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The Different Strategies Used by *Salmonella* to Invade Host Cells

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1. Introduction

Salmonella enterica are members of a Gram-negative enteropathogenic bacteria family, which are able to infect a great diversity of hosts, including human. According to serotypes and hosts, *Salmonella enterica* cause a wide range of food- and water-borne diseases ranging from self-limiting gastroenteritis to systemic typhoid fever. Moreover, no other known bacterial pathogens belonging to a single species show such a remarkable variation in their host specificity. Ubiquitous serotypes such as Typhimurium and Enteritidis tend to produce acute but self-limiting enteritis in a wide range of hosts, whereas host-specific serotypes are associated with severe systemic disease in healthy outbred adults of a single species that may not involve diarrhoea (e.g. Gallinarum in poultry). Host-restricted serotypes are primarily associated with systemic disease in one host (e.g. Dublin in cattle, Choleraesuis in pigs), but may cause disease in a limited number of other species (Velge *et al.*, 2005).

For all these serotypes, the intestinal barrier crossing constitutes a crucial step for infection establishment. As shown in Figure 1, *Salmonella* can induce their own entry into enterocytes, but M cells and CD18-expressing phagocytes also facilitate their translocation through the intestinal epithelium (Watson & Holden, 2010). During gastroenteritis pathology, host colonization is restricted to the intestinal tract. However, *Salmonella* also have the ability to disseminate to extra-intestinal sites at least via CD18-expressing phagocytes, leading to deep organ colonization (Vazquez-Torres *et al.*, 1999).

Bacterial pathogens have developed two different mechanisms to invade non-phagocytic host cells by hijacking physiological cellular processes. Bacteria, such as *Listeria monocytogenes* and *Yersinia pseudotuberculosis* express surface proteins that interact with receptor on the host cell plasma membrane. This interaction promotes an activation of host cell signaling pathways, leading to actin remodelling. This process is referred to as a Zipper mechanism and is characterized by the induction of little protrusive activity and thin membrane extensions (Figure 2A and C) (Cossart & Sansonetti, 2004). Other bacteria, such as *Shigella flexneri*, do not require a receptor but trigger internalization from "inside" via the action of pathogen-effector proteins delivered by specialized protein secretion systems (Schroeder & Hilbi, 2008). Translocated effector proteins effectively allow the bacteria to "hijack" many essential

intracellular processes and induce a massive reorganization of the host actin cytoskeleton, resulting in intense membrane ruffling and internalization of the bacteria. This invasion process is referred to as a Trigger mechanism (Figure 2B and D) (Cossart & Sansonetti, 2004).

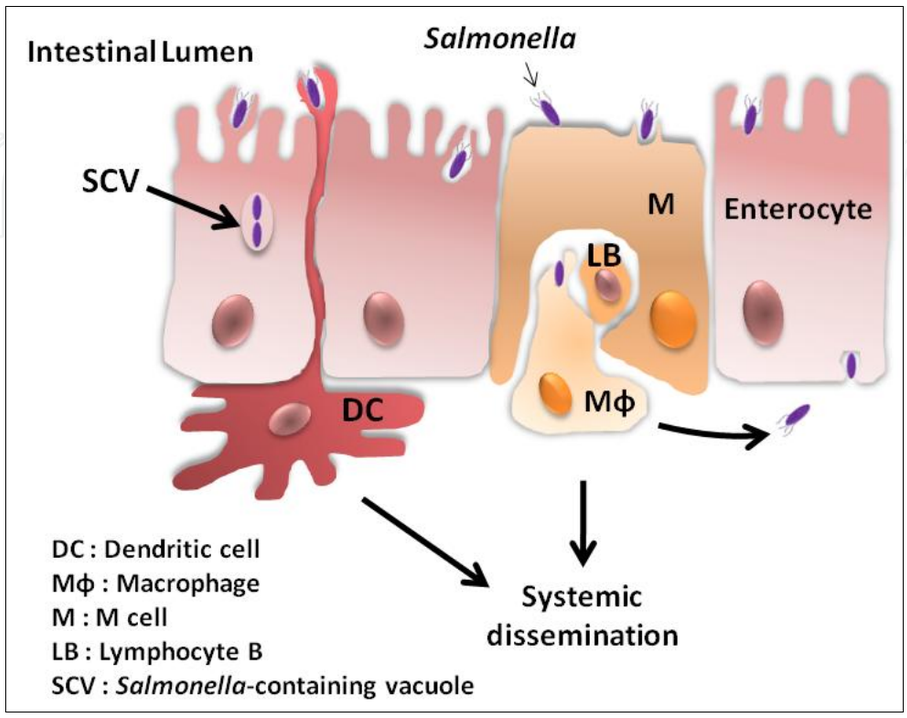


Fig. 1. Intestinal barrier crossing by *Salmonella enterica* through M cells, enterocytes or following a luminal capture by CD18+ phagocytes such as dendritic cells.

The reorganization of actin cytoskeleton at the entry site is a crucial step for Trigger and Zipper bacterial internalization. In eukaryotic cells, actin exists as a globular monomer (G-actin) which can assemble to form a filamentous structure (F-actin). In physiological conditions, actin polymerization requires different steps. First, nucleation of actin which consists in regrouping three actin monomers, is stimulated by cellular factors such as the Arp2/3 complex (Mullins *et al.*, 1998). Once nucleated, the addition of ATP-actin-monomers at the barbed extremity of the filaments allows actin elongation (Pollard *et al.*, 2000). The three-dimensional structure of actin filaments is ensured by capping proteins and other actin-binding proteins such as actinin, gelsolin, and villin that enable bundling of filaments (Bretscher, 1991; Hartwig & Kwiatkowski, 1991). Actin dynamics regulation is closely associated with small Rho guanosine triphosphatase protein (RhoGTPase) activity. RhoGTPases cycle between an inactive guanine di-phosphate (GDP)-bound form and an active guanine tri-phosphate (GTP)-bound form. The switch between inactive and active state is regulated by guanine exchange factors (GEF) which catalyze the exchange of GDP with GTP and GTPase activating proteins (GAP) which hydrolyze GTP into GDP to switch off their active state. When bound to GTP, Rho GTPases target and activate downstream effectors such as proteins from the Wiscott-Aldrich Syndrome protein (WASP) / N-WASP Family, leading to nucleator activation and actin reorganization. All these steps are required during bacterial internalization.

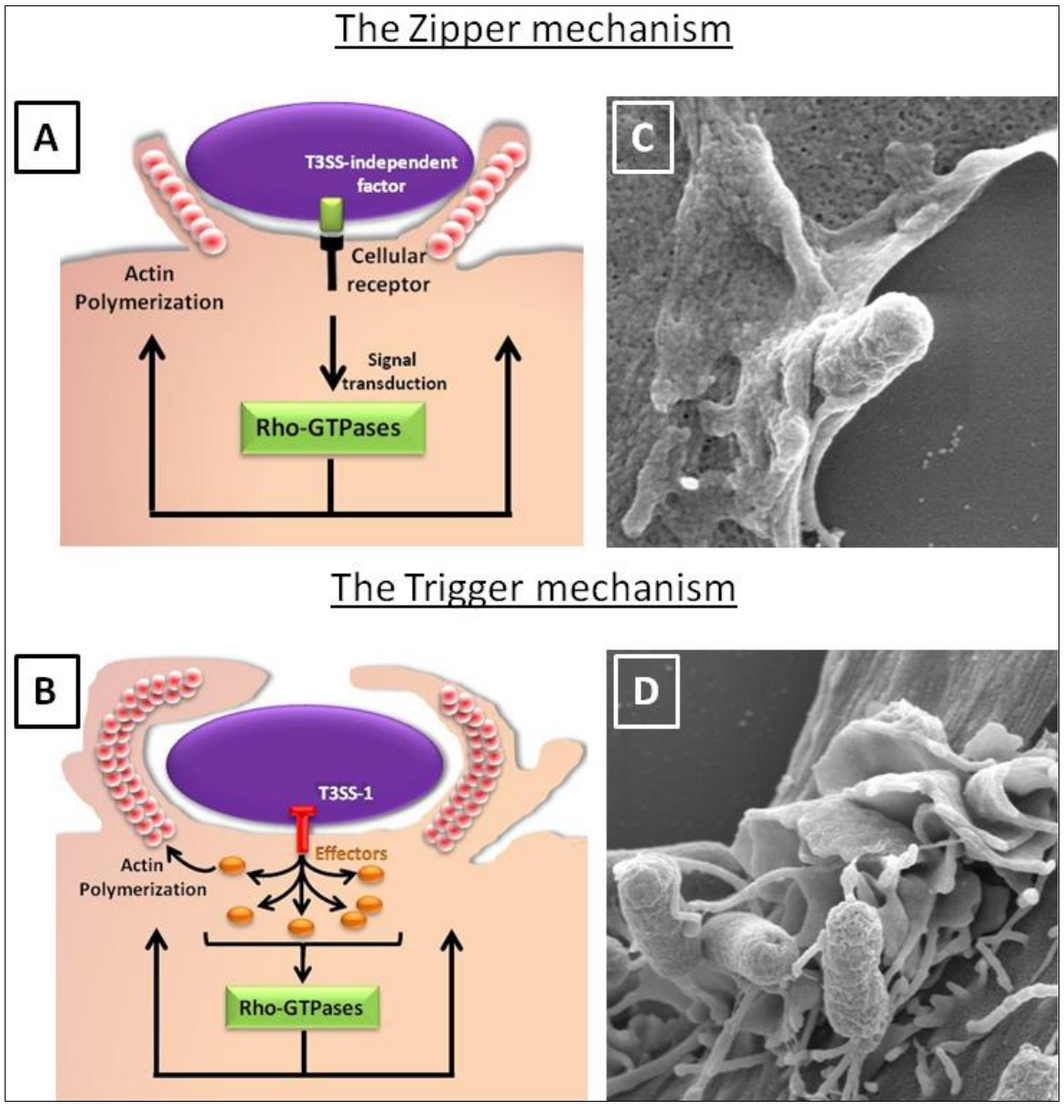


Fig. 2. Models of Zipper and Trigger invasion mechanisms. (A) The Zipper process is initiated by an interaction between a host cell receptor and a bacterial surface protein which allows the activation of RhoGTPases and actin polymerization at the entry site. (B) In contrast, during the Trigger mechanism, RhoGTPases are targeted by bacterial effectors which are directly translocated into host cell via a type–three secretion system, leading to actin polymerization and internalization. Electron scanning microscopy pictures show (C) *S.* Enteritidis invading fibroblasts via a Zipper process which is characterized by weak membrane rearrangements and (D) via a Trigger process which is characterized by intense membrane rearrangements.

The study of host cell invasion by *Salmonella* has been initiated in 1967 by Takeuchi (Takeuchi, 1967). For decades, it was described in the literature that *Salmonella* can enter cells only via a "Trigger" mechanism mediated by a type-three secretion system (T3SS-1) encoded by the *Salmonella* pathogenicity island-1 (SPI-1) (Ibarra & Steele-Mortimer, 2009). Recent data have showed that cell invasion could occur despite the absence of the T3SS-1

(Aiastui *et al.*, 2010; Radtke *et al.*, 2010; Rosselin *et al.*, 2011), indicating that the dominant paradigm postulating that a functional SPI-1/T3SS is absolutely required for cell entry, should be reconsidered. Moreover, the characterization of one T3SS-1-independent invasion pathway revealed that *Salmonella* have also the ability to enter cells via a Zipper process mediated by the Rck invasin (Rosselin *et al.*, 2011). Consequently *Salmonella* are the first bacteria found to be able to invade cells both via a Zipper and a Trigger mechanism.

Here, our current understanding of the different strategies used by *Salmonella* to invade host cells will be summarized and we will focus on how *Salmonella* are able to manipulate the host actin cytoskeleton, leading to discrete or intense membrane rearrangements. The gap of our knowledge about these different entry pathways will be discussed.

2. Invasion mechanism dependent on the T3SS-1

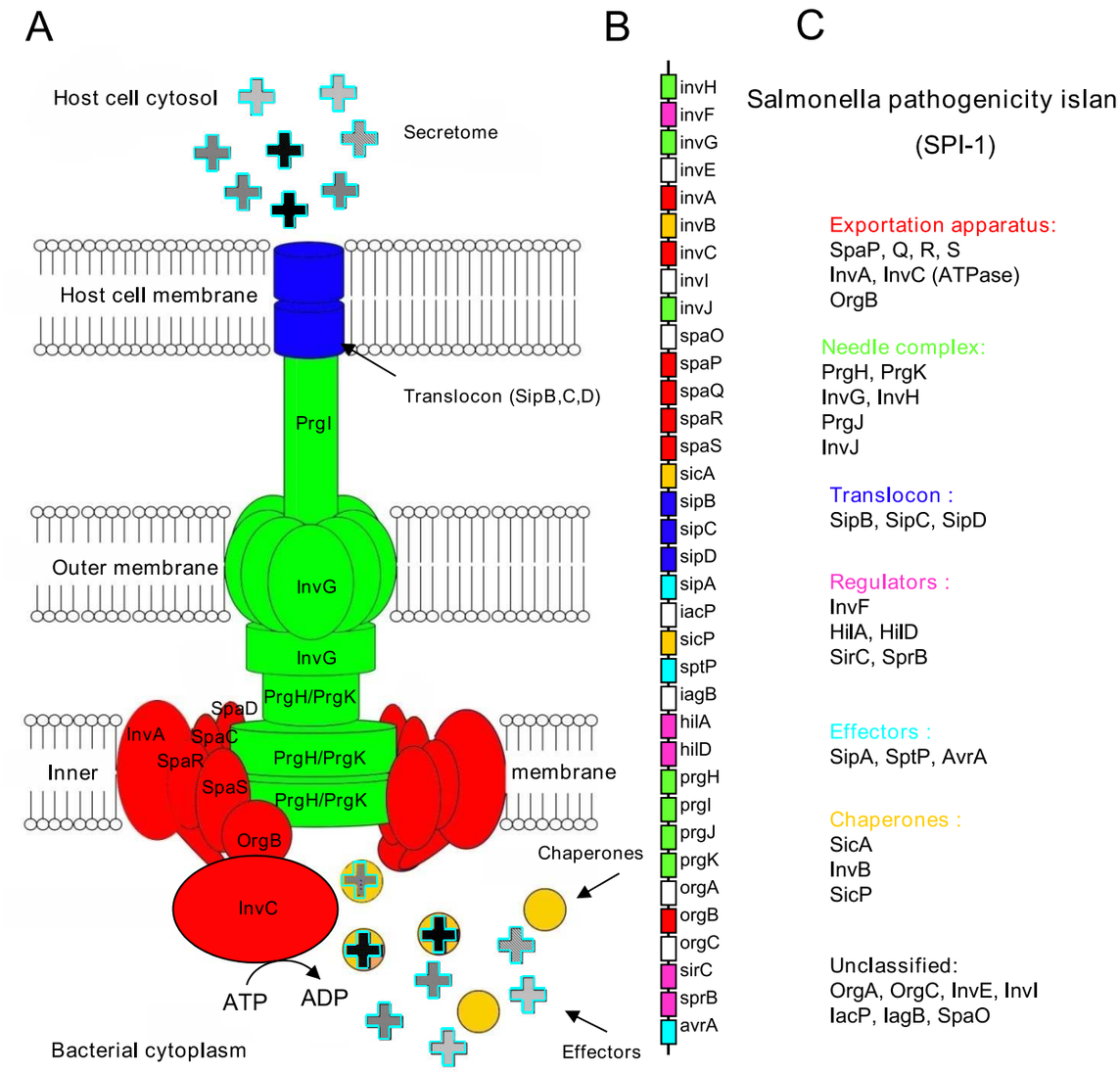
The Type-Three Secretion System (T3SS-1) is the best characterized invasion system of *Salmonella*. It allows bacterial internalization into non-phagocytic cells via a Trigger mechanism which induces massive actin rearrangements and intense membrane ruffling at the entry site (Cossart & Sansonetti, 2004). Under environmental conditions that enable the expression of the T3SS-1, the secretion apparatus is assembled at the bacterial surface and effectors are translocated into the eukaryotic cytosol following an interaction between the bacteria and the host cell (Garner *et al.*, 2002; Hayward *et al.*, 2005).

2.1 T3SS-1 structure

T3SSs are supramolecular complexes that play a major role in the virulence of many Gram-negative pathogens by injecting bacterial protein effectors directly into host cells in an energy-dependent (ATP) manner (Galan & Wolf-Watz, 2006). These complexes cross both inner and outer membranes of bacteria and are able to create a pore in eukaryotic membrane upon contact with a host cell. They are made of an exportation apparatus, a basal body, a needle and a translocon at the tip of the needle (Figure 3A). The structure of these T3SSs shows a high degree of conservation among pathogens (Tampakaki *et al.*, 2004) and the *Salmonella* T3SS-1 apparatus shares in particular a high homology with the T3SS of *Shigella*, also involved in host cell invasion (Groisman & Ochman, 1993).

The basal body of the T3SS anchors the complex into the bacterial inner and outer membranes (Figure 3A). It is composed of PrgH, PrgK and InvG proteins which assemble into an inner ring (PrgH and PrgK) and an outer ring (InvG) (Schraidt & Marlovits, 2011). Anchored to the basal body via its transmembrane part, the needle protrudes from the outer membrane as a long filament of 50 nm length and is composed of the single PrgI protein (Kimbrough & Miller, 2000). At the extremity of the needle, a complex of three proteins (SipB, SipC, SipD), known as the translocon, is able to form a pore in the eukaryotic target cell, allowing the secretion of effector proteins (Mattei *et al.*, 2011). SipB, SipC and SipD proteins (also referred to Ssp proteins) share homology with other translocon proteins such as IpaB, IpaC and IpaD proteins of *Shigella* (Hueck, 1998). SipD has a hydrophilic domain and interacts directly with the PrgI needle protein (Rathinavelan *et al.*, 2011) while the two other proteins of the translocon (SipB and SipC) have a hydrophobic domain and are therefore directly involved in the pore formation (Hayward *et al.*, 2000; Miki *et al.*, 2004). Particularly, it has been shown that the interaction of SipB with cellular cholesterol is necessary for effector translocation (Hayward *et*

al., 2005). Finally, the translocation of T3SS-1 effector proteins requires an exportation apparatus located at the inner membrane level and made of highly conserved proteins among T3SSs (SpaP, SpaQ, SpaR, SpaS, InvA, InvC and OrgB). The unfolded effectors in association with their chaperone are targeted to the exportation apparatus and the ATPase InvC produces the energy necessary to the transport of these proteins through the needle (Akeda & Galan, 2004). The appropriate hierarchy in the secretion process is established by a cytoplasmic sorting platform composed of SpaO, OrgA and OrgB (Lara-Tejero *et al.*, 2011). This platform sequentially loads the secreted proteins by interacting with their chaperones to ensure a specific order of secretion and optimize host cell invasion.



Adapted from Kimbrough and Miller, 2002.
A. Localization of the T3SS-1 structure proteins. **B.** Schematic representation of SPI-1 island encoding the T3SS-1 proteins. **C**. Functional classification of SPI-1-encoded proteins.

Fig. 3. Structure and organization of *Salmonella* T3SS-1.

2.2 Regulation of T3SS-1 expression

During *Salmonella* infection, a crucial step is the crossing of the intestinal barrier. The host environment encountered by the bacteria, and more particularly the small intestine environment, plays a major role in the invasion as it controls expression of the secretion apparatus. Coordination of T3SS-1 expression genes, almost all located on the *Salmonella* Pathogenicity Island 1 (SPI-1) is complex and well-timed. In response to different environmental stimuli, a sophisticated regulatory network controlling the expression of SPI-1 has been established (Figure 4). Our purpose here is not to set up the thorough state-of-the-art on all the regulators involved in *Salmonella* invasion, but to give a general overview of this system (for a review, see (Ellermeier & Slauch, 2007)).

SPI-1 contains 39 genes encoding structural T3SS-1 proteins (*inv/spa* and *prg* operon), translocon proteins (SipB, C, D), some effectors (SipA, SptP and AvrA), some chaperones (SicA, InvB, SicP) and finally four transcriptional regulators (HilA, hilC, HilD and InvF) (Figure 3B and C). Other genes encoding secreted effectors (*sopA, sopB, sopD, sopE, sopE2, slrP, sspH1, sspH2*) are located elsewhere on the chromosome.

HilA is central for SPI-1 transcriptional regulation. This protein activates directly the transcription of *prg*, *inv/spa* and *sip* operons, encoding structural components and some secreted effectors of T3SS-1 respectively. In addition, HilA induces the transcription of *invF*, encoding a transcriptional activator and targeting, among others, *sip* operon, *sopE* and *sopB* genes (Darwin & Miller, 1999). The sequential expression of HilA and InvF regulators allows a hierarchical regulation of invasion genes.

Then, a second crucial level of SPI-1 transcriptional regulation takes place through the regulation of HilA *via* a feed-forward loop, involving three homologous transcriptional regulators: HilC, HilD and RtsA. Each of them binds directly to the *hilA* promoter and is able to activate its own expression. In fact, HilC, HilD and probably RtsA, act as derepressors of *hilA* transcription by counteracting the silencing exerted by nucleoid-structuring proteins such as H-NS or Hha (Akbar *et al.*, 2003; Queiroz *et al.*, 2011). The reason why HilC, HilD and RtsA play such an important role in T3SS-1 expression through *hilA* regulation is that they are at the integration point of a lot of signals that control SPI-1 expression (Figure 4). In this regulatory circuit, it is currently admitted that HilD has a predominant role whereas HilC and RtsA simply act as signal amplifiers. However, it has also been shown that these three regulators are also directly implicated in the regulation of others invasion genes (Akbar *et al.*, 2003; Ellermeier & Slauch, 2004).

Moreover, besides these direct regulators, a great number of other *hilA* regulators, acting mainly through HilD, have been identified. Among them, two-component systems play a major role. They sense environmental conditions and allow the transmission of different signals which modulate T3SS-1 genes expression. Some are able to activate indirectly HilA expression such as BarA/SirA and OmpR/EnvZ, whereas others such as PhoP/PhoQ and PhoB/PhoR repress it. In fact, HilA expression can also be inhibited. HilE has been identified as a negative regulator of *hilA* transcription preventing HilD activity (Fahlen *et al.*, 2000; Baxter *et al.*, 2003). It has been suggested that the two-component systems PhoP/PhoQ and PhoB/PhoR act through HilE to regulate SPI-1 (Baxter & Jones, 2005).

As stated above, environmental signals play a major role in *Salmonella* invasion. Low oxygen tension, high osmolarity, high iron concentration, neutral pH are conditions found in the

ileum, known to be the preferential invasion site of *Salmonella*. Thus, as expected in these conditions, invasion genes are activated through *hilA* expression. In contrast, when *Salmonella* are located at unfavorable sites for invasion in the host organism, the presence of signals such as bile, secreted into the proximal small intestine or cationic peptides, known to exist in macrophages, inhibits T3SS-1 expression (Figure 4).

Although much has already been identified about the regulation of SPI-1, recently, it became more evident that mechanisms regulating this system are more complex than previously thought.

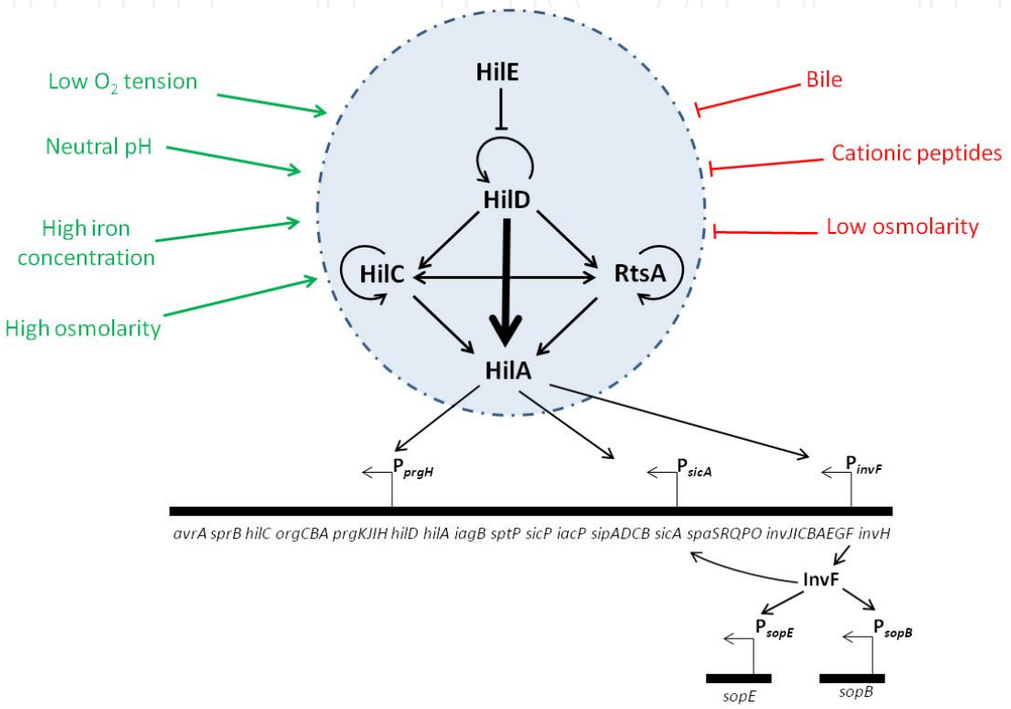


Fig. 4. Environmental and transcriptional regulation of SPI-1 encoded genes. HilA is the major regulator of SPI-1 and is itself regulated by other regulators such as HilC, HilD, RtsA and HilE. When *Salmonella* reach the small intestine, a low O_2 tension, a neutral pH, a high iron concentration and a high osmolarity activate SPI-1 expression. In contrast, the presence of bile or cationic peptides represses its expression.

2.3 Subversion of the cellular machinery during T3SS-1-dependent entry

Among the effectors that are translocated into host cell by the T3SS-1, six are essential to cell invasion (SipA, SipC, SopB, SopD, SopE, SopE2) while the other effectors contribute to a variety of post-invasion processes such as host cell survival and modulation of the inflammatory response (Patel & Galan, 2005). To trigger internalization into cells, effectors manipulate actin cytoskeleton either directly or indirectly. They also manipulate the delivery of vesicles to the site of bacterial entry to provide additional membrane and allow the extension and ruffling of the plasma membrane necessary to promote invasion. In later steps, membrane fission occurs to induce the sealing of the future *Salmonella*-containing vacuole (SCV) and actin filaments are depolymerized, enabling the host cell to recover its normal shape (Figure 5).

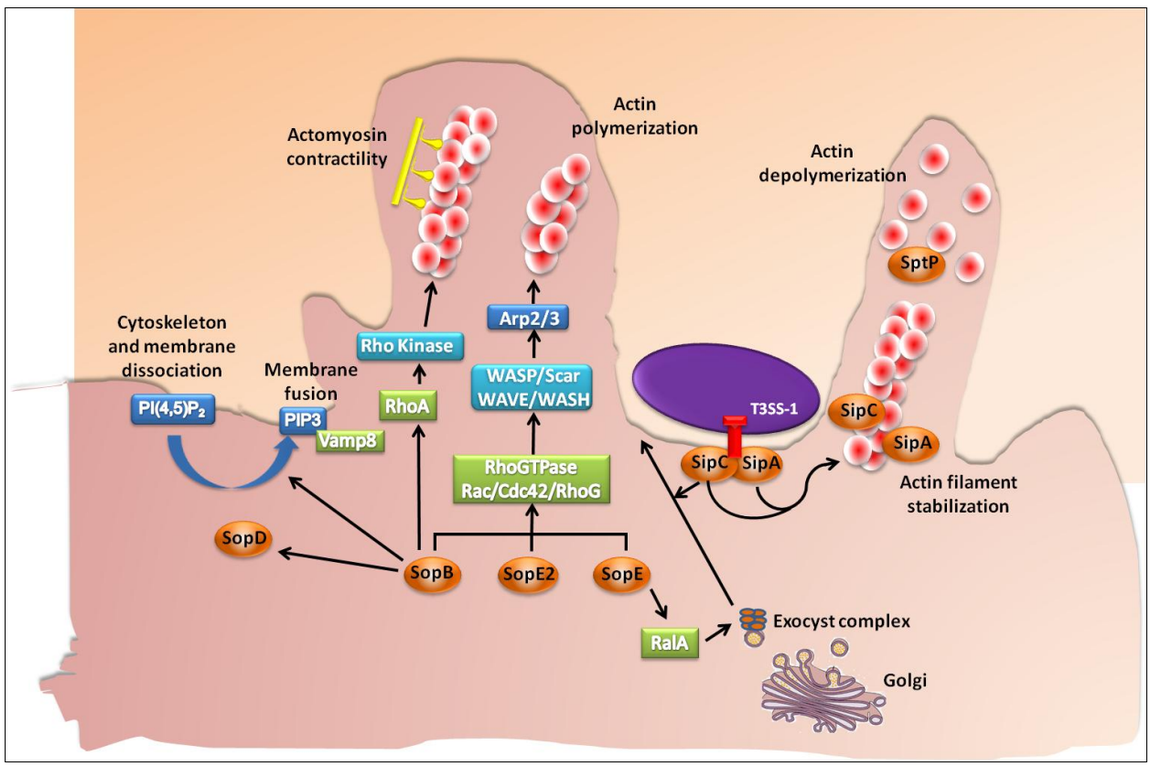


Fig. 5. Intracellular activities of the T3SS-1 effectors: SipA and SipC bind actin directly whereas SopE, SopE2 and SopB stimulate RhoGTPase activity. SipC and SopE also act in cooperation to recruit exocytic vesicles at the entry site. SopB plays diverse roles during invasion: it promotes actomyosin contractility and changes phosphoinositide concentrations to facilitate the dissociation between actin cytoskeleton and membrane at the entry site. SopB also probably triggers the delivery of vesicles to the bacterial entry site in a VAMP-8 dependent way and activate SopD which seems to contribute to the sealing and the formation of the SCV. The last effector SptP turns off the activity of RhoGTPases to allow the host cell to regain its initial shape.

2.3.1 Actin manipulation

Actin polymerization is an essential process induced by T3SS-1 effectors during entry. Some effectors stimulate actin rearrangements indirectly and two effectors, SipA and SipC, manipulate actin directly. SipA and SipC are localized at the eukaryotic plasma membrane during entry (Scherer *et al.*, 2000) but they are also translocated into the host cell cytoplasm (Hueck *et al.*, 1995; Kaniga *et al.*, 1995).

SipC, as part of the translocon, is required for T3SS-1 effector translocation and involved in actin nucleation and bundling. Due to its various functions, a 95% decrease in invasion is observed when *sipC* is deleted (Chang *et al.*, 2005). The C-terminal region of SipC (amino-acids A321-A409) encodes the effector translocation activity (Chang *et al.*, 2005) and its central region (amino-acids N201–S220) binds to actin and induces a rapid nucleation and elongation of actin filaments. More precisely the regions containing the amino acids from H221–M260 and L381–A409 bind to and bundle actin filaments *in vitro* (Myeni & Zhou,

2010). The bundling activity of SipC is essential for internalization as a *sipC* mutant lacking bundling activity is impaired in cell invasion. SipC dimerization / multimerization seems to be required for nucleation and a *sipC* mutant deficient for multimerization and actin nucleation failed to cause severe colitis in a mouse model (Chang *et al.*, 2007).

As SipC, SipA binds directly to actin (amino acids P446-R685) and modulates cytoskeleton dynamics in different ways (Galkin *et al.*, 2002; Higashide *et al.*, 2002). A *Salmonella* Typhimurium *sipA* mutant exhibits a 60-80% decrease in invasion compared to the wild-type strain (Perrett & Jepson, 2009), which can be correlated to the fact that SipA is a multifunctional effector. First, Zhou *et al.* (1999b) have observed that SipA reduces the critical concentration of G-actin in the cytosol required for polymerization. In addition, to facilitate polymerization, SipA also stabilizes actin filaments by displacing ADF / cofilin factor which stimulates actin depolymerization and by protecting F-actin from gelsolin severing (McGhie *et al.*, 2004). Moreover, SipA enhances actin cross-linking both by interacting with T-Plastin, a cellular bundling protein, and by enhancing SipC activity (Zhou *et al.*, 1999a; McGhie *et al.*, 2001). Interestingly, Perrett and Jepson have demonstrated that a *sipA* deletion induces a decrease in host cell invasion but without affecting the frequency of membrane ruffle formation (Perrett & Jepson, 2009). By visualizing the membrane ruffles in the absence of SipA, they have observed that SipA is required to ensure the spatial localization of actin rearrangement beneath invading *Salmonella* for efficient uptake of bacteria.

In contrast to SipA and SipC, SopB, SopE and SopE2 exert their activity into host cells by inducing actin polymerization in an indirect way. They activate RhoGTPases which are key cellular effectors that regulate actin cytoskeleton remodeling (Patel & Galan, 2005).

During *Salmonella* T3SS-1 dependent invasion, SopE and SopE2 effectors mimic GEF activity to activate RhoGTPases. Their activation induces actin polymerization by stimulating downstream cellular proteins such as N-WASP, WAVE and WASH which activate the Arp2/3 nucleator complex (Buchwald *et al.*, 2002; Schlumberger *et al.*, 2003). SopE specifically targets the Rho GTPases Rac and Cdc42 *in vitro* whereas SopE2 only activates Cdc42 (Friebel *et al.*, 2001). The role of Rac in *Salmonella* T3SS-1-dependent entry is well characterized but the role of Cdc42 is controversial. Criss *et al.* (2001) have demonstrated by using dominant negative forms and by pull-down assays that, in contrast to non-polarized cells, Cdc42 is not required and not activated during invasion of MCDK polarized epithelial cells. In the same way, Patel and Galan have observed that the depletion of Cdc42 by RNA interference (RNAi) in COS-2 and Henle-407 had no effect on membrane ruffling and efficient uptake (Patel & Galan, 2006). In contrast, a recent genome-scale RNAi screening in HeLA cells indicated that Cdc42 depletion induced a 70% decrease in invasion, suggesting that it is required for the T3SS-1-dependent invasion process (Misselwitz *et al.*, 2011). All together, it is difficult to conclude about the involvement of Cdc42 during T3SS-1-dependent entry.

Salmonella mutant strains lacking both *sopE* and *sopE2* are still able to invade cells in a SopB dependent way (Zhou *et al.*, 2001). SopB is an inositol phosphatase which shares homology with eukaryotic phopho-inositol (PI) phosphatases (Norris *et al.*, 1998). Like SopE and SopE2, SopB targets a GTPase from the Rho family, RhoG, but in an indirect manner since SopB activates the GEF protein that allows RhoG activation (Patel & Galan, 2006). Once

activated, RhoG induces actin polymerization at the entry site presumably by stimulating the Arp2/3 complex (Patel & Galan, 2006). A *sopB* deletion in *S*. Typhimurium induces a 50% decrease in invasion. But when *sopB* deletion is coupled with a *sopE* deletion, *Salmonella* uptake is drastically impaired (Zhou *et al.*, 2001), demonstrating that all the T3SS-1 effectors work in concert to trigger entry.

Recently, SopB was also shown to manipulate actomyosin contractility to mediate invasion. SopB recruits myosin II by activating RhoA and its Rho kinase downstream effector. In contrast to the process leading to actin polymerization during *Salmonella* entry, myosin II recruitment at the entry site is independent on Arp2/3 nucleator activity (Hanisch *et al.*, 2011).

In addition, different cellular proteins are involved during the T3SS-1 dependent entry of *Salmonella* without the identification of the bacterial effector responsible of this effect. This includes the focal adhesion kinase FAK, the Abelson tyrosine kinase Abl, and Shank3 (Shi & Casanova, 2006; Huett *et al.*, 2009; Ly & Casanova, 2009). During *Salmonella* uptake, FAK acts as a scaffolding protein, but not as a protein tyrosine kinase and its interaction with p130Cas is involved in actin reorganization and membrane ruffle formation (Shi & Casanova, 2006). But how *Salmonella* can nucleate assembly of focal adhesion-like complexes is still unclear and further research is needed to determine if this mechanism involves secreted bacterial effector proteins, other transmembrane host proteins, or both. Another scaffolding protein Shank3 also seems to regulate actin rearrangement during entry but the mechanisms leading to its recruitment and its activation have to be elucidated (Huett *et al.*, 2009). As the experiments were performed using HeLa cells which are highly permissive to the T3SS-1-dependent entry and with bacterial culture conditions that allow T3SS-1 expression, it is more probable that the *Salmonella* entry process inducing Shank3 recruitment is dependent on the T3SS-1. However, a T3SS-1-independent invasion process could also be involved because no mutant deficient for T3SS-1 expression was used as a control to analyze the involvement of Shank3.

Abelson tyrosine kinase Abl is also involved during *Salmonella* invasion as well as its effectors Abi1, a member of the WAVE2 complex, and CrkII (Ly & Casanova, 2009). Abi1 could thus enhance actin polymerization at the entry site but the role of CrkII during the invasion process remains poorly characterized.

2.3.2 Subversion of exocytosis machinery and membrane fusion during entry

Recent data indicate that membrane fusion is a major process involved during entry, suggesting that membrane ruffling requires the addition of intracellular membrane. A study focusing on the subversion of the host exocyst complex during *Salmonella* entry has showed cooperation between SipC and SopE (Nichols & Casanova, 2010). The exocyst is an octomeric complex (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, Exo84) involved in vesicular trafficking which directs post-Golgi vesicules at specific site on the plasma membrane prior to their fusion. Nichols and Casanova have demonstrated that the mature exocyst complex is recruited at the entry site through an interaction between its subunit Exo70 and SipC. A depletion of exo70 or Sec5, another component of the exocyst complex, impairs *S.* Typhimurium invasion. Moreover, they have shown that SopE activates the Ras-related protein RalA, a small GTPase required for exocyst complex assembly (Nichols

& Casanova, 2010). It appears that SopE and SipC effectors manipulate the host exocyst to bring new membrane at the entry site in order to allow the formation of membrane ruffles and internalization.

SopB is also involved in this membrane fusion process through its inositol phosphatase activity. Dai *et al.* (2007) have shown that SopB-generated PI3P at the entry site leads to the recruitment of VAMP8, a host V-SNARE protein that mediates fusion between early and late endosomes. Moreover, depletion of VAMP8 by RNAi induces a decrease in invasion rate of a wild-type *S.* Typhimurium strain which is equivalent to that obtained with a *sopB* mutant. Thus, it seems that SopB promotes invasion by manipulating eukaryotic vesicular trafficking probably to induce fusion of intracellular vesicles to the cell membrane at the entry site.

How these events of vesicle-membrane fusion interact with actin cytoskeleton rearrangements to trigger entry has not been investigated yet. These different processes may synergize to induce internalization since actin dynamics is closely related to the metabolism of phosphoinosides (Honda *et al.*, 1999; Sechi & Wehland, 2000). However, VAMP8 which seems to be a marker of membrane fusion involved during *S.* Typhimurium invasion does not co-localize with F-actin during entry (Dai *et al.*, 2007). In addition, it could be interesting to better characterize the role of the cellular factor IQGAP1 which is required for *Salmonella* uptake and acts following an interaction with actin and the RhoGTPases Rac1 and Cdc42 (Brown *et al.*, 2007). Indeed, IQGAP1 is known to regulate actin architecture and interestingly, it also seems to act as a regulator of exocytosis by interacting with Exo70 (Rittmeyer *et al.*, 2008). IQGAP1 could thus be one of the missing links between actin rearrangement and membrane fusion during *Salmonella* entry. Further studies could overcome this issue.

2.3.3 Phagosome closure and restoration of the host cell normal shape

As described above, inositol phosphatase activity of SopB drives to changes in cellular phosphoinosite concentrations at the bacteria/cell contact. In addition to generate PI3P at the entry site, translocation of SopB into host cells also induces $PI(4,5)P_2$ hydrolysis, which leads to an almost complete absence of $PI(4,5)P_2$ at the membrane invagination regions (Terebiznik *et al.*, 2002). By reducing the local concentration of $PI(4,5)P_2$, SopB destabilizes cytoskeleton-plasma membrane interactions, thus reducing the rigidity of the membrane and promoting invasion by facilitating the fission and the sealing of the future *Salmonella*-containing vacuole. In addition to SopB, SopD also contributes to membrane fission. Boonyom *et al.* have demonstrated that a *sopD* deletion, like the *sopB* mutant, leads to a decrease in membrane fission during invasion and that SopD is recruited at the bacterial invasion site dependently on the phosphatase activity of SopB (Bakowski *et al.*, 2007). Thus, SopD seems to cooperate with SopB and contribute to *Salmonella* uptake by facilitating membrane fission at the entry site leading to the formation of the SCV (Bakowski *et al.*, 2007).

Following the formation of intense membrane ruffling and internalization, the eukaryotic cell regains its normal shape, inducing the closure of the vacuole of endocytosis containing the bacteria. The restoration of actin cytoskeleton is promoted by the effector SptP, a tyrosine phosphatase which inactivates the RhoGTPases Rac-1 and Cdc42 by stimulating their intrinsic GTPase activity (Fu & Galan, 1998; Fu & Galan, 1999). The N- terminal region of SptP interacts with Rac-1 and Cdc42 and a structural study of SptP indicates that this effector mimics the

activity of host cell GAPs factors (Stebbins & Galan, 2000). Interestingly, SptP is regulated by two different mechanisms in order to delay its activity in host cell compared to that of SopE or SipA. A microscopy analysis revealed that SipA is injected earlier than SptP in the host cytoplasm, which would imply that SipA has a higher affinity for the exportation apparatus of the T3SS-1 than SptP (Winnen *et al.*, 2008). Moreover, SptP degradation by the host cell proteasome occurs later than SopE degradation (Kubori & Galan, 2003).

2.4 T3SS-1 contribution to *Salmonella* pathogenesis

The T3SS-1 is the main invasion factor of *Salmonella in vitro*. Nevertheless, its contribution to pathogenesis depends on the model used. *In vivo* studies with *S.* Dublin and *S.* Typhimurium serotypes have demonstrated that the T3SS-1 is essential for intestinal colonization and is required to induce enterocolitis in bovine, rabbit and murine models (Wallis & Galyov, 2000). In contrast, recent studies demonstrate that different serotypes of *Salmonella* lacking T3SS-1 still have the ability to invade *in vitro* cells of diverse origins and can be pathogenic in different *in vivo* infection models (Aiastui *et al.*, 2010; Rosselin *et al.*, 2011). In addition, it was shown that the T3SS-1 is not required for *Salmonella* internalization into a 3-Dimensional intestinal epithelium (Radtke *et al.*, 2010). Moreover, a SPI-1 mutant of *S.* Gallinarum exhibits a reduced invasiveness into avian cells but is fully virulent in adult chicken (Jones *et al.*, 2001). In *S.* Enteritidis and *S.* Typhimurium, the T3SS-1 is not essential during systemic infection of one week-old chicken or BalB/c mouse nor during the intestinal colonization of rabbit ileal loops (Coombes *et al.*, 2005; Jones *et al.*, 2007; Karasova *et al.*, 2010). Moreover, *S.* Senftenberg strains lacking SPI-1 are isolated from human clinical cases, suggesting that the T3SS-1 is dispensable by this serotype for the establishment of infection in humans (Hu *et al.*, 2008).

Taken together, these results indicate that T3SS-1- independent invasion mechanisms also play an important role in *Salmonella* infection and pathogenesis.

3. Invasion mechanisms independent of the T3SS-1

A *Salmonella* mutant, unable to express its T3SS-1 is still able to invade numerous cell lines and cell types and is shown to induce both intense and local membrane rearrangements (Rosselin et al., 2011). However, to date, little is known about the entry factors mediating these T3SS-1 independent invasion mechanisms. Here, we describe and sum up the state-of-art regarding these new invasion systems. Rck, PagN and HlyE are the three invasins identified as involved in *Salmonella* uptake. Moreover, Rosselin *et al.* (2011) have described that others unknown invasion factors exist although they are still not identified.

3.1 The Rck invasin

Among invasins that play a role in *Salmonella* invasion in a T3SS-1-independent way, Rck is clearly the best characterized. Rck is an 17kDa outer membrane protein (OMP) encoded by the large virulence plasmid of *S.* Enteritidis and *S.* Typhimurium (Heffernan *et al.*, 1992; Rotger & Casadesus, 1999). In addition to its ability to induce adhesion to and invasion of eukaryotic cells, Rck confers a high resistance level to complement killing by preventing the formation of the membrane attack complex (Heffernan *et al.*, 1992; Cirillo *et al.*, 1996; Rosselin *et al.*, 2010).

3.1.1 Rck structure

Rck is a member of an outer membrane protein family named "Ail/Lom family". This family consists of five members (Rck, Ail, Lom, OmpX and PagC) which are predicted to have eight transmembrane beta-sheets and four cell surface-exposed loops. Even if these proteins present a similar conformation, they have different functions. Rck and Ail (encoded by a chromosomal gene of *Yersinia Enterocolitica*) share the ability to promote serum resistance and epithelial cell invasion. These proteins do not exhibit homologous regions that could be related to these two identical roles. In Ail, the cell invasion property is associated with loop2 whereas loop3 and more precisely the region from the amino acids G113 to V159 was shown to be the minimal region of Rck required and sufficient for cell adhesion and invasion (Miller *et al.*, 2001; Rosselin *et al.*, 2010).

Another member of this family involved in virulence is PagC which is encoded by a *phoP*-regulated gene on the *Salmonella* chromosome and plays a role in intracellular macrophage survival in *Salmonella* (Miller *et al.*, 1989; Gunn *et al.*, 1995). Others members of this family are OmpX of *Enterobacter cloacae* (Mecsas *et al.*, 1995) and Lom, a protein expressed by bacteriophage λ from lysogenic *E. coli* (Reeve & Shaw, 1979) but none of them have known virulence-associated phenotype.

3.1.2 Rck regulation

A genetic screening performed in *S.* Typhimurium to identify genes regulated by SdiA (*s*uppressor of *d*ivision *i*nhibition), a transcriptional regulator of quorum sensing, has suggested that *rck* belongs to a putative operon called the "*rck* operon" whose expression is activated by SdiA in an Acyl Homoserine Lactone (AHLs)-dependent manner (Figure 6) (Ahmer *et al.*, 1998; Michael *et al.*, 2001).

The *rck* operon contains 6 open reading frames: *pefI*, *srgD*, *srgA*, *srgB*, *rck* and *srgC* (Figure 6). Two genes in this operon, *pefI* (*p*lasmid *e*ncoded *f*imbriae) and *srgA* (*s*diA-*r*egulated *g*ene), affect the expression and function of the *pef* operon located upstream of the *rck* operon and involved in the biosynthesis of the Pef fimbriae. *pefI* encodes a transcriptional regulator of the *pef* operon, and SrgA is a DsbA paralog that efficiently oxidizes the disulfide bond of PefA, the major structural subunit of the Pef fimbriae (Bouwman *et al.*, 2003). These fimbriae are involved in biofilm formation, adhesion to murine small intestine and fluid accumulation in the infant mouse (Baumler *et al.*, 1996; Ledeboer *et al.*, 2006). Also localized on the *rck* operon, *srgD* encodes a putative transcriptional regulator. Recently, it has been shown that SrgD acts in cooperation with PefI to induce a synergistic negative regulation of flagellar genes expression (Wozniak *et al.*, 2009; Wallar *et al.*, 2011). The remaining genes on the *rck* operon have unknown functions and encode a putative outer membrane protein, SrgB, and a putative transcriptional regulator, SrgC.

Another locus regulated by SdiA-AHLs has been identified during screening. This chromosomal locus encodes a single gene named *srgE* (STM1554) (Ahmer *et al.*, 1998). No function for SrgE is described but a computational approach has suggested that SrgE is secreted by the T3SS-1 (Samudrala *et al.*, 2009) (Figure 6).

As *E. coli* and *Klebsiella*, *Salmonella* lack an AHL synthase and thus do not produce AHLs. However, SdiA can detect and bind AHLs produced by others bacterial species (Michael *et al.*, 2001). SdiA is a LuxR homologue and has two functional domains. The C-terminal

region contains a predicted helix-turn-helix motif implicated in DNA binding and a N-terminal domain called "autoinducer domain" that interacts with AHLs. By NMR analysis, Yao *et al.* (2007) have shown that a direct interaction between SdiA and AHLs is required for SdiA folding and function.

Temperature also affects *rck* operon expression. At temperature below 30°C, the transcription of *rck* operon is repressed, while *srgE* is repressed only at temperature below 22°C (Smith & Ahmer, 2003)(Figure 6). As SdiA expression is not temperature regulated, another level of *rck* operon regulation remains to be identified.

In addition, a transcriptomic study has shown that Hha and its paralogue YdgH could be involved in the regulation of the *rck* operon (Vivero *et al.*, 2008).

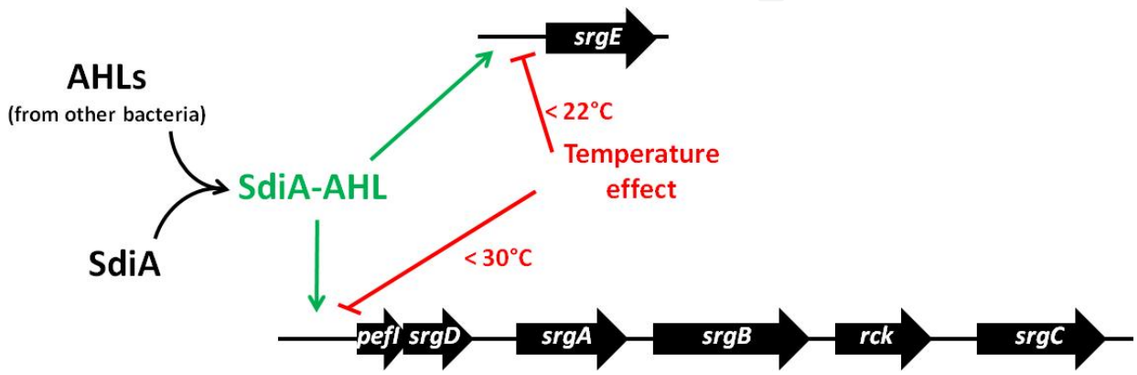


Fig. 6. Regulation of *rck* operon expression. When bound to AHLs, SdiA activates the expression of *rck* operon and *srgE*. Under temperatures that are lower than 30°C or 22°C, the expression of *rck* operon and *srgE* is inhibited, respectively.

3.1.3 Rck-mediated entry mechanism

When a *rck* mutant is grown under swarming conditions known to induce SdiA expression, a 2-3 fold decrease in epithelial cell invasion has been observed compared to the wild-type strain (Rosselin *et al.*, 2010). Moreover, in standard culture conditions, Rck overexpression leads to an increase in invasion.

By using both an initially non-invasive *E. coli* strain overexpressing Rck and latex beads coated with the minimal region of Rck inducing invasion (G113-V159), it was demonstrated that Rck alone is able to induce entry by a receptor-mediated process. This mechanism promotes local actin remodelling and weak and closely adherent membrane extensions (Rosselin *et al.*, 2010). *Salmonella* can thus enter cells through two distinct mechanisms: the Trigger mechanism mediated by its T3SS-1 apparatus and a Zipper mechanism induced by Rck. A model of this Zipper entry process is shown in figure 7. Following an interaction between Rck and its unknown cellular receptor, it was shown by using specific drugs and a dominant negative form that the class I PI3-kinase made of the p85-p110 heterodimer is required for Rck mediated entry. Moreover, Rck induces an increase in the interactions between p85 and phosphotyrosine residues, leading to the class I PI3-Kinase activation. Pharmacological approaches or Akt knockout cells also demonstrate that Akt is necessary to Rck-mediated internalization. Probably by binding to $PI(3,4,5)P_3$, Akt is recruited at the entry site and activated in a PI3-Kinase dependent way (Mijouin *et al.*, 2012).

The GTPase Rho is not involved during the Rck entry process but the use of dominant negatives demonstrates that Rac1 and Cdc42 are required (Rosselin *et al.*, 2010). Moreover, Rac1 is recruited at the entry site and Rck induces an increase in the level of active Rac1, demonstrating that it activates this GTPase (Rosselin *et al.*, 2010; Mijouin *et al.*, 2012). Rac1 activation occurs downstream on the PI-3kinase activity. Finally, overexpression of inhibitory constructs has shown that actin polymerization is dependent on the Arp2/3 nucleator complex during Rck-mediated entry (Rosselin *et al.*, 2010).

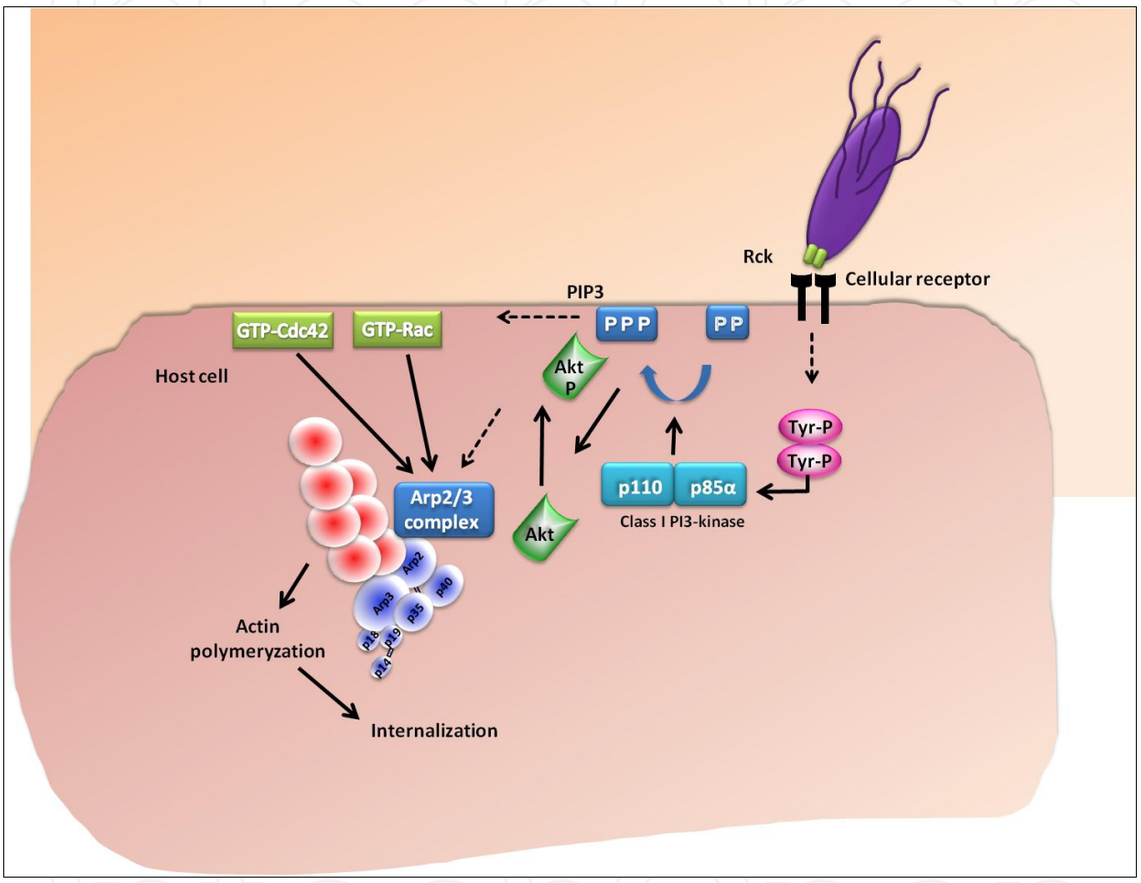


Fig. 7. Model of the cellular signaling pathway induces by Rck and leading to invasion. This Zipper entry process involves different cellular proteins: Class I PI3-Kinase, Akt, Rac and Cdc42, the Arp2/3 complex and actin. Dotted arrows: possible signalling events and/or interactions.

3.1.4 Rck contribution to *Salmonella* pathogenesis

The role of Rck in *Salmonella* invasion is clearly demonstrated *in vitro*, but its role in *Salmonella* pathogenesis is poorly understood. Indeed, *in vivo* conditions allowing *rck* expression are unclear. The fact that Rck is regulated by SdiA, a quorum sensing regulator suggests an intestinal role of this invasin (Ahmer *et al.*, 1998). However, the mechanisms leading to SdiA activation and *rck* expression are not well characterized. SdiA activation has been investigated in different hosts including rabbit, guinea pig, cow, turtle, mouse, pig and chicken but no activated-SdiA has been detected in the gastro-intestinal tract of

these animals, except for turtles which were found to be co-infected with AHLs-producing *Aeromonas hydrophila* (Smith *et al.*, 2008). Another work has demonstrated that SdiA is activated in mice previously infected with *Yersinia enterocolitica*, which is able to synthesize AHLs (Dyszel *et al.*, 2010). However, in these conditions, SdiA activation does not confer a fitness advantage for *Salmonella* intestinal colonization in comparison to a *sdiA* mutant (Dyszel *et al.*, 2010). These results suggest that even if SdiA activation is achieved when AHL-producing strains colonize the gastrointestinal tract, it is not always sufficient to induce the expression of its regulon including *rck*. To assess the role of SdiA and its regulon during intestinal infection, Dyszel *et al.* (2010) have constructed a *Salmonella* strain able to produce AHLs. After co-infection of mice with the AHL-producing *Salmonella* strain and a *sdiA* mutant, it was shown that the constant activation of SdiA confers a selective advantage to *Salmonella*. Moreover, a loss of this selective advantage was observed when all individual SdiA-regulated genes were deleted, including *rck*, suggesting a role during intestinal colonization. Nevertheless, an *in vivo* model allowing a physiological activation of SdiA would be needed to assess the contribution of Rck to intestinal infection.

In addition, as *rck* is also regulated by an unidentified SdiA-independent system (Smith *et al.*, 2008), it is conceivable that Rck invasion mechanism is not restricted to the gastrointestinal tract. Considering that Rck is also involved in the resistance to complement killing, a systemic function of Rck is possible.

3.2 The PagN invasin

In addition to the T3SS-1 and Rck, the PagN outer membrane protein has also been identified as being involved in *Salmonella* invasion (Lambert & Smith, 2008). *pagN*, is localized on the centisome 7 genomic island and is widely distributed among *Salmonella enterica* serotypes (Folkesson *et al.*, 1999). The *pagN* ORF was originally identified during a Tn*phoA* random-insertion screening in *S.* Typhimurium performed to discover PhoP-activated genes (Belden & Miller, 1994).

3.2.1 PagN structure

PagN shares similarity to both the Tia and Hek invasins of *E. coli*, and presents 39% and 42% identity in amino acids with these two invasins, respectively. Tia and Hek are predicted to have eight transmembrane regions, four long exposed extracellular loops and three short periplasmic turns (Mammarappallil & Elsinghorst, 2000; Fagan *et al.*, 2008). Thus, PagN probably adopts a similar conformation as that of Hek and Tia.

3.2.2 PagN regulation

The *pagN* (*phoP*-activated gene) gene is *phoP*-regulated. The PhoP/PhoQ system is a two-component transcriptional regulatory system which modulates transcription of a multitude of virulence genes in *Salmonella*. This regulatory system is composed of the PhoQ sensor kinase (located at the membrane) and the PhoP response regulator. In response to specific stimuli such as acidified macrophage phagosome environment or low Mg^{2+} concentration, PhoQ is auto-phosphorylated and transfers its phosphates to the cytoplasmic DNA-binding protein PhoP, regulating specific genes.

3.2.3 PagN-mediated entry mechanism

Lambert & Smith (2008) have demonstrated that *pagN* deletion in *S.* Typhimurium leads to a 3-fold decrease in invasion of enterocytes without altering cell adhesion. At the cellular level, the PagN-mediated entry process is poorly characterized. It was only shown that actin polymerization is required during invasion (Lambert & Smith, 2008) and that PagN is able to interact with extracellular heparin proteoglycans (Lambert & Smith, 2009). However, proteoglycans cannot transduce a signaling cascade so it is probable that they act as co-receptors for invasion and not as the receptor itself. Moreover, although an interaction between PagN and heparin has been suggested, no clear heparin-binding motif was detected. Moreover, all PagN loops are required for invasion in epithelial cells (Lambert & Smith, 2009).

3.2.4 PagN contribution to *Salmonella* pathogenesis

PagN is required for survival in BALB/c mice (Heithoff *et al.*, 1999) and a *pagN* mutant is less competitive to colonize the spleen of mice compared to its parental strain (Conner *et al.*, 1998). However, the role of PagN in *Salmonella* pathogenesis is still unclear. *pagN* is activated by PhoP and thus maximally expressed intracellularly, a condition in which the SPI-1 island encoding the T3SS-1 is downregulated (Conner *et al.*, 1998; Heithoff *et al.*, 1999; Eriksson *et al.*, 2003). Lambert & Smith (2008) thus postulate that bacteria which exit epithelial cells or macrophages have an optimal level of PagN expression, but have a low T3SS-1 expression and this might facilitate subsequent interactions with mammalian cells that the pathogen encounters after host cell destruction.

3.3 The HlyE invasin

The *hlyE* gene is localized on the *Salmonella* pathogenicity island SPI-18 and is expressed by serotypes associated with systemic infection in humans including *S.* Typhi and *S.* Paratyphi A (Fuentes *et al.*, 2008). The *hlyE* sequence shares more than 90% identity with that of *Escherichia coli* HlyE (ClyA) hemolysin.The HlyE protein is able to lyse epithelial cells when exported from bacterial cell via outer membrane vesicule release (Wai *et al.*, 2003). Recently, Fuentes *et al.* (2008) have demonstratred that HlyE contributes to epithelial cell invasion of *S.* Typhi. However, the cellular events leading to HlyE-mediated invasion have not been characterized.

In vivo studies have shown that HlyE contributes to colonization of mouse deep organs (Fuentes *et al.*, 2008). However, how HlyE participates in establishment of systemic infection of *Salmonella* is not well understood.

3.4 Non-identified invasion factors

Several studies have revealed that invasion systems in *S.* Typhimurium and *S.* Enteritidis are not restricted to the T3SS-1, Rck and PagN. Indeed, a strain which does not express *rck*, *pagN* and the T3SS-1 is still able to significantly invade fibroblasts, epithelial and endothelial cells (Rosselin *et al.*, 2011). Results obtained by Aiastui *et al.* (2010) and van Sorge *et al.* (2011) have reinforced the idea that non-identified invasion factors are involved during entry into these cell types since *Salmonella* strains that cannot express the

T3SS-1 still enter into epithelial cells, endothelial cells and fibroblasts in a significant way. Moreover, invasion analyses of a 3-D intestinal epithelium by *S.* Typhimurium have also highlighted the fact that *Salmonella* possess non-characterized invasion factors (Radtke *et al.*, 2010).

Actin network remodeling and membrane rearrangements induced by these unknown factors have been visualized by confocal and electron microscopy as well as both Zipper-like and Trigger-like membrane alterations (Rosselin *et al.*, 2011). Identification of these factors is required to confirm these observations. It suggests that *Salmonella* express non-identified invasins able to mediate a Zipper process and factor(s) other than the T3SS-1 that induce Trigger-like invasion process(es). Type IV or type VI secretion systems are good candidates to induce Trigger-like cellular structures as they are able to translocate proteins directly into the host cell cytosol and as they are major virulence determinants involved in the pathogenesis of diverse Gram-negative bacteria (Oliveira *et al.*, 2006; Filloux *et al.*, 2008; Blondel *et al.*, 2010).

These observations thus open new avenues for identification of new invasion factors.

4. Conclusions and perspectives

Until recently, it was accepted that *Salmonella* enter cells only via its T3SS-1, which mediates a Trigger entry process. The T3SS-1-dependent invasion system has been widely described in the literature both at the bacterial and the cellular molecular levels. Moreover, the requirement of the T3SS-1 during intestinal and systemic infections has been demonstrated in some animals (Wallis & Galyov, 2000). However, an increasing number of reports describe that different serotypes of *Salmonella* can induce host infection without a functional T3SS-1 (Penheiter *et al.*, 1997; Jones *et al.*, 2001; Karasova *et al.*, 2010). This has been demonstrated with T3SS-1 mutants but also with clinical *Salmonella* strains (Hu *et al.*, 2008). However, the majority of *Salmonella* invasion system studies have focused on the T3SS-1 and we have little information concerning the T3SS-1-independent entry processes. Several invasins including PagN, Rck and HlyE have been recently identified in *Salmonella* and different investigations have provided evidences for other non-identified invasion factors (Aiastui *et al.*, 2010; Radtke *et al.*, 2010; Rosselin *et al.*, 2011; van Sorge *et al.*, 2011). In addition, the vast majority of this information has been obtained in a mouse model and with *S.* Typhimurium and much less data are available for other serotypes especially those adapted to pigs, cattle or poultry which represent major reservoirs of *Salmonella*. *Salmonella enterica* contain, over 2,500 diverse serotypes that have different host ranges, and cause diseases with severity ranging from subclinical colonization to serious systemic disease. Because the essential feature of the pathogenicity of *Salmonella* is its interaction with host cells, the identification of new entry routes could, in part, explain their different host ranges and disease symptoms.

The finding that *Salmonella* serotypes use different cell receptors and cell routes for host infection shows that the contribution of *Salmonella* genes to pathogenesis may be more complex than previously thought. These findings are changing our classical view of *Salmonella* pathogenicity. This new paradigm will modify the understanding of the mechanisms that lead to the different *Salmonella*-induced diseases and could allow us to revisit the host specificity bases.

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