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# Manipulation of the host actin cytoskeleton by *Salmonella* – all in the name of entry

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The invasive pathogen *Salmonella enterica* has evolved sophisticated mechanisms to subvert the cytoskeletal machinery of its host. Following contact with the host cell, it delivers a distinct arsenal of effector proteins directly into the cytoplasm. These bacterial effectors coordinate transient actin rearrangements and alter vesicle trafficking to trigger invasion, without causing overt cellular damage. Recent studies have shed new light on the signaling mechanisms underlying this remarkable host–pathogen interface, in particular, highlighting the unique multi-functional role and temporal regulation of key bacterial effectors.

## Addresses

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## Introduction

*Salmonella enterica* comprise a family of pathogenic Gram-negative bacteria adept at targeting a variety of eukaryotic hosts [1,2]. In humans, *Salmonella* spp. are believed to cause over one billion infections annually, with consequences ranging from acute gastroenteritis (food poisoning) to systemic, often fatal, typhoid fever. Central to their pathogenicity is their ability to enter non-phagocytic cells within the intestinal wall to reach a sheltered niche permissive for replication. Similar to other enteric pathogens, such as *Yersinia* spp. and *Shigella* spp., *Salmonella* has evolved a specialized protein secretion system, termed type III secretion system (TTSS), which delivers several bacterial ‘effector’ proteins directly into the host cell [3]. These virulence factors function in concert to engage host-cell proteins and orchestrate a remarkable series of events that culminate in bacterial entry.

In this review, we discuss recent advances in the understanding of *Salmonella* invasion into non-phagocytic cells. In particular, we focus on the mechanisms by which

bacterial effectors coerce the cytoskeletal machinery of the host cell to facilitate internalization.

## Cytoskeletal remodeling: *Salmonella* style

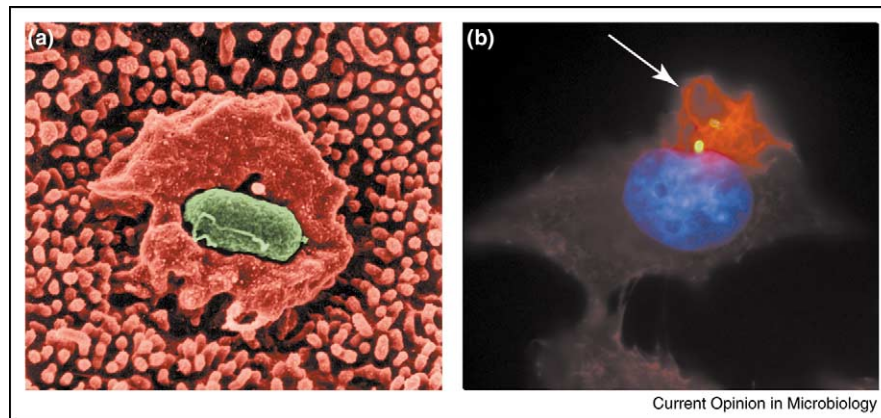
One of the hallmarks of *Salmonella* invasion is the profuse rearrangement of actin at the site of entry (Figure 1) [4–6]. This cytoskeletal remodeling drives localized membrane ruffling and lamellapodial extensions that envelop bacteria and trigger their internalization into membrane bound vacuoles. To date, the coordinated function of at least five distinct effectors (Table 1) are known to contribute to efficient *Salmonella* entry.

## Rho GTPase activation: turning on the switch

Small GTPases of the Rho family are key regulators of eukaryotic cellular architecture [7]. They function as binary switches, cycling between inactive GDP- and active GTP-bound conformations. The cycle is largely controlled by two families of regulatory proteins, the guanine nucleotide exchange factors (GEFs), which catalyze GTP loading, and the GTPase activating proteins (GAPs), which accelerate GTP hydrolysis [8,9]. Many bacterial pathogens have evolved intricate strategies to flip these important switches, ranging from irreversible covalent modifications that are invariably destructive (e.g. glucosylation by Toxin B from *Clostridium difficile* and ADP ribosylation by the C3 toxin of *Clostridium botulinum*) to reversible, less toxic methods by way of effectors that ‘mimic’ host-cell regulatory proteins. *Salmonella* spp. employ the latter approach and encode two closely related type III effectors, SopE and SopE2, which function as genuine RhoGEFs, despite lacking any discernible sequence similarity to eukaryotic proteins [10–12]. Indeed, in a remarkable example of convergent evolution, SopE catalyzes nucleotide exchange by targeting GTPase regions analogous to those bound by mammalian RhoGEFs, thereby presenting the host little scope to evolve resistance to this bacterial toxin [13]. Both SopE and SopE2 exhibit distinct specificities for Rho family members *in vitro* and *in vivo*; SopE is capable of activating Cdc42 and Rac1 whereas SopE2 displays a preference for Cdc42 [10,14]. *Salmonella* strains lacking either SopE or SopE2 display only a modest reduction in cellular invasion suggesting partial functional redundancy *in vivo*, whereas strains lacking both effectors (SopE/E2<sup>-/-</sup>) show a more pronounced, albeit incomplete, invasion defect [15].

The ability of SopE/E2<sup>-/-</sup> mutant strains to remodel actin and retain an invasive phenotype is attributable to a third *Salmonella* type III effector called SopB (also

Figure 1



Interaction of *Salmonella* with intestinal epithelial cells. **(a)** Electron micrograph of *S. typhimurium* infected Madin Darby canine kidney (MDCK) cell. The apical surface of the MDCK cell is pseudo-colored red and displays numerous microvilli (finger-like projections). The bacterium is pseudo-colored green. Invasion disrupts the microvilli and induces localized membrane ruffles that envelop the bacteria leading to engulfment (C Ginocchio, S Olmsted, C Wells and JE Galán; unpublished). **(b)** Immunofluorescence image showing actin cytoskeletal rearrangements (arrow) stimulated by *S. typhimurium* infection of Henle 407 cells. F-actin was stained with rhodamine phalloidin (red), *S. typhimurium* with a FITC-conjugated antibody (green) and DNA with DAPI (blue).

known as SigD) [15]. SopB encodes a phosphatidylinositol phosphatase originally implicated in late events post entry, such as chloride secretion and enteropathogenicity [16,17]. However, its role during invasion became apparent once the redundancy between SopE and SopE2 was established; a triple SopE/E2/B<sup>-/-</sup> mutant is completely defective for invasion [15]. Translocation of SopB into the host cell alters phosphoinositide phosphate (PIP) and inositol phosphate (IP) metabolism, generating several potential secondary messengers. In particular, SopB targets dephosphorylation of PtdIns(3,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> at positions 4 and 5 of the inositol ring [18,19,20,21] and consumes the cellular Ins(1,3,4,5,6)P<sub>5</sub> reservoir to generate Ins(1,4,5,6)P<sub>4</sub>. This phosphatase activity is crucial as point mutations within the active site (e.g. SopB<sup>C460S</sup>) abolish SopB-induced cytoskeletal reorganization [15]. Interestingly, the transient ectopic expression of SopB in COS cells induces very localized actin-rich ruffles, similar to *Salmonella* entry but in marked contrast to the profuse peripheral ruffles generated following

transient SopE overexpression. Furthermore, the SopB-triggered actin changes are completely abrogated by the expression of dominant interfering mutants of Cdc42, but not Rac. However, the signaling mechanisms linking SopB phosphatase function to Cdc42 activation remain unclear. A logical intermediate would be an endogenous eukaryotic RhoGEF of the *Dbl* family, many of which exhibit PIP-mediated regulation.

Therefore, *Salmonella* has evolved two completely independent mechanisms to activate Cdc42 and Rac. SopE and SopE2 ensure direct activation by mimicking eukaryotic RhoGEFs whereas SopB triggers indirect activation by generating PIP fluxes. Eliminating either SopE/E2/B function in *Salmonella* or expressing dominant interfering mutants of Cdc42 and/or Rac within the host cell prevents actin rearrangements and bacterial internalization, highlighting their central role in this process. Recent work has further dissected *Salmonella*-induced GTPase signaling and, surprisingly, advocates disparate functions for

Table 1

**Salmonella effectors contributing to cytoskeletal remodelling.**

Effector	Function	References
<b>SopE/E2</b>	Cdc42 and Rac GEF. Induces membrane ruffling of host cells to promote entry. Ubiquitinated and targeted for rapid degradation (<30 min) post infection.	[10–12,46**]
<b>SopB</b>	Inositol phosphatase. Generates PtdIns(3)P and Ins(1,4,5,6)P <sub>4</sub> upon invasion. Promotes indirect Rho GTPase activation and macropinocytosis.	[15,18,19,20**]
<b>SipA</b>	Induces actin bundling. Inhibits ADF-mediated and gelsolin-mediated actin depolymerization. Potentiates the activity of SipC.	[34,35,36**,37**,38*,39,41,42]
<b>SipC</b>	Translocon component. Inserts into host cell membrane and promotes actin nucleation and bundling.	[32,33]
<b>SptP</b>	Cdc42 and Rac GAP. Mediates recovery of cytoskeletal architecture by antagonizing SopE/E2 activity. Delayed degradation compared with SopE/E2. Facilitates recovery of cellular architecture post invasion.	[43,44,46**]

different Rho-family GTPases, namely a solitary role for Rac in cytoskeletal remodeling and for Cdc42 in the subsequent reprogramming of gene expression (J Patel and J Galán, unpublished).

### Actin nucleation and manipulation

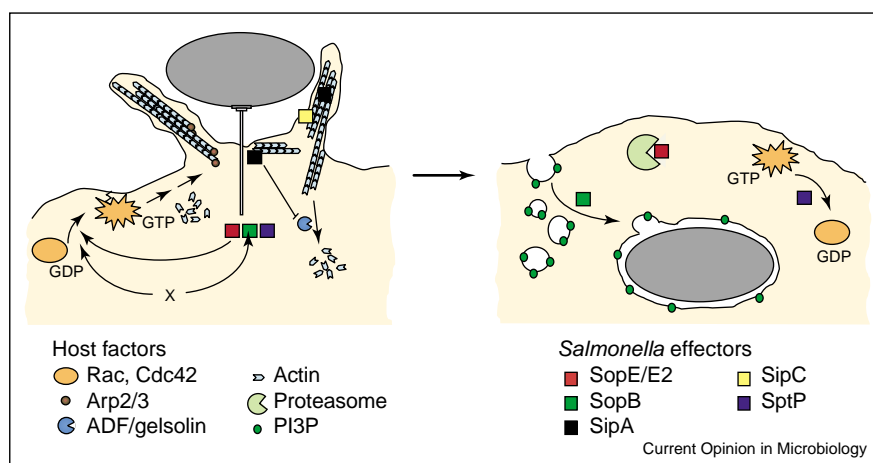
The activation of RhoGTPases by *Salmonella* initiates a complex signaling cascade aimed at altering actin dynamics to promote invasion. This involves the coordinated function of multiple downstream effectors, in a specific temporal and spatial manner, to polymerize the monomeric globular actin (G-actin) into the highly ordered filamentous actin (F-actin) necessary to drive engulfment (Figure 2) [22]. An obvious downstream candidate is the heptameric Arp2/3 complex. This is a ubiquitous, eukaryotic actin-organizer capable of initiating actin nucleation, branching and cross-linking [23]. Indeed, numerous pathogens, including *Listeria monocytogenes*, *Shigella* spp. and enteropathogenic *E. coli*, employ host Arp2/3 to tailor actin remodeling [24,25]. In the case of *Salmonella*, recent studies have shown recruitment of the Arp2/3 complex to the actin ruffles that are generated during entry in both polarized and non-polarized cells [11,26,27]. Moreover, sequestering Arp2/3 function within these cells reduced *Salmonella* invasion by 50–80%, thus arguing for a role in bacterial uptake.

Both Cdc42 and Rac promote actin nucleation via the intrinsically dormant Arp2/3 complex through the family of Wiskott–Aldrich syndrome proteins (WASP) or Scar/WAVE proteins [28]. The synergistic binding of activated

Cdc42 and phosphatidylinositol 4,5-bisphosphate (PI<sub>4</sub>, 5P<sub>2</sub>) to WASP relieves its auto-inhibited, folded conformation, exposing a tripartite VCA (verprolin homology, cofilin homology, acidic; also termed the WA) module at its carboxy terminus capable of binding G-actin and stimulating Arp2/3 [29,30]. The Scar/WAVE family function analogously to bridge between activated Rac and Arp2/3, although they mediate their effect via GTPase induced destabilization of an inhibitory Scar/Pir121/Nap1 complex [31]. Like the Arp2/3 complex, both WASP and Scar proteins display recruitment to actin ruffles upon *Salmonella* invasion [27]. However, dominant interfering mutants of either or both proteins elicit only a moderate decrease (~30%) in *Salmonella* entry, suggesting alternative Arp2/3 dependent or independent mechanisms of actin assembly.

In addition to exploiting the cytoskeletal machinery of the host, *Salmonella* contributes to cellular remodeling through the activity of two type III effector proteins, which are capable of directly engaging actin [6]. One of these proteins, SipC, is an integral component of the translocation apparatus, which has been shown to insert into the host cell plasma membrane upon infection [32]. *In vitro* studies using purified SipC revealed two distinct biochemical activities: an amino terminal domain (SipC<sup>1–120</sup>), sufficient for bundling F-actin, and a carboxy terminal domain (SipC<sup>200–409</sup>), capable of nucleating actin polymerization [33]. Both domains are proposed to initiate actin polymerization and anchor bundled filaments beneath invading bacteria. However, genetically

Figure 2



Multiple host and bacterial effectors function in concert to promote entry. (a) Upon contact, *Salmonella* delivers multiple effectors into the host cell. SopE/E2 and SopB induce RhoGTPase activation (Cdc42 and Rac) leading to actin remodeling, presumably by way of the host-encoded actin nucleation machinery (Arp2/3). *Salmonella* effectors also engage actin directly. SipA promotes F-actin polymerization and bundling. Moreover, it prevents F-actin disassembly by inhibiting host ADF/cofilin and gelsolin. SipC also nucleates actin polymerization. (b) Coincident with internalization, SopB generates phosphatidylinositol 3 phosphate (PI3P) decorated, fusogenic macropinosomes and *Salmonella* containing vacuoles (SCVs), which can coalesce to create a spacious phagosome for *Salmonella* to reside. The rapid degradation of SopE by way of the host proteasome coupled to the prolonged stability of SptP halts RhoGTPase-induced actin remodeling. The mechanisms to counter SipA- and SipC-induced actin remodeling remain unclear.

validating the role of SipC during invasion has been hampered by the need for SipC mutants that are defective in actin changes yet competent for effector translocation into host cells.

The second *Salmonella* effector capable of binding actin directly is SipA. Like SipC, SipA has no sequence similarity to known eukaryotic actin binding proteins (ABPs). Nonetheless, *in vitro* studies have demonstrated G-actin binding through its carboxy terminus and a capacity to reduce the critical concentration of actin required for polymerization [34]. Moreover, SipA increases the stability of F-actin and augments the ability of T-plastin (a eukaryotic ABP) to bundle F-actin filaments [35]. These biochemical activities are supported by crystallography studies showing that SipA adopts a central globular core flanked by two extended, non-globular 'arms' that physically tether actin subunits of opposing filaments and facilitate monomer polymerization [36•]. Recent studies have also begun to address how SipA maintains F-actin stability. SipA perturbs the continual flux of monomer subunits through F-actin (treadmilling) by antagonizing the action of an actin depolymerising factor (ADF/cofilin) [37•,38•]. Indeed, SipA binding to F-actin sterically hinders access of ADF/cofilin and, surprisingly, displaces pre-bound ADF/cofilin, thereby preventing disassembly. SipA further modulates F-actin turnover by opposing the functions of another ABP called gelsolin, namely protecting F-actin from gelsolin-mediated severing and re-annealing gelsolin severed/capped F-actin filaments [37•]. Finally, SipA potentiates the actin nucleating and bundling function of SipC, suggesting functional cooperation between these effectors for the generation of F-actin [39]. By regulating the complex interplay between F-actin assembly and disassembly, SipA could dictate the type of protrusive structures that are generated by the host cell, in a manner similar to that seen recently for capping protein (CP) [40]. Accordingly, SipA mutants, despite being invasion competent, exhibit delayed entry kinetics at the early stages of infection and display diffuse underlying actin rearrangements [34,41,42]. Taken together, the multiple functions of SipA appear to facilitate efficient bacterial uptake by directing the spatial localization of actin foci beneath invading *Salmonella*.

### Regaining cytoskeletal composure: covering ones tracks

The cytoskeletal remodeling events that accompany *Salmonella* invasion are transient and typically reversed 2–3 hours post entry. Remarkably, *Salmonella* actively participates in this recovery process through the function of another type III effector protein called SptP [43]. This modular effector comprises two distinct biochemical activities, both designed to avert potential host cell damage. Its amino terminus encodes a GAP with specific activity towards Cdc42 and Rac. This GAP domain mimics its eukaryotic counterparts, engaging the switch

I and II regions of its cognate GTPase and juxtaposing an invariant arginine residue within the active site to catalyze GTP hydrolysis [44]. By inactivating Cdc42 and Rac, the GAP activity of SptP directly antagonizes the effects of SopE/E2 and SopB, helping the host cell regain its cytoskeletal architecture. A similar mechanism of down-regulating GTPase function is apparent for related type III effectors from *Yersinia* spp. (YopE) and *Pseudomonas aeruginosa* (ExoS).

The carboxy terminus of SptP encodes a tyrosine phosphatase with sequence homology to YopH from *Yersinia* spp. and several eukaryotic tyrosine phosphatases, such as PTP1B. Although the cellular target(s) remains unclear, this phosphatase activity is essential for dampening down the pro-inflammatory nuclear responses generated post infection [45].

### Host degradation of translocated effectors: a new paradigm for regulating invasion

To synchronize invasion, multiple *Salmonella* effectors must engage their cellular targets in a precise, temporal and spatial manner. The spatial localization is inherent, given the restricted nature of the intimate contact between bacteria and their host. However, mechanisms governing the temporal regulation of effectors, especially those with directly opposing functions such as SopE and SptP, are less obvious. Recently, it was demonstrated that although SopE and SptP are delivered equally during the initial infection, SopE undergoes rapid proteasome-mediated degradation following translocation, whereas SptP remains stable [46•]. Further effector chimera studies established that the secretion and translocation domains of these proteins dictated their half-life within the host. Hence, by ensuring prolonged GAP (SptP) activity over GEF (SopE) function, invading bacteria ensure that the RhoGTPase-induced actin rearrangements remain transient. It remains to be seen whether a similar mechanism accounts for the functional regulation of other effectors, although host ubiquitination of SopB has also been reported [47].

### Temporary residence: establishing the *Salmonella* containing vacuole

The entry process concludes with *Salmonella* inhabiting a spacious vacuole within the host [48,49]. SopB plays a key role in assembling this *Salmonella* containing vacuole (SCV) by inducing profuse macropinocytosis events to accompany bacterial uptake. Indeed, *Salmonella* mutants lacking SopB activity invade with minimal macropinocytosis and occupy constricted vacuoles that undergo delayed maturation. Hernandez *et al.* [20•] showed that the inositol phosphatase activity of SopB is necessary to generate PtdIns(3)P decorated, fusogenic vesicles that coalesce with enveloped bacteria. This serves to create a spacious habitat for *Salmonella* to reside in, before establishing its replicative niche.

## Conclusions

The association between *Salmonella enterica* and their hosts has been shaped by extensive co-evolution and offers a fascinating example of how pathogens employ intricate strategies to subvert host cell function. During the past few years we have witnessed remarkable progress in our understanding of the complex interaction between *Salmonella* and its host cells. The knowledge that has been gained by studying these fascinating close encounters promises to enhance our understanding of the eukaryotic cytoskeletal machinery and potentially offers new scope for therapeutics.

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