Chapter

Virulence Determinants of Non-typhoidal *Salmonellae*

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Abstract

Non-typhoidal *Salmonellae* (NTS) belong to *Salmonella enterica* subspecies *enterica* and are common causes of foodborne illnesses in humans. Diarrhea is a common symptom but infection occasionally results in life-threatening systemic involvement. One member of the group, *S. enterica* subspecies *enterica* serovar Typhimurium has been extensively studied in live animal models particularly mice and cattle, leading to a better understanding of the pathogenesis of NTS and the development of diarrhea, respectively. This comprehensive review provides an insight into the genetic regulation of over 200 virulence determinants and their involvement in the four steps of *Salmonella* pathogenesis, namely: attachment, invasion, macrophage survival and replication, and systemic dissemination. There is, however, a paucity of information on the functions of some virulence factors present on the *Salmonella* pathogenicity islands (SPIs). The emergence of next generation sequencing (NGS) technology and the availability of more bacterial genomes should provide further insights into the biology of virulence determinants, mechanisms of NTS pathogenesis and host adaptation of *Salmonella*. The new knowledge should translate into improvement and innovations in food safety, and control of salmonellosis as well as better understanding of zoonotic infections in the context of One Health capturing the risks to humans, animals and the environment.

Keywords: non-typhoidal *Salmonellae*, virulence determinants, Typhimurium, attachment, intracellular survival, systemic dissemination, NGS, food safety, *Salmonella* pathogenicity islands, SPI

1. Introduction

Non-typhoidal *Salmonella* (NTS), a major cause of diarrheal disease globally, is estimated to cause 93 million enteric infections and 155,000 diarrheal deaths each year and is a leading cause of foodborne infections worldwide [1]. In Canada, 88,000 people are estimated to fall ill from foodborne NTS each year (90% credible intervals: 58,532–125,525) [2] with a mean hospitalization of about 925 individuals and 17 deaths [3]. An estimated 1 million cases of NTS infections occur annually in the United States alone, resulting in 19,000 hospitalizations and 380 deaths (http://www.cdc.gov/foodborneburden/PDFs/pathogens-complete-list-01-12.pdf). The genus *Salmonella* consists of Gram-negative, facultative intracellular bacteria and belongs to the Enterobacteriaceae family [4]. Historically, *Salmonella* organisms are serologically characterized using the conventional serotyping method known as the White-Kauffmann-Le Minor scheme which is based on the somatic (O), flagellar (H) and capsular (vi) antigens. Over 2600 serotypes are known to be present in

a wide range of hosts including humans, cattle, pigs, horses, companion animals, reptiles, fish, avian, and insects [5]. The most commonly encountered pathogenic serovars belong to *S. enterica* subspecies *enterica* [6].

Some pathogenic *Salmonella* serovars are restricted to particular host species and are not found in other species. Examples of host-restricted *Salmonella* are serovars Typhi, Gallinarum, and Abortusovis, and they predictably cause systemic infection in their hosts namely, humans, fowls and ovines, respectively [7]. Another group of serovars are host-adapted including Dublin and Choleraesuis and primarily cause

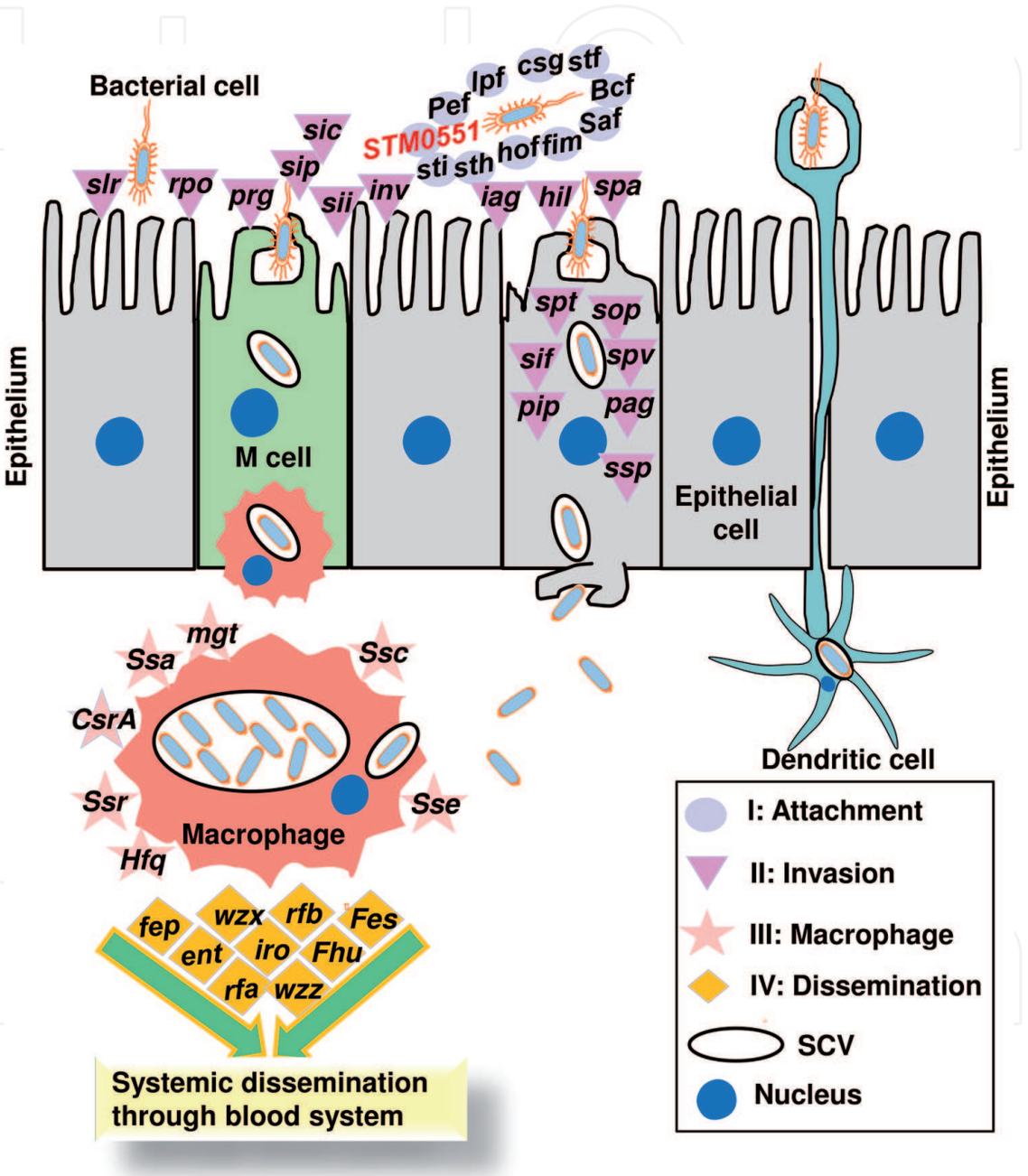

Figure 1.
Pathogenesis of Salmonella following contact with gut epithelium. (I) Salmonella cells attach to the epithelium mainly via adhesins, the representative virulence genes involved are fim, Saf, Bcf, stf, csg, lpf, Pef, sti, sth, hof, as well as a negative regulator of STM0551 (purple circles). (II) Three invasion methods are illustrated: M cells uptake bacteria cells through receptor mediated endocytosis, membrane ruffling and cytoskeletal rearrangement resulting in engulfment; alternatively, bacterial cells can be directly taken up by dendritic cells by phagocytosis. The main virulence factors involved are inv, pip, pag, prg, sap, sip, spa, spv, sop, rop, hil and sii (pink triangles). (III) Salmonella cells taken up by macrophages are localized within a Salmonella containing vacuole (SCV). The representative virulence genes involved in this process are mgt, Ssa, Sse, Ssr, CsrA and Hfq (light red star highlighted). (IV) Phagocyte-mediated systemic dissemination through blood system, mainly to liver, spleen and bone marrow. The virulence genes involved are iro, rfa, rfb, fes, Fhu, fep, ent, wzx and wzz (yellow diamond highlighted).

disease in cattle and pigs respectively, but infrequently cause opportunistic disease in another host species especially humans [7, 8]. The most common non-adapted *Salmonella* are serovars Typhimurium and Enteritidis and they have been studied in live animal models such as mice and cattle, leading to a better understanding of the pathogenesis of NTS and the development of diarrhea [7]. *S. typhimurium* causes a systemic infection in mice that resembles typhoid fever caused by *S. enterica* serovar Typhi in humans [9]. While a vast majority of cases in otherwise healthy, *Salmonella*-infected humans present clinically as a self-limiting gastroenteritis, *S. typhimurium* can cause life-threatening systemic, invasive disease and bacteremia in some patients [10] but the reasons and mechanisms dictating the different disease manifestations in infected humans are not clear.

The advent of microbial whole genome sequencing promises to provide insights to better understand the biology of virulence determinants and mechanisms of NTS pathogenesis. Genomes of *Salmonella* are generated increasingly at a faster rate and deposited in public databases [11]. Further understanding of genome diversity and variation of bacterial pathogens has the potential to improve quantitative risk assessment and assess the evolution of *Salmonella* and emergence of new strains [12]. Mining of the repository of genomes should provide new information expected to complement existing knowledge on virulence genes derived from host infection studies especially involving *Salmonella* mutants. The *Salmonella* Foodborne Syst-OMICS database (SalFoS) was developed as a platform to improve diagnostic accuracy, to develop control methods in the field and to identify prognostic markers in epidemiology and surveillance [13]. Bioinformatics analyses of genomes are expected to reveal the mechanisms of action of virulence genes and help decipher whether there is a dichotomy in the genes contributing to invasive disease compared to restricted pathogenesis in the intestinal tract [14].

This review provides an overview of the genetic regulation of over 200 virulence determinants highlighting their involvement in each of the four steps of *Salmonella* pathogenesis, namely: attachment, invasion, macrophage survival and replication, and systemic dissemination (**Figure 1**). Further analysis of virulence genes will provide us insights in to understanding the mechanisms of invasive disease which appear distinct from gastroenteritis. For instance, the organisms which are responsible for invasive disease have fewer genes because of pseudogenization. Many of these virulence genes have redundant functions; however two *Salmonella* molecules are known to exert a dominant effect in pathogenesis, namely: lipopolysaccharide (LPS) and invasion protein A (invA). Many virulence factors have distinct and unique functions but cooperative crosstalk has been documented at the different steps of infection, e.g., protein products of genes encoded on two *Salmonella* pathogenicity islands (SPI), SPI-2 and SPI-4.

2. Virulence determinants involved in *Salmonella* pathogenesis

2.1 Attachment

In a majority of cases, infection occurs following ingestion of *Salmonella* by the host. Before *Salmonella* can gain entry into the epithelial cell lining the host's gut mucosa, it first needs to attach to the cell. NTS attachment is facilitated by fimbriae, non-fimbriae factors of autotransporter and outer-membrane proteins, which serve as adhesions; up to 20 adhesion molecules have been described so far and it has been demonstrated that the entire adhesiome of *S. enterica* serotype Typhimurium can be expressed [15], which facilitates understanding such a large repertoire of adhesions

contributing to colonization of a broad range of host species and adaptation to various environment within the host.

2.1.1 Fimbrial adhesins

Fimbriae, also known as pili, are thin, filamentous appendages protruding on the bacterial surface and consist of polymerized aggregates of small molecular weight monomers of the fimbrin protein [16]. Characteristically, fimbriae mediate the initial attachment of Gram-negative bacterial pathogens to host cells and surfaces [17]. In *Salmonella*, the initial contact results in relatively weak adherence of the bacteria to intestinal epithelial cells but soon induces *de novo* bacterial protein synthesis which increases the strength and intimacy of the attachment [18]. This process is also accompanied by the development and assembly of a unique secretion apparatus called the Type 3 Secretion System (T3SS) which is required for *Salmonella* to invade epithelial cells [19]. The chromosome of *S. typhimurium* contains 13 fimbrial operons, *afg* (*csg*), *bcf*, *fim*, *lpf*, *pef*, *saf*, *stb*, *stc*, *std*, *stf*, *sth*, *sti*, and *stj* [20–22] (**Table 1** and **Figure 1**). Eight types of fimbriae which have been experimentally investigated [23] are outlined below.

2.1.1.1 Mannose-sensitive Type I fimbriae (Fim)

Mannose-sensitive Type I fimbriae (Fim) are encoded by the *fim ACDHIFZYW* operon and bind to D-mannose-containing receptors on host cell surface as well as the glycoprotein laminin of the extracellular matrix [24]. Type I fimbriae promoted bacterial attachment to epithelial cells, facilitated the invasion of HEp-2 cells and HeLa cells and the colonization of the gut mucosa in chicken, mouse, rat and swine [25, 26]. An immunization experiment using purified Fim protein led to the protection of laying hens against egg contamination and colonization of the reproductive organs by *S. enteritidis* [27]. FimA, FimF, and FimH are necessary for the assembly of Type 1 fimbriae on *S. typhimurium* [24]. Differently, *STM0551* gene plays a negative regulatory role in the regulation of type 1 fimbriae in *S. typhimurium* [28].

2.1.1.2 Plasmid-encoded fimbriae (Pef)

Plasmid-encoded fimbriae (Pef) participate in the attachment of bacteria to the surface of murine small intestine and are necessary for fluid production in the infant mouse similar to the observation with the fimbriae of enterotoxigenic *Escherichia coli* and *Vibrio cholerae* [29]. Expression of *pef* gene is regulated by DNA methylation [30]. Purified Pef specifically binds the trisaccharide Galβ1-4(Fucα1-3) GlcNAc (also known as the Lewis X blood group antigen or Le^x), which are preponderant on the surface of human erythrocytes, skin epithelium and mucosal surfaces [31].

2.1.1.3 Long polar fimbriae (Lpf)

Long polar fimbriae (Lpf) encoded by the *lpfABCDE* fimbrial operon is involved in the colonization of murine Peyer's patches by mediating adherence to M cells, a preferred port of entry for *Salmonella* in mice [32]. Mutation of the *lpfC* gene which encodes the fimbrial outer membrane usher attenuated the virulence of *Salmonella typhimurium* in orally exposed mice as shown by a 5-fold increase in the number of organisms needed to kill 50% of test animals (i.e., LD_{50}) when compared to the wild type organism. Lpf is also involved in the early stages of biofilm formation on host epithelial cells [33] and participate in intestinal persistence in mice [34].

Virulence genes	Location*	Functions
BcfABCDEFGH	Chromosome	Contribute to long-term intestinal carriage and bovine colonization
csgABCDEFG	Chromosome	Curlin subunit; assembly and transport component in curli production; DNA-binding transcriptional regulator
fimCDFHIWYZ	Chromosome	Adhesion to epithelial cells; biofilm formation
hofBC	Chromosome	Type IV pilin biogenesis protein
lpfABCDE	Chromosome	Biofilm formation, contribute to long-term intestinal carriage
misL	SPI-3	An extracellular matrix adhesion involved in intestinal colonization
pefA	Plasmid	Adhesion to crypt epithelial cells; induction of proinflammatory response
ppdD	Chromosome	Putative major pilin subunit
SafC	Chromosome	Salmonella atypical fimbria outer membrane usher
ShdA	CS54	Outer membrane
StdB	Chromosome	Contribute to long-term intestinal carriage
stfACDEFG	Chromosome	Not required for long-term intestinal carriage of mice
sthABD	Chromosome	Outer membrane fimbrial usher. Putative fimbrial subunit and chaperone protein
StiABC	Chromosome	Putative fimbrial subunit/usher/chaparone
STM0551	Chromosome	Downregulates fimbrae protein expression and acts as a negative regulator of virulence
STM4595	Chromosome	Unknown function

**SPI-3 and CS54 are genomic islands on Salmonella chromosome.*

Table 1.
Location and function of the major proteins and virulence determinants contributing to Salmonella attachment.

Lpf synthesis is regulated by an on–off switch mechanism (phase variation) to avoid host immune responses [35].

2.1.1.4 Thin aggregative fimbriae

Thin aggregative fimbriae also known as curli [36] with the designation Agf/Csg, are encoded by the *agf/csgBAC* gene cluster [37]. The thin aggregative fimbriae for Enteritidis which is known as SEF 17 is responsible not only for the auto-aggregative phenotype of the bacteria, but for fibronectin binding [38] and has been shown *in vitro* to bind immortalized small intestinal epithelial cells from mice [36]. Mutation in *agfB* resulted in a 3- to 5-fold increase in the oral LD_{50} of Typhimurium for mice [39].

2.1.1.5 Bovine colonization factor (Bcf)

Bovine colonization factor (Bcf) is encoded by genes in the *bcf* gene cluster. The fimbrial usher protein encoded by *bcfC* is required for colonization of bovine but not murine Peyer's patches in oral infection models of calves and mice [40]. The *bcf* gene together with five other fimbrial operons—*lpf*, *stb*, *stc*, *std*, and *sth*—are reported to be required for long-term intestinal carriage of Typhimurium in genetically resistant mice [34].

2.1.1.6 Salmonella atypical fimbriae (Saf)

Salmonella atypical fimbriae (Saf) are encoded by the chromosomal *safABD* operon. A group of BALB/c mice immunized subcutaneously with SafB/D- and recombinant cholera toxin B subunit (rCTB)-conjugated micro-particles had significantly lower CFU counts than the untreated control group [41]. Two additional functions - poly-adhesive and self-associating activities – were attributed to the Saf pili and appear to contribute to host recognition and biofilm formation [42].

2.1.1.7 Typhimurium fimbriae std and stf operons

Std operon is required for adherence to human colonic epithelial cells and for cecal colonization in the mouse by binding to cecal mucosa receptors containing α(1, 2) fucose residues [34, 43]. *Stf* fimbriae share homology with the MR/P fimbriae of *Proteus mirabilis* and *E. coli* Pap fimbriae [44]. *StfA* expression is induced during infection of bovine ileal loops [45].

2.1.1.8 Enteritidis fimbrial SEF14

Enteritidis fimbrial SEF14 contributes to colonization of chicken intestine, liver, spleen and reproductive organs [46, 47]. The fragment encoding genes responsible for SEF14 biosynthesis contain three genes, *sefABC*. The putative adhesion subunit encoded by *sefD* is essential for efficient uptake or survival of Enteritidis in macrophages, as the *sefD* mutants were not readily internalized by peritoneal macrophages compared with the wild-type bacteria soon after intraperitoneal infection of mice [48]. The *sefD* mutant was severely attenuated after both oral and intraperitoneal infection of BALB/c mice (approximate LD50: $>10^4$ (mutant) vs. <10 (wild type)) [48]. In the mouse model, egg-yolk derived anti-SEF14 antibodies afforded passive protection [49].

2.1.2 Non-fimbrial adhesins

Four distinct non-fimbrial intestinal colonization factors have been identified:

2.1.2.1 MisL

MisL encoded within the SPI-3, is an outer membrane fibronectin-binding autotransporter protein which is induced upon bacterial contact with the intestinal epithelial cells, and is required for colonization of the murine cecum and for intestinal persistence. MisL binds fibronectin and collagen IV via its passenger domain [50].

2.1.2.2 ShdA

ShdA gene is located in the 25-kb pathogenicity island called CS54 which is present only in *S. enterica* subspecies *enterica* [51]. ShdA is a large fibronectin/collagen I-binding outer membrane protein which is induced *in vivo* in the murine caecum [52]. It is required for Typhimurium colonization in the murine caecum and Peyer's patches of the terminal ileum [53] and for efficient and prolonged shedding of the organism in feces [51].

2.1.2.3 BapA

BapA is a huge surface-associated protein and secreted via its downstream type I secretion system, BapBCD. BapA contributes to murine intestinal colonization and

subsequent organ invasion. Mice orally inoculated with *BapA*-deficient strain survived longer and have a significant reduction in mortality rate than those inoculated with the wild-type strain [54].

2.1.2.4 SiiE

SiiE is a SPI4-encoded protein and works as the substrate protein of the T1SS. SiiE is secreted into the culture medium but mediates contact-dependent adhesion to epithelial cell surfaces. SiiE codes for a giant non-fimbrial adhesion of 600 kDa and consists of 53 repeats of immunoglobulin domains; this is a T1SS-secreted protein that functions as a non-fimbrial adhesion in binding to eukaryotic cells [55].

2.2 Intestinal phase: invasion and intracellular survival

Shortly after adhesion to a host cell, *Salmonella* invasion proceeds as a consequence of the activation of host cell signaling pathways leading to profound cytoskeletal rearrangements [56]. These internal modifications dislocate the normal epithelial brush border and induce the subsequent formation of membrane ruffles that engulf adherent bacteria in barge vesicles called *Salmonella* containing vacuoles (SCVs), which is the only intracellular compartment where *Salmonella* cells survive and replicate [57, 58]. Simultaneously, induction of secretory response in the intestinal epithelium initiates recruitment and transmigration of phagocytes from the submucosal space into the intestinal lumen. Alternatively, *Salmonella* cells may be directly engulfed by dendritic cells from the submucosa. Taken up During SCV maturation, *Salmonella* induces *de novo* formation of an F-actin meshwork around bacterial vacuoles, a process which is termed vacuole-associated action polymerization (VAP) and is important for maintenance of the integrity of the vacuole membrane [59]. Furthermore, intracellular *Salmonella* can induce the formation of long filamentous membrane structure called *Salmonella*-induced filaments (SIFs) [60], which may lead to an increased availability of nutrients within the SCV [61]. A fraction of SCVs transcytose to the basolateral membrane. Once across the intestinal epithelium, *Salmonella* are engulfed by phagocytes and internalized again with SCVs, triggering a response similar to that reported inside epithelial and M cells to ensure bacterial survival and replication [62]. The pathogenic bacterium must at this stage employ many virulence strategies to evade the host defense mechanisms (**Figure 1**).

The majority of the virulence determinants are located within highly conserved SPIs on the chromosome, while others are either on a virulence plasmid (pSLT) or elsewhere in the chromosome. To date, 21 SPIs have been identified in *Salmonella*, and the generalist *S. typhimurium* and the invasive *S. typhi* genomes share 11 (SPIs-1 to 6, 9, 11, 12, 13 and 16). Two SPIs namely SPI-8 and 10 were initially found in *S. typhi* and without counterparts in *S. typhimurium* chromosome; SPI-14 is specific to *S. typhimurium*, while *SPIs*-7, 15, 17 and 18 are specific to *S. typhi*; and SPIs-19, 20 and 21 are absent in both of them [63]. Because of the prominence of the SPIs in pathogenesis, the virulence factors encoded on the major SPIs, SPI-1 to SPI-5 are described below, and their respective functions summarized (**Tables 2** and **3**).

2.2.1 SPI-1 mediates contact-dependent invasion of the intestinal epithelium and enteropathogenesis

SPI-1 codes for several effector proteins that trigger invasion of epithelial cells by mediating actin cytoskeletal rearrangements and hence internalization of the

Virulence genes	Location*	Functions
Crp	Chromosome	cAMP-regulatory protein
hilACD	SPI-1	Promote phop-repressed prgHIJK, sipA, sipC, invF, and orgA; activates the expression of the *hilA* gene
Hnr	SPI-2	SPI-2 regulator (transcriptional and post-transcriptional)
HtrA		Resistance to periplasmic stress
IacP	SPI-1	Posttranslational modification
iagB	SPI-1	Invasion
invABCEFGIJ	SPI-1	Secretion and chaperone; promote sipBCDA, sigD and sicA
msgA	Chromosome	Unknown function
ompR/envZ	SPI-2	Regulates *ssrAB* expression
orgABC	SPI-1	Pathogenesis; secretion
phoR/Q	SPI-2	Regulates *ssrAB* expression; down-regulates the transcription of its master regulator HilA, control *mgtC*
pagACDP	SPI-11	Resistance to AMP, macrophage cytotoxicity
pipABB2CD pipC (*sigE*)	SPI-5	Pathogenesis, effector protein; sif extension; SCV maturation and positioning
prgHIJK	SPI-1	Secretion
Prc		Resistance to periplasmic stress
rpoES rpoS (*katF*)	SPI-2	SPI-2 regulator (transcriptional and post-transcriptional); controls the transcription of the regulatory gene *spvR*; expression of rpoS is induced after entry of *Salmonella* into macrophages or epithelial cells, or in vitro during the stationary growth phase
rtsA	Chromosome	Activates the expression of the *hilA* gene
sapABCDF		Resistance to AMP, macrophage cytotoxicity
sifA	SPI-2	Sif formation in epithelial cells and maintenance of SCV membrane integrity
siiCDEF	SPI-4	Translocation; adhesion to apical side of polarized epithelial cells; involved in T3SS-1 dependent invasion
sicAP	SPI-1	Chaperone for sipBC
sipA (*sspA*)	SPI-1	Stabilization and localization of actin filaments during invasion, stabilization of VAP, correct localization of SifA and PipB2, SCV perinuclear migration and morphology, promote inflammatory response and fluid secretion
sipBCD (*sspBCD*)	SPI-1	Adhesion to epithelial cells, early macrophage pyroptosis, macrophage autophagy; Adhesion to epithelial cells
SpaSRQPO	SPI-1	EscU/YscU/HrcU family type III secretion system export apparatus switch protein; antigen presentation protein SpaO
sptP	SPI-1	Disruption of the actin cytoskeleton rearrangements by antagonizing SopE, SopE2, and SigD, downregulate inflammatory response
sirA	SPI-1	SirA/BarA encoded outside SPI-1 activates HilA
slrP	Chromosome	Adhesion to epithelial cells
slyA	SPI-2	Regulates resistance to oxidative stress
sspH1H2	Phage	Localize to the mammalian nucleus and inhibits NF-κB-dependent gene expression; SCV maturation and positioning

Virulence genes	Location*	Functions
sodABD		Resistance to oxidative stress
SopABDD2EE2 sopB (*sigD*)	SPI-5	Chloride secretion; promote actin cytoskeletal rearrangements, invasion and inhibition of apoptosis of epithelial cells, induction of proinflammatory response and fluid secretion, SCV size, instability, maturation and positioning, nitrate respiration, outgrowth in the intestine; inhibition of vesicular trafficking; replication inside macrophages; sif formation
spaOPQRS	SPI-1	Secretion
SprB	SPI-1	Regulation of transcription, DNA-templated
spvABCD	Plasmid	Modifies actin and destabilizes the cytoskeleton of infected cells; SCV maturation and positioning; induction of apoptosis; Host cell signaling
SsJ		Resistance to oxidative stress
STM2231	SPI-2	SPI-2 regulator (transcriptional and post-transcriptional)
YejABEF	Chromosome	Resistance to AMP, macrophage cytotoxicity
ymdA	Chromosome	Stress response

**SPI1–5 are genomic islands on Salmonella chromosome.*

Table 2.
Location and function of the major proteins and virulence determinants contributing to Salmonella invasion.

bacteria. These effectors are translocated into host cell by means of a Type III Secretory System or T3SS-1 [64], which is made up of proteins encoded by the SPI-1, such as *inv*, *spa*, *prg* and *org* [65]. Naturally occurring mutants of *Salmonella* have been found in the environment with a deletion of a vast DNA segment of SPI-1 locus and are deficient for *inv*, *spa*, and *hil* hindering their ability to enter cultured epithelial cells [66]. Mutations leading to a defective secretory function of T3SS-1 led to a 50-fold increase in LD_{50} following oral administration of Typhimurium in the mouse model [67]. The *prg/org* and *inv/spa* operons encode the needle complex, whereas the *sic/sip* operons encode the effector proteins and the translocon (SipBCD), a pore-forming structure that embeds in the host cell membrane and delivers these effectors to the host cytosol. In addition, several chaperones are also encoded within SPI-1. For example, SlrP mediate ubiquitination of ubiquitin and thioredoxin [68] and one of the SPI-1 regulons, STM4315 (*rtsA*) interferes with the interactions of *S. typhimurium* and host cells [69]. In general, the expression of SPI-1 genes is subject to control by complex regulatory mechanisms involving local regulators such as HilA, iagB and InvF which are necessary for host invasion by *Salmonella* and induction of gastroenteritis [70, 71]. For example, *prgHIJK*, *invA*, *invJ*, and *orgA* are primarily regulated by HilA [71]. In addition, two major global regulatory networks, SirA/BarA and PhoP/PhoQ, indirectly regulate the expression of the invasion-associated genes via HilA [72, 73].

2.2.2 SPI-2 is essential for survival and replication in macrophage

The SPI-2 is composed of two segments. The smaller portion contains the *ttrRSBCA* operon, which is involved in tetrathionate reduction, and seven open reading frames (ORFs) of unknown function. The expression of these genes may contribute a growth advantage over the microbiota [74]. The larger portion of this island was shown to be critical for the ability of *Salmonella* to survive and replicate

Virulence genes	Location	Functions
CsrA		RNA chaperones
Hfq	SPI-2	SPI-2 regulator (transcriptional and post-transcriptional), RNA chaperones
mgtABCD	SPI-3	A hydrophobic membrane protein; Mg^{2+} transporter (Mg^{2+}-transporting P-type ATPase)
SsaABCDEFGHIJKLMNOPQRSTUV *ssaB* (*spiC*), *ssaC* (*spiA*), *ssaD* (*spiB*), *ssaR* (*yscR*).	SPI-2	Regulate the secretion of translocon proteins under conditions that simulate the vacuolar environment; interferes with vesicular trafficking; intracellular bacterial proliferation; secretion
sscAB	Chromosome	Putative type III secretion system chaperone protein or pathogenicity island effector protein
sseABCDFGIJL	SPI-2	Translocation; sif formation in epithelial cells; SCV maturation and positioning; SCV membrane dynamics; nuclear response-gene expression;
ssrAB (*ssrA/SpiR*)	SPI-2	Regulates SPI-2 gene expression

Table 3.
Location and function of the major proteins and virulence determinants contributing to Salmonella macrophage survival and replication.

inside host cells—both epithelia cells and macrophages—within the SCV [75]. Non-functional SPI-2 mutants are unable to colonize internal target organs such as spleen and liver of mice, although they penetrate the intestinal barrier as efficiently as the wild type strain [76]. These mutants were attenuated by at least five orders of magnitude compared with the wild type strain after either oral or intraperitoneal inoculation of mice [75]. The SPI-2 related events are triggered by the action of effector proteins with its own T3SS known as T3SS-2, which also encodes its proper translocon machinery named SseBCD [77]. Gene sequence similarity to the known components of other T3SS has been used to propose functions for *SsaN*, *SsaR*, *SsaS*, *SsaT*, *SsaU* and *SsaV* as coding for putative proto-channel components, SsaD/SpiB, SsaJ, SsaK and SsaQ appear to code for basal components, whereas SsaC/SpiA may code for an outer ring protein [78]. Generally, SPI-2 contains four types of virulence genes: *ssa* encodes T3SS-2 apparatus; *ssr* encodes regulators; *ssc* encodes the chaperones and *sse* encodes the effectors (**Table 2**) [79, 80].

2.2.3 SPI-3 contributes to intramacrophage proliferation

Unlike SPI-1 and SPI-2, only four ORFs within SPI-3 have been shown to contribute to replication in macrophages via a high-affinity Mg^{2+} uptake system [81]. The *mgtC* gene encoding a 22.5-kDa hydrophobic membrane protein, is the major virulence gene factor found within this locus, and is responsible for growth in Mg^{2+} limiting environment, intramacrophage survival, and systematic virulence in mice [82]. The transcription of *mgtC* is followed by activation of PhoP-PhoQ in response to low Mg^{2+} levels [81].

2.2.4 SPI-4 is involved in colonization

The fourth SPI contributes to *Salmonella* colonization in the intestine of cattle, but not of chicks [83]. Loss of SPI-4 attenuates the oral but not intraperitoneal virulence of serovars Typhimurium and Enteritidis in mice [84]. Three genes namely *SiiC*, *SiiD*, and *SiiF* produce proteins that form the type 1 secretion system (T1SS); the fourth gene, *siiE* codes for a giant non-fimbrial adhesion exported by the T1SS and mediates contact-dependent adhesion to polarized epithelial cells rather than to non-polarized cells. In contrast, SiiA and SiiB are not secreted but represent inner membrane proteins whose function is unknown [55, 85]. Recently, transmembrane mucin MUC1 was shown to be required for *Salmonella* siiE-mediated entry of enterocytes via the apical route [86].

2.2.5 SPI-5 is associated with enteropathogenicity

The SPI-5 locus is well characterized in the serovar Dublin infection in calves. This bovine-adapted serovar primarily causes bacteremia rather than gastroenteritis in humans. This region comprises six genes namely, *pipD*, *orfX*, *sopB* (also known as *sigD*), *pipC* (also known as *sigE*), *pipB*, and *pipA* [87]. Four gene products which include three SPI-5 Pip proteins (PipD, PipB, PipA) and one SPI-1 SopB protein are involved in secretory and inflammatory responses in bovine ligated ileal loops but they do not appear to play a significant role in the development of systemic infection in mice inoculated by the intraperitoneal route [87, 88]. Furthermore, it has been found that SigE serves as a chaperone for the *S. typhimurium* invasion protein, SigD [89].

2.2.6 Crosstalk between SPI-1 and SPI-2 gene products to promote Salmonella survival and virulence

The SPI-2 genes are activated after *Salmonella* gains access into the SCV [76]. T3SS-2 secretes multiple effector proteins into different subcellular fractions where they interfere with various host cellular functions to establish a replication-permissive environment [90]. The identified effectors are encoded within SPI-2 (e.g., SpiC, SseF and SseG) and outside SPI-2 (e.g., SifA, SseI, SseJ and SspH 2) [23]. These SPI-2-encoded effectors together with some of SPI-1-encoded effectors (e.g., SipA, SipD, SopA, SopE, SopB) that persist in the host cytosol after invasion, are distributed in different cellular compartments including the vascular membrane of SCV and Sif, host cytosol, cytoskeleton, Golgi apparatus, and nucleus. These molecules influence distinct intracellular events and collectively contribute to establish a *Salmonella* replicative niche in macrophages [91]. These intracellular events include: inhibition of endocytic trafficking, evasion of NADPH oxidase-dependent killing [92, 93], induction of a delayed apoptosis-like host cell death [94], assembly of a meshwork of F-actin around the SCV [59], accumulation of cholesterol in the SCV [95], and interference with the localization of inducible nitric oxide synthase to the SCV [96]. Efficient replication has been found to be associated with two phenotypes involving host microtubule cytoskeleton and its motor proteins, Golgi apparatus-associated juxtanuclear positioning of SCV [97–99] and Sifs formation which appear as tubular membrane extensions of SCVs enriched in lysosomal glycan proteins [100].

2.2.7 Joint regulation between SPI-1 and SPI-4

The functional relatedness between SPI-1 and SPI-4 is reflected by their co-regulation by the same set of key regulators, for example, a transcriptional activator

SprB encoded within SPI-1 and regulated by HilA under similar environmental conditions; SprB directly activates SPI-4 gene expression and weakly represses SPI-1 gene expression through HilD [101].

2.3 Intramacrophage survival and replication

Similar mechanisms occur inside epithelial cells after intestinal invasion and once bacteria have been internalized by macrophages. Briefly, *Salmonella* cells are localized in the SCV once engulfment is completed. Preserving the SCV membrane integrity plays a crucial role in allowing *Salmonella* replication inside these intracellular niches. These procedures are regulated by T3SS-2 transporting action and its translocon machinery, namely SseBCD complex [77]. Hence, the required effectors which are encoded both inside and outside SPI-2 facilitate the success of *Salmonella* intramacrophage survival. The SPI-2 gene expression is triggered in response to a number of environmental signals mimicking the vacuolar environment of SCV, including stationary growth phase, low osmolarity [102], low concentrations of Mg^{2+}, Ca^{2+} or PO_3 [103, 104], and low pH [76]. The expression of SPI-2 genes is coordinately regulated at both transcriptional and post-transcriptional levels. During the transcription of SPI-2 genes, many two-component regulatory systems are involved, including SsrA-SsrB, OmpR-EnvZ and PhoP-PhoQ as well as transcriptional regulators, namely SlyA and the alternative sigma factor of RNA polymerase RpoE. The main regulatory proteins that act post-transcriptionally are the RNA chaperons, including Hfq, CsrA, and SmpB. The *mgtC* gene located in SPI-3 has been shown to contribute to replication in macrophages. All the mentioned virulence determinants can be found in **Table 3** and **Figure 1**.

2.4 Systemic infection/dissemination

Internalization of the infecting *Salmonella* within SCV is followed by systemic spread through other target organs, such as the spleen and liver. As a prerequisite for spread, the bacterial cells must evade the innate immune system. During this process, serum resistance or resistance to complement-mediated serum killing is a major virulence factor for the development of systemic salmonellosis. It involves three major factors, namely LPS, outer membrane proteins PagC and Rck and siderophores (**Table 4** and **Figure 1**).

2.4.1 LPS constitutes a chemical and physical protective barrier for the cell

LPS of Gram-negative bacteria, a major component of the outer membrane, constitute a chemical and physical protective barrier for the cell. LPS consists of the hydrophobic lipid A, a short non-repeating core oligosaccharide and a long distal repetitive polysaccharide termed O-antigen or O-side chain [105]. Complete LPS is characterized by long O-antigen which confers the smooth (S) phenotype on *Salmonella*. The O-antigen is a major component associated with serum resistance. Incomplete LPS devoid of O-antigen leads to rough (R) phenotype, which is of low virulence [106]. Naturally occurring infections are caused by S-phenotype *Salmonella*, which are resistant to complement killing [107, 108]. There is a correlation between the amount, structure, and chain length of the O-antigen and virulence [109]. The long O-antigen of LPS confers on the organism the ability to resist complement-mediated serum killing by sterically hindering the insertion of the membrane attack complement complex (C5b-9) into the bacterial outer membrane [107, 108].

Virulence genes	Location	Functions
cirA	Chromosome	Colicin I receptor
entABCDEF	Chromosome	Enterobactin synthase
fepABCDEG	Chromosome	Outer membrane receptor; iron-enterobactin transporter binding protein
Fes	Chromosome	Salmochelin secretion/degradation
FhuABCDE	Chromosome	Enterobactin/ferric enterobactin esterase
foxA	Chromosome	Ferrioxamine B receptor precursor
FruR	SPI-2	DNA-binding transcriptional regulator
FUR	Chromosome	Ferric uptake regulator
iroBCDE	Chromosome	Salmochelin glycosylation, transport and processing
MsbA	Chromosome	Lipid transporter ATP-binding/permease protein
rfaBCDFGHIJKLPQYZ	Chromosome	LPS core biosynthesis protein; transcriptional activator; O-antigen ligase
rfbBDFGHIJKMNOPUVX	Chromosome	Glucose biosynthesis pathway; O-chain glycosyltransferase; O-antigen transporter
rfc	Chromosome	O-antigen polymerase
STM0719	Chromosome	Unknown function
wzxCE	Chromosome	Colanic acid exporter; putative LPS biosynthesis protein
wzzBE	Chromosome	LPS chain length regulator and biosynthesis protein
yibR	Chromosome	Unknown function
ybdAB	Chromosome	Enterobactin exporter EntS

Table 4.
Location and function of the major proteins and virulence determinants contributing to Salmonella dissemination.

Surface expression of O-antigen involves multiple steps: O-antigen biosynthesis in the inner membrane (*rfb*), translocation across the inner membrane by Wzx flippase (*wzx*), polymerization (*wzz*, *rfc* and *rfe*) and ligation on to the preformed Core-Lipid A complex by WaaL ligase (*rfaL*). The Core-Lipid A is translocated independently by the ATP-binding cassette (ABC) transporter MsbA [110, 111]. Complete LPS molecules are then transported to the surface across the periplasm and outer membrane by the Lpt (LPS transport) pathway [111]. Defects in any of the above steps would affect the surface display of the O-antigen and its function. The mutants defective in the biosynthesis of LPS core encoded by the *rfa* loci or the O side chain by the *rfb* loci, are significantly attenuated with a LD50 at least 100 times higher than the parental strain in chickens subcutaneously infected with Enteritidis [112].

Typhimurium possesses two functional *wzz* genes responsible for regulating the chain length of the O-antigen [113]. One is *wzzST* encoding a long LPS with 16–35 O-antigen repeat units and the other *fepE* gene coding for a very long LPS estimated to contain more than 100 repeat units [113]. Either gene product is sufficient for complement resistance and virulence in the mouse model of infection, which reflects a degree of functional redundancy of these two *wzz* genes [113]. Double mutation of these two *wzz* genes resulted in relatively short, random-length

O-antigen and the mutant displayed enhanced susceptibility to complement-mediated killing and was highly attenuated in mice [113]. The transcription of *wzzST* gene is independently activated by two-component systems of Typhimurium, PmrA/PmrB (PmrA, sensor; PmrB, response regulator) and RcsC/YojN/RcsB (RcsC, sensor; YojN, intermediate phosphotransfer protein; RcsB, response regulator) [114]. PmrA/PmrB is activated through two pathways: one is directly activated through its cognate sensor PmrB in response to Fe^{3+} and the other is dependent on the PhoP/PhoQ two-component system in response to low Mg^{2+}. The RcsC/YojN/RcsB is activated in the presence of low Mg^{2+} plus Fe^{3+} [114]. In addition, mutants in a number of genes (*rfaG*, *rfaI*, *rfaL*, *rfaQ*, *rfaP*, *rfbC*, *rfbD*, *rfbJ*, *rfbM*, *rfbP*, *yibR*) necessary for LPS biosynthesis/assembly had severely impaired movement on swimming motility agar [115].

2.4.2 PagC and Rck confer resistance to the complement-mediated bacterial activity

In addition to LPS, two outer membrane proteins, the 18-kDa PagC [116] and the 17-kD Rck [117], confer a high level of resistance to the complement-mediated bactericidal activity. These two proteins share homology with virulence-associated outer membrane protein Ail from *Yersinia* that blocks formation of the complement membrane attack complex on the bacterial surface. Similarly, complement resistance mediated by Rck is associated with a failure to form fully polymerized tubular membrane attack complexes [117]. One strain of Typhimurium which contains a single mutation in *pagC* had a virulence defect and decreased survival in cultured murine macrophages and 100-fold reduction in intraperitoneal virulence in mice [118].

2.4.3 Siderophores are important for bacterial growth in serum in the extracellular phase of salmonellosis

Iron is an essential element for the growth of most bacteria through its involvement in a variety of metabolic and regulatory functions [119]. Studies with different iron concentrations in growth media demonstrated an effect on gene expression of the iron acquisition systems encoded both on the chromosome and plasmids at both transcriptional and translational levels [120]. Siderophores which are bacterial molecules that bind and transport iron are important for bacterial growth in serum in the extracellular stage of *Salmonella* systemic infection. They are not required after bacteria reside in SCV where siderophore-independent iron acquisition systems are sufficient for iron uptake during intracellular stage. *Salmonella* produce two major types of siderophores, high-affinity catecholate consisting of salmochelin and enterobactin the latter also known as enterochelin and a low-affinity hydroxamate known as aerobactin which is expressed under iron-restricted conditions [121]. The synthesis, secretion, and uptake of salmochelin requires genes clustered at two genetic loci, the *fepA* gene cluster and *iroBCDEN* operon. The *fepA* gene cluster includes most *ent* genes for synthesis and export [122]. The *iroBCDEN* operon encodes gene products for enterobactin glycosylation (IroB, glycosyltransferase), export (IroC, ABC transporter protein), and utilization (IroD, esterase; IroE, hydrolase; IroN, outer membrane receptor) [122]. Mutants deficient in *iroB* or *iroC* exhibit reduced virulence during systemic infection of mice via intraperitoneal route, as indicated by lower bacterial load in liver and a delayed time of death [122]. Moreover, the enterobactin metabolite, 2, 3-dihydroxybenzoyl serine (DHBS), can also be used by *Salmonella* as sources of iron, albeit at much lower affinities, by recognizing the three catechelate receptors, FepA, IroN and Cir. The three receptors demonstrate a significant degree of functional redundancy. The Typhimurium

double mutant Δ*fepA iroN* were similarly virulent to the parental strain after intragastric gavage inoculation of mice, while the triple mutant *ΔfepA iroN cir* was attenuated as indicated by a significantly reduced cecal colonization and no measurable spread to the liver [123, 124].

Furthermore, *Salmonella* also utilize xenosiderophores as iron sources by utilizing the outer membrane receptors, including FhuA, FhuE, and FoxA. For example, utilization of ferrioxamines B, E, and G by Typhimurium is dependent on the FoxA receptor encoded by the Fur repressible *foxA* gene. A strain carrying the foxA mutation exhibited a significantly reduced ability to colonize rabbit ileal loops and was markedly attenuated in mice challenged by either intragastric gavage or intravenously route strain compared to the foxA+ parent [125]. The best characterized regulator for iron uptake is the iron-dependent repressor Fur that acts together with the co[-]repressor ferrous iron (Fe(II)) to regulate genes involved in the iron uptake process in response to iron restriction, including *fhuA*, *fhuB*, *fepA*, *fes*, *fepD*, *entB*, *fur*, *foxA*, *hemP*, and *fhuE* [126, 127].

3. Future directions

The advent of next generation sequencing (NGS) has provided an opportunity to verify or improve on knowledge gained from *in vitro* and *in vivo* analyses of *Salmonella* mutants which were designed for the purpose of understanding gene function and mechanism of action. Recently, Rakov et al. [14] carried out bioinformatics analysis of 500 *Salmonella* genomes and identified 70 allelic variants virulence factors which were associated with different pathogenesis outcomes, i.e. gastrointestinal vs. invasive disease. However, the causative relationship between a putative virulence factor and disease outcome using a genomics based tool is yet to be attained. To that end, we propose the development of a comprehensive genome based tool such as a NGS AmpliSeq assay that can be used to simultaneously interrogate the presence and potential expression of over 200 virulence genes of *Salmonella* identified in this communication. The tool can be used to evaluate differences in strains and correlate the output with virulence phenotype derived from epidemiological or experimental observations which can be developed simultaneously or based on historical documentation. The tool could be used in assessing the potential risk posed by a strain of *Salmonella* given the fact that the serovars obtained from the environment are often distinct with those involved in human diseases. The technology appears suitable for dissecting the complexity associated with the redundancy and pleiotropic nature of some of the currently known virulence genes. In addition, NGS based analysis of virulence genes should provide new insights on *Salmonella* evolution and a better tool for analyzing epidemiological data that could translate to a reduction in the burden on human health posed by this important foodborne and zoonotic pathogen.

4. Conclusions

This review provides an outline of over 200 identified virulence determinants and details of their involvement in the four steps of *Salmonella* pathogenesis, namely: attachment, invasion, intramacrophage survival/replication and systemic dissemination. The genetic regulation of only some of the virulence determinants have been elucidated in live animal models such as mice and cattle, and this has enriched our understanding of the pathogenesis and mechanism of diarrhea and systemic disease. The majority of the current evidence on pathogenesis

and virulence determinants of NTS was derived from murine model of serovar Typhimurium infection with and only a few studies focused on NTS infection in humans. For this reason, the relevance of published observations is often called into question. Linking clinical, epidemiological and experimental observations on the nature and severity of diseases caused by *Salmonella* organisms with the presence of a large number of virulence genes currently may not garner enough predictive ability to infer virulence or pathogenetic potential of a strain. Still, the increasing availability of a large number of *Salmonella* genomes in the public databases is proving to be a timely resource. Next generation sequencing and the twin subject of bioinformatics represent an unprecedented opportunity to verify past observations and help improve our understanding of *Salmonella* virulence towards a coherent and comprehensive understanding of the mechanism of *Salmonella* pathogenesis. What is required is a robust laboratory tool that can be used to analyze the large number of virulence genes in an isolate using the tools of whole genome sequencing. We expect that a tool such as an AmpliSeq assay for *Salmonella* virulence could be developed to generate accurate and reliable information that can be fed into a quantitative risk assessment framework. This could usher a new era of risk management customized for a *Salmonella* strain involved in an outbreak and should translate to impactful outcomes in the areas of improved food safety, evaluation of zoonotic diseases and reducing the burden of human salmonellosis.

Acknowledgements

RG is funded by Genome Canada. DO's research program has received funding support from Genome Research and Development Initiative of the Government of Canada, Ontario Ministry of Agriculture, Food and Rural Affairs, Canadian Security and Science Program of the Department of National Defense and the Canadian Food Inspection Agency.

Conflict of interest

The authors declare no conflict of interest.

Acronyms and abbreviations

AMP	antimicrobial peptides
invA	invasion protein A
LPS	lipopolysaccharide
NTS	non-typhoidal *Salmonella*
NGS	next generation sequencing
SalFoS	*Salmonella* Foodborne Syst-OMICS database
SPIs	*Salmonella* pathogenicity islands
SIFs	*Salmonella*-induced filaments
SCV	*Salmonella*-containing vacuole

DOI: http://dx.doi.org/10.5772/intechopen.88904

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