquitinase that is required for delayed cytotoxicity of macrophages and full virulence in the mouse model [88]. One of the targets of the deubiquitinase activity of SseL is I κ B α [105]. Suppression of I κ B α ubiquitination and degradation by SseL suppresses NF- κ B activation. This effect was detected by expressing SseL in HEK293T cells by transfection and also in RAW264.7 cells and primary cultured mouse macrophages by infection with wild-type and *sseL* mutant strains [105]. However, it was not detected in a previous report using J774 murine macrophages [88]. SseL binds to oxysterol-binding protein (OSBP) [154] via a region within OSBP that contains a predicted coiled-coil and an FFAT motif [354]. OSBP is a lipid-binding protein that has been implicated in the regulation of various cellular processes, including nonvesicular cholesterol transport, signaling, lipid metabolism, and vesicular trafficking. Although OSBP is not a substrate for the activity of SseL, this interaction could be connected to a proposed role of SseL in interfering with lipid metabolism: SseL prevents the accumulation of lipid droplets during infection of gallbladder epithelial cells. This phenotype depends on the deubiquitinase activity of this effector, suggesting a mechanism by which *Salmonella* can intercept host lipid homeostasis by the direct modification of cellular ubiquitination patterns [355]. In addition, SseL counteracts a cellular response to *Salmonella* infection that leads to the formation of T3SS2-dependent ubiquitinated aggregates and aggregates-like induced structures. Accumulation of these aggregates is at least in part due to the combined actions of SifA and SseJ. SseL deubiquitinates these aggregates and prevents recruitment of the autophagy markers p62 and LC3, leading to a reduction in autophagic flux during infection [115]. Since lipid droplet metabolism has been associated with autophagy [356], a relationship between the interference with lipid droplet metabolism and inhibition of selective autophagy by SseL could exist.

4.24. SspH1 and SspH2. SspH1 is encoded by the prophage Gifsy-3 [181], which is present in only a few *S. enterica* serovar Typhimurium strains. SspH2 is encoded in the vicinity of a phage remnant, suggesting that it was acquired also by lysogenic conversion [283], and is conserved among most *Salmonella* serovars [249]. SspH1 is translocated through T3SS1 and T3SS2, whereas SspH2 is specifically translocated via T3SS2 [249], but both share a similar N-terminal translocation signal that is also present in several other effectors [142]. These effectors also share with other effectors like SlrP a central region containing leucine-rich repeats [249]. These motifs are present in many proteins and can participate in protein-protein interactions [357]. In fact, the leucine-rich repeat domain of SspH1 has been shown to mediate interaction with a mammalian serine/threonine protein kinase called PKN1 [104], an interaction that could explain the nuclear localization of SspH1 and its role in the inhibition of NF- κ B-dependent gene expression [304]. In contrast, SspH2 colocalizes with VAPs, and two host interacting partners identified for SspH2, filamin and profilin, related to the actin cytoskeleton, interact through the C-terminal and the N-terminal domains of the effector, respectively [312]. The N-terminal domain of SspH2 is also important for targeting of the effector to the host plasma membrane, a process that requires S-palmitoylation [345]. Biochemically, SspH1 and SspH2 belong to a family of E3 ubiquitin ligases whose activity resides in their C-terminal domain [103, 251, 358]. Other members of this family are SlrP from *Salmonella* and IpaH9.8 from *Shigella*. SspH1 can ubiquitinate its host partner PKN1 *in vitro*, although the functional significance is unknown [251]. No putative substrates have been identified for SspH2.

4.25. SteA, SteB, and SteC. These proteins were identified in a screen, based on the generation of CyaA fusions, to find genes encoding effectors in *S. enterica* serovar Typhimurium [143]. SteA can be translocated into epithelial cells and macrophages through T3SS1 and T3SS2 [143, 359] and localizes to the *trans*-Golgi network in both transfected and infected human epithelial HeLa cells and to *Salmonella*-induced membrane tubules containing *trans*-Golgi markers [143, 360]. No functional studies have been published about SteB. SteC is a kinase whose activity is necessary for actin meshwork formation in the vicinity of SCV. Overexpression of SteC in host cells causes extensive alterations to the actin cytoskeleton [361], also in a yeast model [152]. In the yeast model, SteC inhibits signaling at the level of the GTPase Cdc42 through binding to the exchange factor Cdc24. Interestingly, SteC is also able to bind to human Vav1, a member of the family of Rho GEFs involved in key biological functions, including actin cytoskeleton reorganization, activation of ERK and JNK, and development and activation of immune cells [362].

4.26. Other Effectors. Six new effectors were identified in a proteomic study of the secretome of an *ssaL* mutant strain [145]: CigR, GtgA, GtgE, SpvD, SteD, and SteE. All of them are translocated through T3SS2, and GtgE, SpvD, and SteE

are also substrates of T3SS1, but, except for GtgE, very little is known about the functions of these effectors.

CigR is encoded in the SPI3 island [363] and induced 4 h after infection of macrophages [194]. GtgA and GtgE are Gifsy-2 prophage-encoded proteins [181, 364]. GtgE is a protease that cleaves Rab29. This is a Rab GTPase that is recruited to the *S. enterica* serovar Typhi-containing vacuole and is necessary for the export of typhoid toxin, which is exclusively encoded by the human-specific *S. enterica* serovars Typhi and Paratyphi. Interestingly, these serovars do not encode GtgE, since they are not Gifsy-2 lysogens. In contrast, *S. enterica* serovar Typhimurium and other broad-range serovars produce GtgE, and this effector, secreted through T3SS1, prevents the recruitment of Rab29 to the SCV [365]. SpvD is encoded within the *Salmonella* virulence plasmid and was previously predicted to be a T3SS effector using a computational approach [366]. Whereas *steD* is located outside a pathogenicity island, and *steE* is in the Gifsy-3 prophage [181], which is present specifically in strain 14028 but not strains LT2 or SL1344 from *S. enterica* serovar Typhimurium. Both genes significantly contribute to virulence in mice [145].

Abbreviations

CU:	Chaperone-usher
GAP:	GTPase-activating protein
GDI:	Guanine nucleotide dissociation inhibitor
GEF:	Guanine nucleotide exchange factor
IL:	Interleukin
iNOS:	Inducible nitric oxide synthase
LNT:	LAMP1-negative tubule
MAPK:	Mitogen-activated protein kinase
MKK:	MAPK kinase
NF- κ B:	Nuclear factor κ B
PI:	Phosphatidylinositol
PMN:	Polymorphonuclear leukocytes
SCV:	<i>Salmonella</i> -containing vacuole
SIF:	<i>Salmonella</i> -induced filament
SIST:	<i>Salmonella</i> -induced secretory carrier membrane protein 3 (SCAMP3) tubule
SPI:	<i>Salmonella</i> pathogenicity island
STE:	<i>Salmonella</i> translocated effector
SVAT:	Spacious vacuole-associated tubule
T1SS:	Type I secretion system
T2SS:	Type II secretion system
T3SS:	Type III secretion system
T4SS:	Type IV secretion system
T5SS:	Type V secretion system
T6SS:	Type VI secretion system
VAP:	Vacuole-associated actin polymerization
WRC:	WAVE regulatory complex.

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