

The functional interface between *Salmonella* and its host cell: opportunities for therapeutic intervention

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***Salmonella* is a facultative intracellular pathogen that causes diseases ranging from self-limiting enteritis to typhoid fever. This bacterium uses two type III secretion systems to deliver effector proteins directly into the host cell to promote infection and disease. Recent characterization of these virulence proteins and their host-cell targets is uncovering the molecular mechanisms of *Salmonella* pathogenesis and is revealing a picture of the atomic interface between this pathogen and its host. This level of analysis provides the possibility of designing novel therapeutics to disrupt infection and disease processes at the molecular level.**

***Salmonella* pathogenicity and type III secretion**

Salmonella enterica, the cause of food poisoning and typhoid fever, has evolved a complex functional interface with its host [1]. This interface, which is the product of evolutionary forces operating over a long period of time, is characterized by its fine-tuning rather than by its potential to inflict harm on the host. From the side of the pathogen, a central theme that drives the organization of this interface is one of structural and functional mimicry of host-cell proteins [2]. In contrast to the less-refined approach used by bacterial products that introduce covalent, non-reversible modifications to their cellular targets [3], the protein ‘mimics’ of *Salmonella* enable the precise and reversible manipulation of cellular functions, thus minimizing the disruption of host-cell homeostasis. Using a specialized protein injection device, known as the type III secretion system (TTSS), *Salmonella* delivers into the host cell a battery of effector proteins that are able to mimic the function of a variety of host-cell proteins [4]. Some of these effector proteins, although structurally highly divergent from their mammalian counterparts, have convergently evolved to carry out functions that are virtually identical to those of the mammalian proteins. However, mimicry is also accomplished by proteins that share extensive primary amino acid sequence similarity with their host counterparts and therefore might have been horizontally acquired during evolution.

After oral ingestion, most often through the consumption of tainted food or water, *Salmonella* reaches the intestinal tract, comes into contact with the intestinal epithelium and stimulates its own uptake into epithelial cells. After internalization, *Salmonella* actively modulates host vesicular trafficking pathways to avoid delivery to lysosomes and to establish a specialized replicative niche (Figure 1). These events are mediated by the bacterially encoded effector proteins that are sequentially delivered by two TTSSs encoded in different regions of the *Salmonella* chromosome: *Salmonella* pathogenicity island 1 (SPI-1) and 2 (SPI-2) [4,5]. However, it should be noted that, although central to pathogenicity, these steps constitute just a subset of the events that lead to bacterial pathogenesis. During the past few years, the interaction of *Salmonella* with host cells has been the subject of intense research. As a result, details of the functional, and even the atomic, interface between *Salmonella* and its host cells are beginning to emerge. In this article, we will discuss the events that lead to the uptake of *Salmonella*, the survival and replication of *Salmonella* within mammalian cells, and the possibilities for the development of new therapeutic strategies.

Manipulation of the actin cytoskeleton to enter intestinal epithelial cells

The ability of *Salmonella* spp. to enter host cells is central to establishing a successful infection. In vertebrates, *Salmonella* spp. use a variety of mechanisms to breach the intestinal wall and reach a sheltered niche that is permissive for replication. The mechanisms of cell entry are characterized by the profuse rearrangement of the actin cytoskeleton at the site of bacteria–host-cell contact (Figure 1a) [6–8]. This localized remodeling of the host cytoskeleton drives membrane ruffling and lamellopodial extensions that envelop external bacteria, leading to the entry of the bacteria into membrane-bound vacuoles. Entry into non-phagocytic cells is absolutely dependent on the delivery of specific effector proteins directly into the host cell via the SPI-1-encoded TTSS. These virulence factors function in concert to engage host-cell proteins and orchestrate a complex series of events that culminate in entry of the bacteria.

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Targeting the RhoGTPase switch

To date, five distinct *Salmonella* effectors, each capable of manipulating the cytoskeletal machinery within the host, are known to drive engulfment. One key cellular target is the Rho family of small GTPases, which are central regulators of eukaryotic cytoskeletal dynamics [9]. RhoGTPases function as molecular switches, alternating between active GTP-bound and inactive GDP-bound conformations. This binary exchange of nucleotides induces defined structural changes, primarily at two regions within the GTPase: switch I and switch II [10]. These regions comprise specific invariant amino acids that, in the presence of Mg^{2+} , form a high-affinity binding pocket to stabilize the bound guanine nucleotide. For example, in the activated GTP-bound state, conserved threonine and glycine residues within switch I and II, respectively, are spatially oriented to form hydrogen bonds with the γ -phosphate oxygens. *In vivo*, the GTPase cycle is regulated by a vast array of accessory proteins, including guanine nucleotide exchange factors (RhoGEFs), which catalyze activation, GTPase-activating proteins (RhoGAPs), which accelerate GTP hydrolysis leading to inactivation, and guanine nucleotide dissociation inhibitors (GDIs), which control GTPase cycling between membranes and the cytosol [9]. These accessory proteins engage their cognate GTPase, in particular targeting the switch I and II regions, and insert residues intended to destabilize the bound nucleotide and induce cycling.

Salmonella has evolved a sophisticated strategy to reversibly modulate the RhoGTPase switch. It encodes two closely related type III effector proteins, SopE and SopE2, which, following translocation, directly engage and activate two Rho family members, Cdc42 and Rac [11,12]. Remarkably, SopE and eukaryotic RhoGEFs appear to use a near identical catalytic mechanism, despite lacking any sequence or structural homology [13,14]. Indeed, SopE locks the switch I and II regions of Cdc42 in a conformation that is analogous to that seen with eukaryotic RhoGEFs and their substrates. However, the catalytic core of SopE displays significant differences from eukaryotic RhoGEFs. SopE contains a unique $^{166}GAGA^{169}$ motif that inserts between the switch regions of Cdc42 creating an environment that is incompatible with high-affinity nucleotide binding. In particular, the GAGA loop instigates a 'push-pull mechanism' whereby switch I is pushed aside, removing residues that are important for nucleotide and phosphate binding, whereas switch II is pulled towards the nucleotide binding site such that it sterically interferes with the stabilizing Mg^{2+} ion [10]. Eukaryotic RhoGEFs use an array of amino acids, typically with larger side-chains than the GAGA loop, to similarly destabilize the nucleotide-binding pocket. The net result is a dramatic reduction in the affinity of the GTPase for guanine nucleotides, creating a transient binary complex of GEF and nucleotide-free GTPase. The high intracellular ratio of GTP:GDP coupled with the solvent-accessible nucleotide-binding site ensures that the 'empty' GTPase is rapidly loaded with GTP. Once activated the switch I and II regions of the GTPase regain a structure that is incompatible with GEF binding, thereby concluding the exchange reaction. The capacity

of SopE to mimic eukaryotic RhoGEFs represents a remarkable example of convergent evolution and ensures that the host has little chance of developing resistance to this virulence factor.

A second *Salmonella* type III effector, termed SopB, also targets the RhoGTPase switch. This effector has phosphoinositide phosphatase activity, which was implicated originally in late events post-entry into the cell, such as fluid secretion and inflammation [15,16]. However, subsequent studies revealed functional redundancy with SopE and SopE2 in driving GTPase-mediated actin rearrangements [17]. Translocation of SopB into cells alters cellular phosphoinositide phosphate (PIP) and inositol phosphate (IP) metabolism [18–20]. In particular, SopB catalyses dephosphorylation of $PtdIns(3,5)P_2$, $PtdIns(4,5)P_2$ and $PtdIns(3,4,5)P_3$, primarily at positions 4 and 5 of the inositol ring. Indeed, entry of *Salmonella* coincides with SopB-mediated elimination of $PtdIns(4,5)P_2$ at the base of ruffles and subsequent enhancement of vesicle fission [21]. In addition, SopB consumes the cellular $Ins(1,3,4,5,6)P_5$ reservoir to generate $Ins(1,4,5,6)P_4$. These changes are propagated, presumably via an endogenous eukaryotic RhoGEF, to Cdc42 and/or Rac and subsequently localize actin remodeling. This indirect GTPase activation contrasts with the direct GTPase stimulation mediated by SopE and SopE2. However, despite their differing biochemical activities, SopE, SopE2 and SopB have key redundant roles during internalization. Indeed, *Salmonella* strains that lack just one of these effectors display only a modest reduction in entry, whereas a triple $\Delta sopE/E2/B$ mutant is completely abrogated for cytoskeletal remodeling and entry [17].

Hijacking the cytoskeletal machinery: mechanisms of actin nucleation

By locally activating Cdc42 and Rac, *Salmonella* can exploit the downstream cytoskeletal machinery of the host to drive actin nucleation and promote its engulfment. In fact, recent studies advocate a role for the heptameric Arp2/3 complex, a ubiquitous eukaryotic actin organizer that can initiate actin nucleation, branching and cross-linking [22]. Moreover, two proteins, WASp and Scar, which can bridge Cdc42 and Rac activation to the regulation of Arp2/3, have also been implicated in *Salmonella* entry [23]. However, alternative mechanisms of actin assembly are also likely to contribute because blocking Arp2/3, WASp or Scar function, by expression of dominant-interfering mutants, elicits only a moderate decrease in *Salmonella* invasion [23]. Indeed, *Salmonella* encodes two type III effector proteins, SipA and SipC, which can engage actin directly [7]. SipA has several biological activities, including a capacity to nucleate filamentous actin (F-actin) and promote F-actin bundling [24–26]. Moreover, SipA can stabilize F-actin by directly antagonizing the action of depolymerizing factors within the cell, such as ADF/cofilin and gelsolin [27,28]. The second actin binding protein from *Salmonella*, SipC, comprises *de novo* actin polymerization and F-actin bundling activity, in addition to its distinct role in mediating the translocation of effector proteins [29,30]. Together, SipA and perhaps SipC are believed to promote

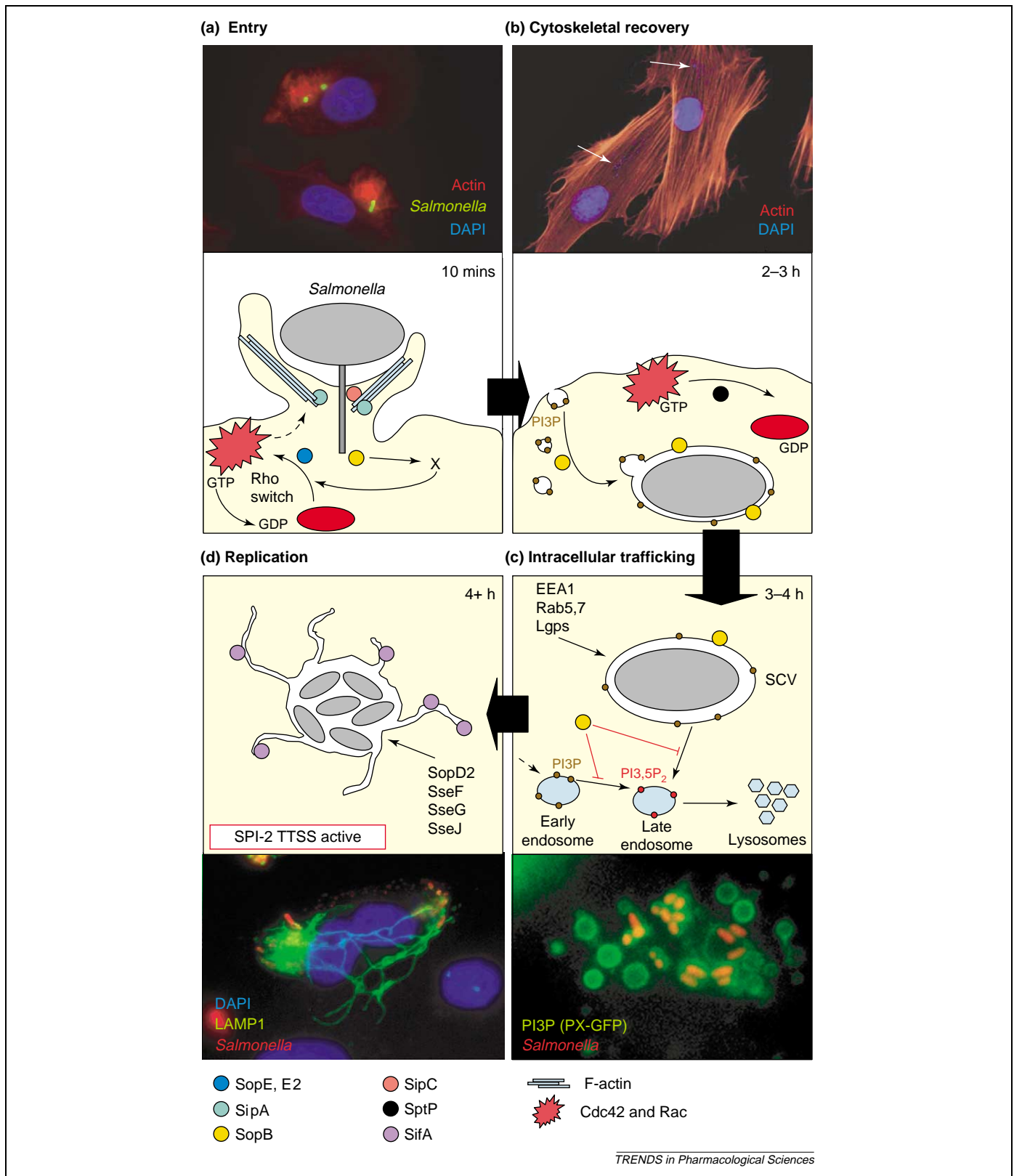


Figure 1. Interaction of *Salmonella* with epithelial cells. **(a)** On contact with the host cell, *Salmonella* delivers multiple effector proteins to manipulate the cytoskeletal machinery of the host and trigger entry. SopE, SopE2 and SopB target the RhoGTPase switch and promote activation of the Rho family members Cdc42 and Rac, leading to actin remodeling. SopE and SopE2 function as GEFs and directly catalyze GTPase activation, whereas SopB functions indirectly, presumably via a host-encoded GEF (X). SipA and SipC engage actin directly to induce actin nucleation and polymerization. The immunofluorescence image shows actin remodeling induced by *Salmonella typhimurium* infection of Henle-407 cells. **(b)** The GTPase-activating protein (GAP) activity of SptP inactivates the RhoGTPase switch, leading to cytoskeletal recovery. Internalized *Salmonella* occupies a spacious vacuole generated by the fusion of SopB-induced vesicles. The immunofluorescence image shows recovery of cytoskeletal homeostasis following invasion of *S. typhimurium* (arrows) into Ref52 cells. **(c)** Following internalization, the *Salmonella*-containing vacuole (SCV) selectively interacts with the host endocytic machinery. The SCV initially acquires the early endosome markers EEA1 and Rab5, which are sequentially replaced by the late endosome and lysosome markers Rab7 and lysosomal glycoproteins (Lggs). However, the SCV does not fuse directly with either the endosomes or the lysosomes, in part, because of the phosphoinositide phosphatase activity of SopB. The immunofluorescence image shows intracellular *Salmonella* residing within phosphatidylinositol 3-phosphate

efficient bacterial uptake by ensuring the spatial localization of actin foci beneath invading *Salmonella*.

Closing the door on entry: the role of SptP

The actin remodeling events initiated by *Salmonella* are transient and typically reversed 2–3 h post-entry (Figure 1b). Remarkably, *Salmonella* actively helps the host cell to regain its normal cellular architecture through the action of SptP [31]. This type III effector protein comprises two functionally independent domains, both designed to minimize excessive cellular damage. The C-terminus encodes a tyrosine phosphatase. Although its cellular substrate(s) remains unclear, this activity is necessary for downregulating the pro-inflammatory nuclear responses that accompany invasion [32].

The N-terminus of SptP folds to mimic a eukaryotic RhoGAP, thus extending the repertoire of *Salmonella* effectors that target the RhoGTPase switch [33]. Similar to other RhoGAPs, SptP accelerates the intrinsic GTP hydrolysis activity of GTPases [34]. Upon binding Rac, it stabilizes the switch I and II regions and initiates hydrolysis by positioning a nucleophilic water molecule, via the conserved Gln61 residue, in the correct geometry to attack the γ -phosphate of GTP. Unlike SopE, the catalytic core of SptP is identical to its eukaryotic counterparts and comprises an invariant arginine residue (Arg209), termed the 'arginine finger', which protrudes into the active site of the GTPase and neutralizes the negative charge that develops at the β -phosphate during the transition state [33]. In an interesting deviation from eukaryotic GAPs, the arginine finger of SptP is contained within a well-organized α -helix and not in a conventional loop structure.

The GAP activity of SptP specifically inactivates Cdc42 and Rac and hence directly counters the cytoskeletal remodeling functions of SopE, SopE2 and SopB. Given their antagonistic nature, the correct temporal regulation of these effectors is essential for efficient entry of *Salmonella*. Recent studies addressed the signaling pathways involved and revealed a regulatory mechanism based on differing protein half-lives following translocation [35]. In contrast to SptP, SopE was found to undergo rapid proteasome-mediated degradation in the host cell, ensuring only a temporary activation of the RhoGTPase switch.

Hence, the entry process concludes with cytoskeletal homeostasis for the host cell and intracellular residence for *Salmonella* within a membrane-bound compartment.

Modulation of vesicular trafficking to prevent delivery into lysosomes

The building of the replicative niche of *Salmonella* starts during the internalization event itself. Through the activity of the SPI-1 TTSS effector SopB, *Salmonella* induces the formation of abundant large macropinosytic

vesicles, most of which do not contain bacteria (Figure 1b) [19]. These vesicles are highly fusogenic because of the rapid acquisition of PtdIns(3)P [19], which results in the recruitment of components of the vesicular fusion machinery such as Rab5 and EEA1 [36]. In fact, PtdIns(3)P is already present in the nascent phagosomes because of its localized generation at the plasma membrane through the SopB-mediated degradation of PtdIns(3,4,5)P₃ [17]. The homotypic fusion of the *Salmonella*-generated vesicles leads to the formation of large *Salmonella*-containing vacuoles (SCVs), which do not fuse with lysosomes and thereby avoid destruction. Such diversion from the canonical endocytic pathway is at least in part due to the activity of the phosphoinositide phosphatase activity of SopB, which presumably exerts its function by preventing the conversion of PtdIns(3)P to PtdIns(3,5)P₂, a crucial signal that mediates the transition of early and late endosomes into multivesicular bodies and lysosomes [37,38].

To establish a successful infection, *Salmonella* must avoid delivery to lysosomes and create a niche that is suitable to support its replication. During the early stages of infection, the SCV acquires the late endosomal marker Rab7, followed by the lysosomal glycoproteins LAMP1, LAMP2 and LIMP1/LAMP3, and the vacuolar ATPase (Figure 1c) [39]. The acquisition of these early and late endosomal markers reveals that the SCV can interact with the host endocytic network and suggests that *Salmonella* does not evade destruction by simply removing its vacuoles from the endocytic pathway. Indeed, *Salmonella* requires extensive contact with the host to obtain the membrane and nutrients it will require to survive and replicate. However, the SCV membrane never acquires the lysosomal marker mannose-6-phosphate receptor and the proteolytic enzymes of the lysosome do not gain access to the lumen of the SCV [39], indicating that the SCV does not fuse directly with either late endosomes or lysosomes. Therefore, *Salmonella* can interact selectively with the host-cell endocytic machinery to specifically control the process of vacuolar maturation and to carefully modulate the composition and contents of the SCV.

Establishing a replicative niche: engaging SPI-2

Continued remodeling of the SCV membrane is necessary for effective replication of intracellular bacteria. This modulation requires the function of the SPI-2 TTSS, whose expression is induced ~3 h after infection [5]. The SPI-2 TTSS translocates a second set of effector proteins across the SCV membrane, and this function is essential for the establishment of systemic infection. Strains that lack the SPI-2 TTSS are defective for replication within macrophages and are avirulent in mice [40].

At the time SPI-2 TTSS expression is induced, polymerized actin is observed around the SCV; inhibition of actin by latrunculinB blocks bacterial replication and causes the loss of the vacuolar membrane from around

(PI3P)-labeled vesicles. PX-GFP, a chimera of green fluorescent protein and the PI3P binding (PX) domain of p40phox, a subunit of NADPH oxidase, was used as to label PI3P. (d) As infection progresses, the *Salmonella* pathogenicity island 2 (SPI-2) type III secretion system (TTSS) delivers additional effector proteins and bacterial replication is initiated. SifA is essential for the formation of *Salmonella*-induced filaments (Sifs), elongated membrane tubules that emanate from the SCV. SifA, SopD2, SseF, SseG and SseJ localize to the SCV and to Sifs, and participate in membrane dynamics. The immunofluorescence image shows characteristic Sifs extending from the SCV, visualized with LAMP1. Abbreviations: DAPI, 4'-6-diamidino-2-phenylindole (used to stain DNA); PI3,5P₂, phosphatidylinositol (3,5)-biphosphate.

the bacteria [41]. This actin accumulation around the SCV is dependent on the function of the SPI-2 TTSS, although the actual effector(s) that is responsible for the modulation of these actin dynamics has not been identified. The SPI-2 effector SspH2 has recently been demonstrated to colocalize with the SCV-associated polymerized actin, suggesting that this effector has a role in these late-stage actin dynamics [42]. However, *Salmonella* mutants that lack SspH2 are still capable of inducing actin polymerization around the SCV, indicating that other effectors are involved in this process.

Three to 4 h after infection, the SCV moves to a perinuclear position, in close proximity to the Golgi apparatus [43]. This intracellular positioning of the SCV is dependent on SseG, a SPI-2 TTSS effector that possesses a domain that can physically interact with Golgi membranes. The interaction of the SCV with Golgi membranes might have implications for bacterial replication because *Salmonella sseG* mutants are defective for intracellular growth.

The *Salmonella* replicative compartment, ~5–6 h after internalization, is characterized by the presence of long membrane tubules that extend outwards from the SCV (Figure 1d) [44]. These structures are called *Salmonella*-induced filaments (Sifs). Sifs are similar in membrane composition to the SCVs and are dependent on microtubules for their formation [45,46]. The physiological relevance of Sifs is unknown but their formation is an indicator of a successful late-stage infection and the initiation of bacterial replication.

Five effectors of the SPI-2 TTSS, SifA, SopD2, SseF, SseG and SseJ, have been implicated in modulating the SCV membrane. These effectors localize to the SCV membrane and to Sifs in infected cells, suggesting that they might have a direct role in the manipulation of membrane dynamics [45,47–51]. SifA, in particular, is essential for the integrity of the SCV [50]; bacteria that lack SifA are unable to maintain an intact vacuole and are released into the cytosol of host cells [52]. Together with the host protein SKIP (SifA and kinesin-interacting protein), SifA mediates Sif formation and the Golgi proximal localization of SCVs [53]. SopD2, SseF and SseG have also been implicated in Sif formation; infection of cells with strains with mutations in any of the genes encoding these proteins leads to a reduction in the quality and quantity of the Sifs formed [49,54]. Consistent with the important role that these five effectors have in the establishment of an intracellular replicative niche, bacteria that lack these proteins have a replication defect in macrophages that is correlated with reduced virulence in the mouse model of infection [48–51,55].

The precise mechanisms by which these effectors promote SCV maturation are unknown. SseF and SseG can localize to microtubules in infected cells, suggesting that these proteins might disrupt microtubule-dependent trafficking events within the host, for the benefit of SCV maturation [56]. SseJ contains a domain with homology to an acyltransferase, and thus it is possible that this protein directly modulates the lipid composition of the SCV membrane [48]. Because expression of SifA, SopD2 or SseJ on their own can cause aggregation and swelling of

endosomes, it is also possible that these effectors could increase the fusogenicity of the SCV itself [45,47,48,54].

Establishment of systemic infection is likely to be complex and most probably requires additional virulence functions and effectors. The SPI-2 TTSS effectors PipB, PipB2 and SifB localize to the SCV and to Sifs, but have not been implicated in the formation of either structure, nor do they appear to have a role in intracellular replication [51,57]. Conversely, *Salmonella typhimurium* that lacks SopD, the homolog of SopD2, has a replication defect in macrophages and is attenuated in the mouse model of infection but does not localize to the SCV and has no role in the formation or maintenance of either the SCV or Sifs [54]. Although many of the bacterial effector proteins responsible for the formation of the *Salmonella* intracellular replicative niche have been identified, no mechanistic details of this process have yet emerged. Detailed studies of these events are likely to uncover exciting biology.

Defining the atomic host–pathogen interface: a new dawn for novel therapeutic approaches to infectious disease?

Research during the past few years has begun to define, in great detail, the interface between *Salmonella* and its host. Indeed, the availability of the high-resolution structures of virulence organelles and their components [58–61] or those of bacterial proteins in complex with their target host-cell proteins [13,26,33,62] has already provided a unique glimpse of the true atomic interface between *Salmonella* and its host. What emerges from this level of analysis is the possibility of novel therapeutic strategies aimed at selectively and specifically disrupting these interactions. For example, specialized organelles involved in bacterial virulence, such as the type III or type IV secretion systems, offer unique targets for therapeutic drug design. In fact, research efforts in this direction are well underway [63,64]. Because the function of these organelles is not essential for bacterial growth *in vitro*, drugs aimed at inhibiting their function would most likely interfere with bacterial pathogenicity rather than survival and therefore they should be less likely to generate resistance. In addition, because these virulence organelles are largely absent from non-pathogenic bacteria, the potential use of such drugs would not result in disruption of the normal flora, a recurrent problem with the use of conventional antimicrobials. Perhaps even more intriguing is the possibility of targeting the function of the virulence factors. Because some of the virulence factors exhibit enzymatic activity, drugs aimed at specifically inhibiting these activities might provide a useful anti-pathogen strategy [65–67]. Another strategy would be to target the atomic interfaces between virulence factors and their host target proteins. Superficial analysis might suggest that the fact that most bacterial virulence effector proteins target essential host-cell processes might pose an insurmountable challenge to the design of therapeutic agents. However, what is emerging from the analysis of the atomic interface between *Salmonella* effector proteins and their target host-cell proteins is that the actual interfaces offer unique features that could be amenable for

specific targeting. For example, although the bacterial GAP (SptP) and GEF (SopE) function in a manner that closely resembles their eukaryotic cell counterparts, the actual interfaces of these proteins with their targets is significantly different from those of the eukaryotic proteins with their cognate GTPases [13,33]. Targeting the specific interface between the pathogen and the host might enable the design of anti-infectives with advantages over conventional antibiotics. For example, compounds that specifically target the host-pathogen interface might be less likely to generate resistance. Although these potential drugs would not kill the pathogens, they would significantly hamper their ability to infect or cause disease, enabling the host defense mechanisms a chance to eliminate the pathogen. However, given the often-redundant nature of virulence proteins, it is possible that this approach will require the simultaneous targeting of various virulence factor-host interfaces.

Although this is clearly only the beginning of an exciting area of investigation, the *Salmonella*-host interface has already provided a paradigm that can be explored to validate this novel concept of therapeutic intervention to treat and combat infectious diseases. As the details of the atomic interfaces between other pathogens and their hosts emerge, the scope of these strategies can be expanded to develop selective strategies against a wide range of infectious agents.

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Current Opinion in Pharmacology

The October 2005 issue of *Current Opinion in Pharmacology* focuses on two areas of pharmacology. The first, edited by David Payne and Alisdair MacGowan, discusses the impact of resistance mechanisms that have spread to significant levels globally, what can be done to better predict the timescale and the extent of future resistance levels, and the prospects for the delivery of new anti-infective agents. The second, edited by Patrick Iversen, focuses on novel and innovative approaches that might lead to the development of drugs from flexible platform technologies. The issue includes:

Current challenges in antimicrobial chemotherapy: the impact of extended-spectrum β -lactamases and metallo- β -lactamases on the treatment of resistant Gram-negative pathogens

Marion S. Helfand and Robert A. Bonomo, pp. 452–458

Predicting the future *Streptococcus pneumoniae* resistance landscape

Robertino Mera, pp. 459–464

Prospects and challenges of developing new agents for tough Gram-negatives

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