Food Research International 45 (2012) 842–851

Contents lists available at SciVerse ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Review

Salmonella Typhimurium general virulence factors: A battle of David against Goliath?

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ARTICLE INFO

Article history:
Received 23 February 2011
Accepted 12 August 2011

Keywords:
Salmonella Typhimurium
Host-interaction
SPI
Virulence
TCS

ABSTRACT

The genus *Salmonella* is the most common agent causative of foodborne diseases. Although genus *Salmonella* members are genetically close microorganisms, they show wide variations in host-specificity, virulence and disease manifestations. Salmonellosis caused by contaminated water or food is usually present as two clinical forms: typhoid fever and nontyphoidal diseases. The latter producing gastroenteritis is frequently caused by *Salmonella* Typhimurium and *Salmonella* Enteritidis. The nontyphoidal *S.* Typhimurium infection involves the following steps: bacterial adhesion, invasion, SCV maturation and replication. During these steps there is a strong interaction between host and pathogen, in which the pathogen must resist different host defense mechanisms. In each of these stages the bacteria modulate the expression of diverse groups of genes, most of which are encoded in *Salmonella* pathogenicity islands (SPI). This modulation is in general under control of the so-called two-component systems (TCS). The TCSs are capable of sensing different environmental conditions and trigger a physiological response. Currently, the risk of contracting salmonellosis disease has been considerably increased due to the emergence of new serovars showing multiple-drug resistance that presents a high risk to human health. In this work, we summarize the new advances in the study of host–pathogen interactions during the *Salmonella* infection that leads to the establishment of the disease. This finding highlights the role of the *S.* Typhimurium secretion systems and effectors during infection. In addition, we mentioned some strategies that could be explored in order to take control of *Salmonella* infections.

Contents

1. Introduction

Salmonella spp., a ubiquitous Gram-negative bacterium, is a worldwide spread facultative enteropathogenic bacteria (Aarts et al., 2010; Betancor et al., 2010; McGhie, Brawn, Hume, Humphreys, & Koronakis, 2009; Thiele et al., 2011). In the course of evolution, this organism has had to survive to environmental changes by modulating the genes expression. The adaptation of *Salmonella* to these environmental changes is essential for pathogenicity, which affects the human health as well as the global economy.

According to the Centers for Disease Control and Prevention (CDC) and the World Health Organization, Food and Agriculture

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doi:10.1016/j.foodres.2011.08.009

Organization of the United Nations (2004) members of the genus *Salmonella* are divided into only two species: *Salmonella enterica* and *Salmonella bongori* (Brenner, Villar, Angulo, Tauxe, & Swaminathan, 2000). *S. enterica* has six subspecies (i.e., *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*) each of which consists of various serovars, having identified more than 2500 (Sanchez-Jimenez, Cardona-Castro, Canu, Uzzau, & Rubino, 2010; Soyer, Orsi, Rodriguez-Rivera, Sun, & Wiedmann, 2009). There are different ways to name each serovar, such as *S. enterica* subspecies *enterica* serovar Typhimurium, also referred as *Salmonella* Typhimurium or simply *S.* Typhimurium (Brenner et al., 2000). *S. enterica* subspecies *enterica* is a widely spread pathogen mainly present as common inhabitants of humans as well as animals (Ibar et al., 2009; Ribeiro et al., 2006; Stevens, Humphrey, & Maskell, 2009).

Although members of the genus *Salmonella* are genetically close, there are wide variations in the host-specificity, virulence and disease manifestations. The acquisition or loss of certain genes, plays an important role in the evolution of different serovars, which are classified by the antigen type present in the cell membrane lipopolisacharide (LPS) like the somatic O, flagellar H or Vi antigen as well as by proteomic analysis (de Jong & Ekdahl, 2006; Dieckmann, Helmuth, Erhard, & Malorny, 2008).

Salmonellosis includes different clinical cases such as: i) typhoid-like disease, whose infectious agents, *S.* Typhi and *S.* Paratyphi, may cause human death, and ii) non-typhoid disease, limited to infection in the lining of the small intestine causing gastroenteritis mainly by *S.* Enteritidis and *S.* Typhimurium, although the latter causes systemic disease in mice (Raffatellu et al., 2006).

The most frequent source of infection is raw or poorly cooked foods, especially foods containing egg, standing out the importance of controlling food processing. (Betancor et al., 2010; Bone et al., 2010; Braden, 2006). In recent decades, *S.* Enteritidis and *S.* Typhimurium topped the list of emerging pathogens and re-emerging in Latin America (Khakhria, Woodward, Johnson, & Poppe, 1997), being an endemic disease in Argentina (Eiguer, Caffer, & Fronchkowsky, 1990). The Division of Sanitary Surveillance of Rio Grande do Soul, Brazil, found more than 8000 people infected by *Salmonella* during the 1997 to 1999 period (Costalunga & Tondo, 2002). Similar events were also reported in Mexico and Colombia as well as Canada, New Zealand, Asia, the northern region of Africa United States, England and Wales (de Jong & Ekdahl, 2006; Gordon, 2009; Smith et al., 2010).

To date, the complete genomes of human-virulent serovars like *S.* Typhi CT18, *S.* Typhimurium LT2 and *S.* Paratyphi A ATCC 9150 have been sequenced, showing a difference of about 200,000 bp between them (Sabbagh, Forest, Lepage, Leclerc, & Daigle, 2010). The difference in DNA length, attributed to presence or absence of virulence plasmids, prophages and phages, makes a difference in the severity of the disease caused by each of these pathogenic serovars in humans (Sabbagh et al., 2010). In this sense, it has been reported that *S.* Typhimurium lacks genes required for the Vi antigen biosynthesis, although they are present in *S.* Typhi (Virlogeux, Waxin, Ecobichon, & Popoff, 1995). However, *S.* Typhimurium harbors a virulence plasmid containing the *spv* operon, required for intramacrophage survival, and the fimbrial operon (Sabbagh et al., 2010). Infection studies of *S.* Typhimurium carried out on mice allowed the identification of most of the virulence factors. Therefore, this serovar has been used as a model to elucidate the steps of typhoid fever pathogenesis and the host defense mechanisms (Boyle, Brown, Brumell, & Finlay, 2007; Monack, Bouley, & Falkow, 2004; Tran et al., 2010). Recent advances in creating an animal model for *S.* Typhi infection were reported by Stephen Libby and coworkers. They created a humanized mouse by transplanting stem cells from human umbilical cord blood and the resulting knockout mice, NOD-*scid*-IL2R (null), showed a hypersusceptibility to *S.* Typhi infection. (Libby et al., 2009). Similar models have been developed by others investigators (Mian, Pek, Chenoweth, Coombes, & Ashkar, 2011; Song et al., 2010).

The nontyphoidal *S.* Typhimurium infection process involves the following steps: bacterial adhesion, invasion, SCV maturation and replication, where the pathogen must overcome the defense mechanisms of the host in order to develop an intracellular lifestyle (Dorman, 2009). This adaptation occurs by the coordinated expression of many genes in response to specific signals present in the host. The two-component regulatory systems (two component system, TCS) are one of the most important mechanisms by which bacteria are able to respond to such variations (Majdalani & Gottesman, 2005; Stock, Robinson, & Goudreau, 2000). A large number of genes encoding virulence factors have been identified by the role they play in *Salmonella* pathogenesis. Interestingly, most of these genes are located closely to each other in the bacteria genome, in groups denominated pathogenicity islands (SPI, *Salmonella* Pathogenicity Islands) (Mills, Bajaj, & Lee, 1995). About 21 SPIs have been indentified in *Salmonella*, which were classified from SPI-1 to SPI-21. However, only 12 SPIs are present in *S.* Typhimurium: SPI-1 to 6, 9, 11, 12, 13, 14 and 16 (Haneda, Ishii, Danbara, & Okada, 2009; Saroj, Shashidhar, Karani, & Bandekar, 2008; Shah et al., 2005). The SPI are generally adjacent to tRNA genes and the GC composition differs from the bacterial chromosome (Hensel, 2004; Marcus, Brumell, Pfeifer, & Finlay, 2000). Although exhaustive investigations have been based on the study of the SPIs, only the gene product of five of them were well characterized by the role and time of function during infection, especially SPI-1 and SPI-2 genes, while the precise role of the rest is less known.

2. Two component signal transduction systems

For a long time, it has been considered that the two-component systems are the principal pathway of signal transduction developed by prokaryotic organisms, to connect determined environmental signals to specific cellular responses (Alves & Savageau, 2003; Maeda, Sugita, Sugita, & Omata, 2006; Ulrich & Zhulin, 2010). These TCSs control diverse physiological processes like the pathogenesis pathway, in response to extra or intracellular signals for an environmental change adaptation (Ulrich, Koonin, & Zhulin, 2005; Wuichet, Cantwell, & Zhulin, 2010). This kind of signal transduction mechanism has been described in all the bacterial pathogens, except for *Mycoplasma* species which possess a much reduced genome, as well in lower eukaryotes (Alex & Simon, 1994; Majdalani & Gottesman, 2005; Parkinson & Kofoid, 1992; Wuichet & Zhulin, 2010).

These systems consist of two conserved elements: the sensor, an internal membrane histidine protein kinase (histidine kinase, HKs) that senses environmental changes; and the regulator, a cytoplasmic transcription factor that regulates the response by DNA-binding (RR) (Stock et al., 2000). The sensor, by its periplasmic sensor domain, is capable of recognizing a protein or a small ligand to activate the system (Falke, Bass, Butler, Chervitz, & Danielson, 1997). Due to the wide range of the sensor ligand-binding domain reported, *Salmonella* can modulate the gene expression under different environmental changes (Anantharaman & Aravind, 2000; Aravind & Ponting, 1997; Galperin, Nikolskaya, & Koonin, 2001; Ponting & Aravind, 1997; Wuichet & Zhulin, 2010; Zhulin, Taylor, & Dixon, 1997). The response regulator (RR) produces, through its conserved aspartate phosphorylation, the adaptive response of bacterial cells modulating transcription of certain genes or operons (Gao, Mukhopadhyay, Fang, & Lynn, 2006; Stock et al., 2000).

The OmpR/EnvZ, RtsAB, PhoPQ, SsrAB, PmrAB and RcsCDB are the most important TCSs that control the virulence factors during the *Salmonella* infection (Beuzon, Unsworth, & Holden, 2001; Bijlsma & Groisman, 2003; Delgado, Mouslim, & Groisman, 2006; Ellermeier & Slauch, 2003; Fass & Groisman, 2009; Garcia-Calderon, Casadesus, & Ramos-Morales, 2007; Garmendia, Beuzon, Ruiz-Albert, & Holden, 2003; Gunn, Ryan, Van Velkinburgh, Ernst, & Miller, 2000; Jones, 2005; Mouslim, Delgado, & Groisman, 2004). The function and description of each one are shown below according to its role in the stages of *Salmonella* infection.

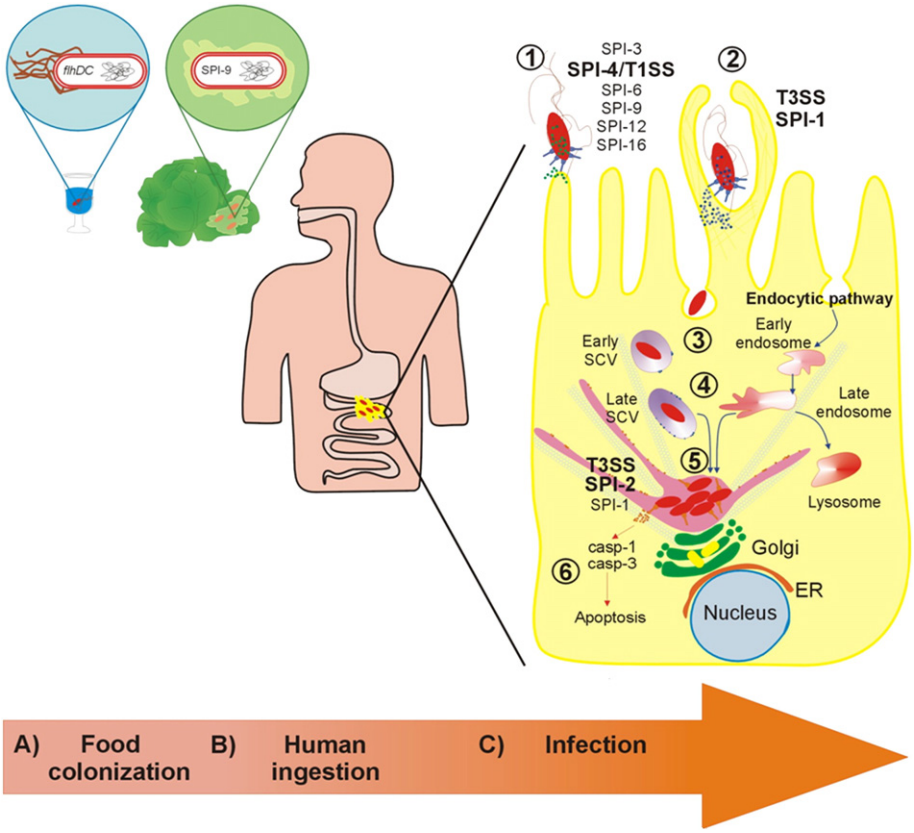

Fig. 1. Schematic representation of *Salmonella* infection. (A) *Salmonella* is able to freely live in many environmental conditions like conventional or unconventional sources (B) Once enter into the organism through contaminated water or foods, reaches the small bowel and traverse the intestinal mucus layer. (C) After the bacteria encountering the intestinal epithelial cell, the infection is established. To facilitate adhesion, *Salmonella* induce the expression of fimbriae and adhesins that are encoded in six SPI (1). The uptake of *Salmonella* induces several rearrangements in the cytoskeleton leading to membrane ruffles. This step is regulated by the expression of SPI-1 genes, and the translocation of the effectors into the cell is produced by the T3SS (2). The bacteria entry to the cell in an early SVC (3), that maturates to a late SVC (4). Several hours after invasion *Salmonella* initiated the replication (5). Finally, the bacteria induce the cell host apoptosis (6).

3. Infection of host cells by *Salmonella* Typhimurium: physiological and genetic processes

As we mentioned before, *Salmonella* enters the host by ingestion of contaminated water or food (Fig. 1A). Stomach acidity decreases the number of ingested pathogens, however the surviving population reaches the intestinal lumen where they can infect epithelial cells. At this point a host–pathogen interaction is initiated, the infection is established and the pathogen reaches the appropriate localization to survive in the host (Fig. 1B). These steps are the result of coordination in time and space of many virulence-gene expression encoded mainly in the pathogenicity islands (Fig. 1C). After overcoming the epithelial barrier and killing the host cells, *Salmonella* can spread into visceral organs through phagocytic cells of the lymphatic system thus causing the systemic infection (Dougan, John, Palmer, & Mastroeni, 2011). In epithelial cells the establishment of *Salmonella* infection covers the steps of adhesion, invasion, SCV maturation and replication.

3.1. Adhesion

Salmonella adhesion to host cells is the crucial step of infection which enables bacteria to colonize the host intestine (Fig. 1C, Step 1). It has been demonstrated that even when *Salmonella* can infect a healthy host, the probability of colonization increases during periods of host immunosuppression or physiological stress (Lambert, 2009). After ingestion, *S.* Typhimurium colonizes the mucosal cells and Peyer's patches. The presence of pathogens activates the signaling pathways of host cells, inducing broad range responses, such as inflammatory cytokines, which act as a physical barrier for new infections. These host-receptors interact with several adhesions factor of pathogens, including fimbrial, flagella, lipopolysaccharide (LPS) and capsule (Wagner & Hensel, 2011; Wanner & Chang, 1987). At least 13 different fimbriae and 3 nonfimbrial adhesins for *S.* Typhimurium colonization have been found, which are transiently expressed in a very controlled way in terms of time and space.

Six of the twelve SPIs present in *S.* Typhimurium are involved in bacterial adhesion to the host cell prior to the onset of the disease, they are:

1. The SPI-3 encoding for a protein belonging to the autotransporter family called MisL, which enables *S.* Typhimurium to bind fibronectin and appears to be an extracellular matrix adhesion factor involved in intestinal colonization (Dorsey, Laarakker, Humphries, Weening, & Baumler, 2005).
2. The SPI-4 harbors the *siiE* gene encoding for a nonfimbrial adhesion factor and the *siiABCDEF* operon, which encodes for the type one secretion system (T1SS) (Fig. 1C, Step 1). This secretion system, as well as T3SS, is an intricate machinery composed of many membrane-bound proteins assembling a structure called needle complex, similar to the flagellar complex, by which the bacterial effector proteins are delivered into host cells (Kubori, Sukhan, Aizawa, & Galan, 2000; Misselwitz et al., 2011). Even though the molecular function of T1SS is the translocation of effector proteins, it was recently demonstrated that it is also required for injecting the adhesin into the host cells. Moreover, it was reported that SiiC, SiiD and SiiF proteins, subunits of the T1SS, could function as a TolC-like outer membrane protein, a membrane fusion protein, and a transport ATPase,

respectively (Gerlach, Jackel, Geymeier, & Hensel, 2007; Gerlach et al., 2007). The SPI-4 genes are regulated, like those of SPI-1, by BarA/SirA TCS, in response to low oxygen, high osmolarity and a slightly alkaline pH value. Another protein that regulates the SPI-4 genes is the HilA SPI-1 encoded regulator; suggesting that both, SPI-1 and SPI-4, may be closely regulated and induced by the same environmental signals (Gerlach, Jackel, Geymeier, & Hensel, 2007). In addition, it has been demonstrated that *siiE* expression is negatively regulated by an atypical TCS, the Rcs phosphorelay system composed of the RcsC sensor, the RcsD phosphor-transfer and the RcsB response regulator (Majdalani & Gottesman, 2005). This system is activated by an unknown signal, probably present in all of those conditions that induce the RcsB phosphorylation. Remarkably, this system participates in the regulation of many virulence factors, such as flagella, biofilm and invasion effectors (see below) (Delgado et al., 2006).

3. The SPI-6, about which little is known, comprises genes encoding for a type six secretion system (T6SS), and the *safABCD* fimbrial operon (Lambert & Smith, 2008).
4. The SPI-9 contains three genes encoding for a T1SS and one for a large protein of 386 kDa (BapA). These genes show a 40% nucleotide identity to SPI-4 *siiCDEF* genes (Morgan, Bowen, Carnell, Wallis, & Stevens, 2007). Latasa et al. (2005) demonstrated that BapA participates in biofilms formation as well as host colonization and it is also secreted by T1SS (Gerlach, Jackel, Stecher, et al., 2007; Latasa et al., 2005). The *bapA* gene is under the positive control of the RpoS alternative sigma factor, required for starvation response as well in a variety of stressful conditions, like low pH, heat or cold shock, oxidative damage, hyper- or hypo-osmolarity and DNA damage. In addition, this gene is positively regulated by the mentioned Rcs phosphorelay system (Garcia-Calderon et al., 2007), while it is negatively controlled by RstA/RstB TCS involved in the bacterial resistance to different β-lactamic antibiotics (Hirakawa, Nishino, Yamada, Hirata, & Yamaguchi, 2003; Latasa et al., 2005).
5. The SPI-12 in *S.* Typhimurium is located next to the *proL* tRNA gene and contains the *oafA* gene, which encodes an O-antigen acetylase protein (Hansen-Wester & Hensel, 2002; Slauch, Lee, Mahan, & Mekalanos, 1996).
6. SPI-16, the least characterized SPI, contains three ORFs which could be mediating O-antigen glycosylation and cell surface variation (Sabbagh et al., 2010).

Although the role of the SPI-12 and -16 genes has not been demonstrated at the stage of adhesion, we include here both SPIs assuming that the O-antigen is also important to interact with the specific-host barrier. The O-antigen is the most exposed part of the bacteria LPS, which is extended from the cell surface and is anchored to the outer core (Kawasaki, in press).

In *S.* Typhimurium the LPS is composed of three principal domains: a hydrophobic lipid A, a short non-repeating core oligosaccharide and a distal polysaccharide termed O-antigen (O-ag). The O-ag consists of repeating polysaccharide chains of galactose, rhamnose and mannose (Raetz & Whitfield, 2002). According to the number of repetitive subunits linked to lipid A-core, the O-antigen can be classified as short or S region with 11 to 17 monosaccharides; a long or L region of 16 to 35 subunits, and very long or VL, with 35 to 100 or more subunits. The length of the polymer is responsible for the different host-interaction responses (Holzer, Schlumberger, Jackel, & Hensel, 2009). The long and very long forms of O antigen are involved in the resistance to neutrophils action, phage lysis, bactericidal action of serum complement and cationic peptides protection, and play an important role during the bacterial adaptation and the intestinal colonization (Burns & Hull, 1998; Groisman & Casadaban, 1987; Holzer et al., 2009; Joiner, 1988; Nevola, Stocker, Laux, & Cohen, 1985; Okamura & Spitznagel, 1982). *bapA* SPI-9 and the gene encoding for the O-antigen L-chain length, wzz_{st}, are positively regulated by the Rcs system (Delgado et al., 2006), suggesting that both are needed simultaneously. In addition, wzz_{st} gene is also positively controlled by PmrA/PmrB TCS involved in the lipopolysaccharide (LPS) modifications, polymyxin B and iron resistance, and in mice virulence (Delgado et al., 2006; Gunn et al., 2000). This system is directly activated by the presence of iron in the medium sensed by PmrB (Wosten, Kox, Chamnongpol, Soncini, & Groisman, 2000), and indirectly activated by low magnesium concentration through the PhoP/PhoQ regulatory system (Kato & Groisman, 2004).

Another putative pathogenicity island described in the *S.* Typhimurium adhesion, is the CS54 located between *xseA* and *yfgJ* genes, which harbors 5 genes (*shdA*, *ratB*, *ratA*, *sinI* and *sinH*) implicated in intestinal colonization (Kingsley et al., 2003). In addition, the *S.* Typhimurium genome contains 13 operons with homology to fimbrial biosynthesis genes, including *agf* (*csg*), *fim*, *pef*, *lpf*, *bcf*, *saf*, *stb*, *stc*, *std*, *stf*, *sth*, *sti* and *stj* (Humphries et al., 2003). Little is known about the function of these gene products, but it was determined that 6 operons (*lpf*, *bcf*, *stb*, *stc*, *std*, and *sth*) are required for intestinal persistence in mice (Weening et al., 2005). Moreover, the *fim* operon encodes type I fimbriae required for *Salmonella* biofilm formation in a BarA/SirA regulated pathway. Therefore, it might help the bacteria to persist within the host (Boddicker, Ledeboer, Jagnow, Jones, & Clegg, 2002; Brandl, 2006; Teplitski, Al-Agely, & Ahmer, 2006; Weening et al., 2005). Since BarA/SirA TCS is able to modulate genes involved in the bacteria adhesion encoded in the SPI-4 and those involved in biofilm formation, we can assume that both processes are together regulated to ensure the pathogen lifestyle.

Finally, full adherence of *S.* Typhimurium to M cells is brought about by a significant production of flagellum, the propulsion and motility apparatus, probably mediated by chemotactic responses (Marchetti, Sirard, Sansonetti, Pringault, & Kerneis, 2004; Stecher et al., 2008). This bacterial membrane component is associated to three promoter classes that controls more than 30 transcriptional hierarchy expressed genes encoding structural and assembly related proteins (Chevance & Hughes, 2008). The class 1 promoter controls the transcription of the flagellar master *flhDC* operon, while the products of two early genes encoded in this operon modulate the expression of class 2 promoters. This second promoter controls the expression of *flg*, *flh* and *fli* operons, required to assemble the flagellum hook-basal body. FliA protein functions as positive regulator of the last promoter class (Claret et al., 2007). The class 3 promoters are required for transcription of filament subunits, the MotA and MotB motor force generation proteins and components of the chemosensory system (Chevance & Hughes, 2008). So this huge transcriptional cascade is under control of different signals present in the host that converge in the control of *flhDC* operon transcription. One of the most important regulators involved in the flagella control is SlyA, showing a positive effect in the expression of this machinery. Other regulators act decreasing expression of *flhDC* operon, such as RcsCDB and RtsAB regulatory systems, PefI/SrgD complex, CsrB/CsrC and the uncharacterized EcnR regulatory protein (Delgado et al., 2006; Ellermeier & Slauch, 2003; Spory, Bosserhoff, von Rhein, Goebel, & Ludwig, 2002; Teplitski et al., 2006; Wang, Zhao, McClelland, & Harshey, 2007; Wozniak, Lee, & Hughes, 2009).

3.2. Invasion

After the pathogen is bound to the host cell surface, a new step of infection is initiated, the invasion (Fig. 1C, Step 2). This step is carried out in *S.* Typhimurium by a successful mechanism encoded and regulated by SPI-1 genes named "trigger process", where many new bacterial effectors hardly induce re-arrangement of the mammalian cell cytoskeleton in the interaction site, driving to a ruffling process that internalizes the bacteria cell (Ly & Casanova, 2007). In epithelial cell *Salmonella* is enclosed within an intracellular phagosomal compartment termed SCV by means of *Salmonella*-containing vacuole (Steele-Mortimer, 2008).

To establish this infection step, *S.* Typhimurium used the gene products of the best-characterized SPI-1. SPI-1 contains at least 29 genes involved in the synthesis of: a) invasion effectors like SipA (or Ssp,

Salmonella secreted protein), SipB, SipC, AvrA (avirulence factor A) and SptP (tyrosine phosphatase protein); b) HilA regulator protein controlling SPI-1 genes expression; c) the type 3 secretion system components (T3SS), like Inv, Spa and Prg proteins; and d) chaperones required for the delivery of the effectors (Fig. 1C, Step 2) (Marcus et al., 2000; McGhie et al., 2009; Sabbagh et al., 2010). The SipC and SipA proteins are responsible for inducing actin-cytoskeletal rearrangements which facilitate the entry of bacteria into epithelial cells and shoot the signal transduction cascades leading to migration of polymorphonuclear leukocyte (PMN) through the intestinal epithelium (Hayward & Koronakis, 2002; Lee et al., 2000). The role of SipB has not been determined, but this appears to have a dual function, effector and translocator for other SPI-1 effector proteins (like SptP, AvrA). In the host cell, SopE activates the receptors Cdc42 and Rac, also leading to actin-rearrangements and production of pro-inflammatory cytokines (Andrews-Polymenis, Baumler, McCormick, & Fang, 2010; Hapfelmeier et al., 2004; McGhie et al., 2009; Zhou, Mooseker, & Galan, 1999).

Specific regulation of SPI-1 genes is controlled by *hilA* gene product, a locus of this pathogenicity island, in response to environmental and genetic factors (Lucas et al., 2000). According to DNA binding domain, HilA regulator has been classified as a member of the OmpR/ToxR transcriptional activators family, able to bind to *prg*, *inv*/*spa*, and *sip* operon promoters (Jones, 2005). Due to the importance of the SPI-1 in the invasion process, the regulation of *hilA* gene expression has been the subject of a large number of research works. The *hilA* gene expression is induced in a BarA/SirA TCS-dependent manner by the intestinal lumen conditions: low oxygen, high osmolarity and mild alkaline pH. In addition, *hilA* produces the activation of T3SS/SPI-1 and SPI-1 effectors (Bajaj, Lucas, Hwang, & Lee, 1996). Moreover, the RstA/RstB TCS and HilD and HilC protein positively control the *hilA* expression (Jones, 2005).

Following the engulfment of *Salmonella*, the host cell cytoskeleton is repaired by a SptP-mediated process opposed to the activity of SopE (Fig. 1C, Step 3) (McGhie et al., 2009). The low Mg^{2+} concentration within vacuoles (SCV) represses the *hilA* expression by PhoP/PhoQ system (Groisman, 2001), turning off the T3SS/SPI-1 activity. Furthermore, it has also been reported that the RcsCDB phosphorelay system represses the transcription of *hilA*, *invF* and *invG* SPI-1 genes under unknown signal (Mouslim et al., 2004). Despite of the PhoP and RcsB regulators, *hilA* is repressed by complex networks, suggesting that while the SPI-1 genes must be strongly inhibited, the SPI-2 genes expressions are activated by PhoP at the same time in order to establish the subsequent stages of systemic infection (Jones, 2005).

3.3. SCV maturation

After *Salmonella* is engulfed into SCV, these vacuoles pass through a maturation process. The secreted effectors from SopE and SopB T3SS/SPI-1 and SpiC T3SS/SPI-2 are used as endosomal stage markers. In this sense, it has been postulated that SopB is required to divert SCV trafficking from endosomal maturation and to delay SCV-lysosomal fusion (Fig. 1C, Step 3 and 4), while SpiC is required for fusion inhibition of the late endosomes/lysosomes with SCV (Bakowski, Braun, & Brumell, 2008; Mallo et al., 2008).

S. Typhimurium SPI-2 harbors more than 40 genes, including those encoding the SsrA/SsrB TCS (*ssr* regulatory operon), the T3SS/SPI-2 (*ssa* operon); the secretion system chaperones (*ssc* operon) and the secretion system effectors (*sse* operon) (Hensel, Nikolaus, & Egelseer, 1999; Marcus et al., 2000; Ochman, Soncini, Solomon, & Groisman, 1996). Even though the signal that induces activation of SsrA/SsrB TCS to control *sse*, *ssa* and *ssc* genes expression is unknown, it has been well documented that this system is modulated by osmolarity and pH change in a OmpR/EnvZ-dependent pathway (Beuzon et al., 2001; Fass & Groisman, 2009; Lee, Detweiler, & Falkow, 2000).

Once the SCV maturation is finished, the vacuole travels through the host-cytoplasm to reach a perinuclear region near the Golgi (Fig. 1C, Step 4). The SCVs localization allows it the capture of nutrients from endocytic and exocytic transport vesicles in a pathway involving SifA, SseG and SseF SPI-2 effectors (Kuhle, Abrahams, & Hensel, 2006). Here, the SPI-3 effectors are also involved. SPI-3 contains the *mgtCB* operon encoding for the intramacrophage survival protein MgtC and for the high affinity Mg^{2+} transporter protein MgtB, which is under the PhoP/PhoQ TCS regulation (Blanc-Potard, Solomon, Kayser, & Groisman, 1999; Groisman & Ochman, 2000).

3.4. Bacteria replication

The bacterial replication starts after the SCV is located in the perinuclear region and when enough nutrients have been incorporated, the bacteria replication starts (Fig. 1C, Step 5). The main feature of this *Salmonella* virulence step is the presence of Sifs structures (*Salmonella*-induced filaments) which are specific tubulovesicular extensions. These structures are rich in lysosome-associated membrane proteins of late endosomes/lysosomes (known as Lamps, LIMPs or lgps). In fact, it has been postulated that they are produced by a fusion of SCV with late endosomes/lysosomes.

These Sifs structures are extended from SCV throughout the microtubule network (Rathman, Sjaastad, & Falkow, 1996; Stein, Leung, Zwick, Garcia-del Portillo, & Finlay, 1996). Although the function of Sifs during infection remains unknown, it has been demonstrated that SifA activity as well as PipB2, SseJ, SseF and SseG SPI2-effectors are required. Probably, the function of SifA is the inhibition of the microtubule-based motor kinesin recruitment on the SCV, while PipB2 enhances the extension of Sif tubules from the SCV/MTOC (microtubule organizing center). A similar Sif enhancement is carried out by SseF and SseG, while SseJ and SpvB act repressing the Sif tubule formation (Drecktrah et al., 2008; Drecktrah, Knodler, Howe, & Steele-Mortimer, 2007).

4. Resistance to the host-defense mechanisms

During this step, a host anti-bacterial cascade is activated as defense barrier. The host-defense includes the acidification of phagosome lumen, the activation of cationic proteins and production of antimicrobial peptides such as defensins. In order to overcome this barrier, the pathogen responds by activating the global regulator PhoP/PhoQ TCS. This system is involved in acid tolerance and resistance to cationic peptides, inducing the activation of the *pagD* and *pagC* SPI-11 genes required for intramacrophage survival (Sabbagh et al., 2010). In addition to SsrAB, PhoP/PhoQ system controls the expression of SPI2-T3SS effectors, which in turn reduces the exposure of *Salmonella* to the host defense battery such as antimicrobial defense, antigen presentation and reactive oxygen and nitrogen species generation (Bijlsma & Groisman, 2005; Deiwick, Nikolaus, Erdogan, & Hensel, 1999; McCollister, Bourret, Gill, Jones-Carson, & Vazquez-Torres, 2005).

It has been demonstrated that the PhoP regulator induces in *Salmonella* the expression of the PmrA encoding gene. PmrA is the regulator of the PmrA/PmrB TCS (Kato & Groisman, 2004; Kox, Wosten, & Groisman, 2000). The PmrA/PmrB system is directly activated by iron presence in a PhoP/PhoQ-independent pathway. PmrA/PmrB modulates the *pbgP*, *pmrF*, *ugd*, *pmrC*, *cptA* and wzz_{st} gene expression in order to modify the LPS, at lipid A and O-antigen levels, leading to cationic peptides resistance (Chamnongpol, Dodson, Cromie, Harris, & Groisman, 2002; Delgado et al., 2006; Groisman, Kayser, & Soncini, 1997; Tamayo, Prouty, & Gunn, 2005; Wosten et al., 2000; Wosten & Groisman, 1999). Changes in lipid A produce an LPS with a lower pro-inflammatory potential. As iron is limited in eukaryotic host, we can speculate that PmrA regulator only can modulate gene expression in a Mg^{2+} limitation-PhoP fashion within SCV (Nairz et al., 2009). However, it has been recently discovered that macrophage-phagosomes contain iron, at concentrations that are able to affect the activities of metal-responsive promoters. Consistent with this finding, the PmrA regulator could also be activated independently of PhoP during macrophage-bacterial survival (Taylor, Osman, & Cavet, 2009). In addition, the LPS is also

modified by the RcsCDB system activating the wzz_{st} gene transcription. wzz_{st} encodes for O antigen length determinant protein, associated with serum complement resistance (Delgado et al., 2006).

5. Host cell death and *Salmonella* dissemination

Many studies have postulated that the pathogen is able to cause cell death and systemic spread after replication, by mechanisms that involve the SPI-1 effectors in a number of cell types (Knodler, Finlay, & Steele-Mortimer, 2005). This hypothesis was supported by the observation that the SipB SPI-1 effector induces a direct activation of host caspase-1, resulting in cell death in a cytokines-dependent pathway (Fig. 1C, Step 6) (Layton & Galyov, 2007). In epithelial cells, but not in macrophages, the interaction of the SopB SPI-1 effector with the Akt host protein produces a lag in the apoptosis pathway (Knodler et al., 2005). Moreover, accumulated evidence suggests that T3SS/SPI-2 effectors are also involved in programmed cell death by different mechanisms, activated at different times after *Salmonella* infection (Hueffer & Galan, 2004). In this sense, it was postulated that T3SS/SPI-2 effectors along with the SpvB protein, following activation of Toll-like receptor, favor the pro-apoptotic responses (Hueffer & Galan, 2004; Knodler et al., 2005; Knodler, Winfree, Drecktrah, Ireland, & Steele-Mortimer, 2009; Patel, Hueffer, Lam, & Galan, 2009).

6. Host-defense against *Salmonella* infection

As is well known, *S.* Typhimurium is a human and animal intracellular "facultative" pathogen, able to live freely in many environmental conditions like in water and food. Depending on the colonized source, certain genes must be regulated in order to have enough nutrients for bacterial survival. In order to eliminate the pathogen, the host activated the innate immune response which is initiated by the cytokine and chemokine regulators. The production and secretion of these immune regulators are usually indicated by activation of the Toll-like receptors (TLR) and nucleotide binding-like receptors (NLRs) (Akira & Takeda, 2004; Franchi, Warner, Viani, & Nunez, 2009).

After ingestion the free-live bacteria become intracellular pathogen, able to initiate the non-phagocytic epithelial cell infection. Here the flagellar T3SS system could be induced to allow the microorganism motility until *Salmonella* reaches the intestinal lumen. *Salmonella* is attached to the epithelial cell surface by adhesins, biofilm and O-antigen capsule production, and turn off the flagellar motility (Fig. 1A). Such activities could be simultaneously controlled by a specific TCS, like the RcsCDB, or by a combination of more than one TCS (Delgado et al., 2006; Groisman & Mouslim, 2006; Pescaretti, Lopez, Morero, & Delgado, 2010; Pescaretti, Morero, & Delgado, 2009; Soncini, Garcia Vescovi, Solomon, & Groisman, 1996).

The *Salmonella* biofilm formation as well as other bacterial adhesion effectors such as LPS, flagella and capsule is able to induce inflammation by B lymphocytes and by the neutrophil activation pathway, which induces cell proliferation and cytokines secretion to remove pathogens by means of phagocytosis (Fig. 1B) (Edwards, Fisher, Presta, & Bodary, 1998; Hultgren, Jones, & Normark, 1996; Kawasaki, in press). It has been demonstrated that *Salmonella* flagellin the activator of the proinflammatory cytokine IL-8, is recognized by the Toll-like-5 receptor (TLR5) expressed in the basal surface of epithelial cells (Uematsu & Akira, 2009; Zeng et al., 2003). While the LPS from *Salmonella* outer membrane is sensed by Toll-like receptor 4 (TLR4) and is unable to induce the proinflammatory cytokine IL-8 (Takeuchi & Akira, 2010; Zeng et al., 2003).

Salmonella survives this attack and binds to epithelial cells by induction of T3SS/SPI-1 effectors, which are extracellulary secreted into the host cell cytoplasm (Fig. 1C). These *Salmonella* effectors stimulated the nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) of many different host cells types (Franchi et al., 2009; Inohara & Nunez, 2003). Specifically, the induction of NOD1 or NOD2-*Salmonella* dependent is promoted by the SPI-1 effectors like SopE, to activate the NF-κB and MAPKs pathway and produces inflammation (Franchi et al., 2009; Inohara & Nunez, 2003). The SopE effector can also stimulates the action of MAP kinases (mitogen-activated protein), Erk (extracellular signal-regulated kinase), JNK (terminal kinase) and p38 driving membranes ruffling (Galan & Zhou, 2000; Hall, 1998).

Following engulfment, the epithelial cell response against SCV is associated with the endocytic pathway (Fig. 1C, Step 3 and 4). Finally, *Salmonella* replication (Fig. 1C, Step 5) within SCV kills host cells in a SipA and SipB dependent apoptotic way, activating caspase-3 and 1, respectively (Fig. 1C, Step 6) (Andrews-Polymenis et al., 2010; Srikanth et al., 2010).

Broz et al. (2010) reported that the *Salmonella* within macrophage activates the NLRP3 and NLRC4-dependent caspase 1. Moreover, it has been demonstrated that the NLRC4 responds to SPI-1 T3SS as well to SPI-2 T3SS-secreting flagellin (Broz et al., 2010; Mariathasan et al., 2004). However, *Salmonella* can survive in macrophages thus facilitating its systemic dissemination (McGhie et al., 2009). It was also established that many other host factors are stimulated after 6 h of infection, these include natural resistance-associated macrophage protein 1(Nramp1), gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α) and inducible nitric oxide synthase (iNOS) at later time points (Andrews-Polymenis et al., 2010). The IFN-γ induces the host protective immunity against the SCV presence, by reactive oxygen and nitrogen species generation. These species act by repressing the SPI2-T3SS effectors expression in order to avoid the *Salmonella* replication step (McCollister et al., 2005).

7. Prevention and control of *Salmonella*: strategies for development of vaccines and antibiotics

S. Typhimurium is adapted to colonize human hosts and cause clinical pathologies characterized by gastroenteritis associated with intestinal inflammation and diarrhea. The conventional source of human contamination has been related to water, poultry or poultry product. However, recent evidences taken from salmonellosis outbreaks in many countries have demonstrated that this microorganism has also adapted to new environments leading to unconventional food sources for *Salmonella* contamination. These new sources include peanut butter, vegetables, vegetarian snack food, dry puffed breakfast cereal, microwaveable pot pies, and hot peppers (Tauxe, 2009). From it observation is possible to highlight the importance of exerting stricter controls of food-processing to ensure the complete elimination of bacteria, thereby reducing the incidence of future outbreaks.

The ability of the pathogen to colonize new sources is explained by the presence of bacterial genetic determinants that can be modulated under environmental changes. Phylogenetic studies have suggested that the virulence evolution of *S.* Typhimurium at molecular levels was carried out by horizontal transference of multiple genetic elements, like pathogenicity islands, plasmids, and prophages (Sabbagh et al., 2010). So, the presence of two T3SS systems (T3SS/SPI-1 and T3SS/SPI-2), with the same function with a different substrate of virulence proteins, there is strong evidence that the horizontal gene transfer can occur, under two different events in this case (Hensel, 2004). Such an evolution process could be also responsible for the emergence of multidrug-resistant *S.* Typhimurium strains, which are able to form a strong biofilm on food surfaces like ground beef, sausages, cheese and other dairy products (Kim & Wei, 2007). The increased occurrences of this *S.* Typhimurium strain have become another public health concern that has to be considered. Therefore, the development of new diagnostic tools, vaccines and antimicrobial chemotherapies targeting specific pathogenesis factors that show a sporadic distribution could be the strategy for successful control and prevention of *Salmonella* infection. However, it has been

demonstrated that the use of specific-*S*. Typhimurium factors in this prevention could not be extrapolated to relate *S*. Typhi control. Therefore, more general infection pathways such as secretion system formation or global virulence regulatory systems would be an appropriate way to address this issue.

Two kinds of vaccines can be developed to prevent *Salmonella* infection. The first one using a purified effector-protein combined with biomolecules as adjuvants. The identification and function of each virulence secreted effector in *Salmonella* pathogenesis carried out in many different cells as well as in mice, indicated that the MisL, SopB and SopB are all good candidates for vaccines development. However, in most of cases the challenge of a single effector vaccine could not guarantee enough protective effects, because it is involved in specific host-interaction. In this way, many investigators exploited the used of these effector proteins to design *Salmonella* recombinant vaccines (Cheminay & Hensel, 2008; Husseiny, Wartha, & Hensel, 2007; Pulickal & Pollard, 2007).

The second type of vaccine is the development of live-attenuated strains, where target genes are eliminated and the resultant mutants are supplied in combination with adjuvant to facilitate its absorption. These attenuated vaccines have an advantage over the first type, because they are able to decrease or eliminate the induction of disease symptoms, maintaining immunogenic activity. For instance, inhibition of SopB phosphoinositide phosphatase activity attenuates *Salmonella*, which is an interesting case because this effector is involved in cytoskeletal reorganization induction and inhibition of the epidermal growth factor receptors degradation (Patel & Galan, 2005). Some studies have attempted to elaborate this second type of vaccines by removing genes encoding for T3SS functional formation and/or for global transcriptional regulator TCS from genomes, with concomitant blockage of more than one host-interaction hence guaranteeing the host-protection. In addition, it has been demonstrated that T3SS structural components are well conserved in many bacterial pathogens, while TCSs were found in all three superkingdoms genomes making them attractive targets for improved vaccines and new antimicrobial peptides (Muller, Feldman, & Cornelis, 2001). Mouslim et al. (2004) described the Rcs system as a good candidate for live-vaccine development, they reported that the *rcsC* sensor gene point mutation resulted in constitutive activation of the system dramatically attenuating *Salmonella* virulence, displaying a defective invasion in non-phagocytic cells and poor survival within macrophages, while retaining the ability to protect mice upon challenge with wild-type strain (Mouslim et al., 2004).

The antimicrobial chemotherapy industry is constantly growing as a battleground against emergence of new multidrug-resistant strains. A generic review of the latest strategies employed is well described (Chalon et al., 2011). Good examples of these strategies can be found in the following works. Firstly, Hudson et al. (2007) demonstrated that the small-molecule inhibitors like salicylanilides inhibit *S*. Typhimurium T3SS-1, preventing secretion effectors and invasion of cultured epithelial cells (Hudson et al., 2007). Secondly, Pomares, Delgado, Corbalan, Farias, and Vincent (2010) showed that the dual combination of membrane-permeabilizing $(KFF)_3K$ synthetic peptide and microcin J25 (MccJ25) inhibits *in vitro S*. Typhimurium cell growth and its replication within macrophages, turning this combination into a potential therapeutic agent against pathogenic *Salmonella* strains (Pomares et al., 2010) In addition, a chymotrypsin-susceptible MccJ25 derivative, MccJ25 (G12Y), was described as a good candidate for use in food preservation. This modified MccJ25 is able to inhibit the growth of pathogenic *S. enterica* Newport as well as *E. coli* O157:H7, when they are present in skimmed milk and egg yolk (Pomares et al., 2009).

Even when a wide number of investigations have been carried out to find the best way to control the Salmonella infection, a joint effort by research groups and government organizations must be accomplished in order to prevent the pathogen from contaminating the foodchain and coming into contact with more humans worldwide. That means finding David's rock to be able to defeat Goliath.

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