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RESEARCH TOPICS

SALMONELLA HOST-PATHOGEN INTERACTIONS

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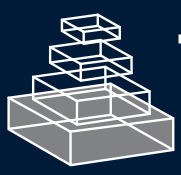

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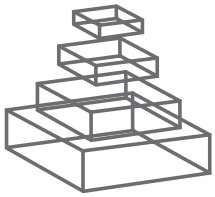

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ISSN 1664-8714
ISBN 978-2-88919-005-8
DOI 10.3389/978-2-88919-005-8

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SALMONELLA HOST-PATHOGEN INTERACTIONS

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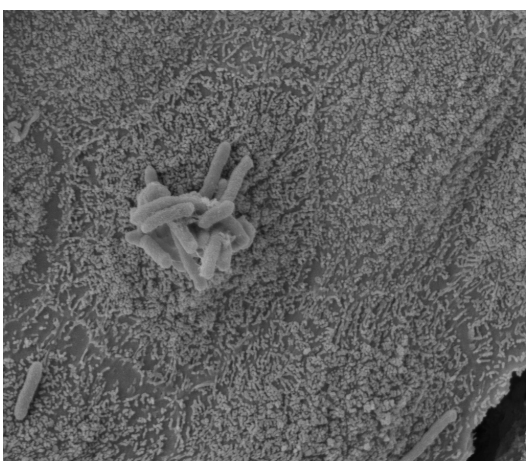

This Research Topic provides updated information on several important areas of Salmonella host cell interaction. Articles begin on topics outside of the host cell, examining the transit to and interaction with the epithelium, including pattern recognition. Once inside the host cell, topics of manuscripts include examination of the Salmonella-containing vacuole, its ability to resist intracellular killing, and the bacterial gene induction within the host cells responsible for survival. Finally, knowledge concerning carriage in and transmission from the infected host are discussed.

Table of Contents

frontiers in
MICROBIOLOGY

EDITORIAL
published: 19 September 2011
doi: 10.3389/fmicb.2011.00191

Salmonella host–pathogen interactions: a special topic

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Salmonellae are Enterobacteriaceae that cause a spectrum of diseases in humans and animals, including enteric (typhoid) fever and gastroenteritis (Coburn et al., 2007; Andrews-Polymenis et al., 2010). Most human *Salmonella* infections result in gastroenteritis and are caused by *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) or *Salmonella enterica* serovar Enteritidis acquired from contaminated food. Non-typhoidal *Salmonella* serotypes, such as *S.* Typhimurium, have a broad host range and infect a variety of animals. Interestingly, while these serotypes cause gastroenteritis in humans, they cause a systemic illness similar to typhoid fever in the mouse and cow (Andrews-Polymenis et al., 2010). In contrast, *S.* Typhi, the etiologic agent of typhoid (enteric) fever, infects only humans. Typhoid fever is a human systemic disease that is responsible for an estimated 21 million new infections annually resulting in approximately 600,000 deaths worldwide (Crump et al., 2004). It is an important health problem in developing countries and poses a significant risk to travelers. This illness has a mortality rate of 2–3% even with adequate antibiotic therapy, as antibiotic resistance of *S.* Typhi is a significant problem (Bhutta et al., 1991). Since the host range of *S.* Typhi is restricted to humans, the murine model system (*S.* Typhimurium infection of susceptible inbred mice) has been widely used to study typhoid fever pathogenesis and immunity in the laboratory. Examining the progression of naturally acquired infection, the bacterium passes through the stomach and into the small intestine, preferentially invading, as do many other intestinal pathogens, through M-cells located in Peyer's patches in the distal ileum. After passage through the epithelium, the bacterium encounters, infects, replicates within, and disseminates (for systemic infections) primarily within macrophages. In this special topic, we explore the details of this infectious process through comprehensive reviews and summaries of key, recent publications.

One of the first contacts Salmonellae make in the intestine, particularly the colon, is with other bacteria or normal flora. Ahmer and Gunn (2011) describe the factors involved in overcoming "colonization resistance," mediated by normal flora and the gut innate immunity. In addition to mechanisms of innate immune defense subversion, they discuss various models and techniques that have been utilized to examine the S. Typhimurium-intestinal microbiota interplay. A key concept brought forth is that *S.* Typhimurium has been shown to be unable to colonize the mouse intestine in the absence of inflammation, as the normal flora in the non-inflamed state is able to effectively outcompete an avirulent (lacking inflammatory capacity) *Salmonella* intruder.

In addition to the normal flora, *Salmonella* must overcome numerous other gut barriers to infection, and must utilize its own weapons to enter through the non-phagocytic intestinal epithelial layer. Hallstrom and McCormick (2011) describe how *Salmonella* overcomes these hurdles and exploits the host defenses for their own purposes. They walk the reader through intestinal entry, the mucus layer, the intestinal epithelium [with focused discussion of M-cells as well as a paracellular pathway of entry (area between adjacent epithelial cells; tight junctions)], and into phagocytes where they are trafficked to the mesenteric lymph nodes.

Type III secretion systems are key to the virulence of several Gram-negative and enteric pathogens. The accepted dogma is that proteins are secreted by these systems directly from the cytoplasm of the bacterium to the cytosol of the host cell. However, the review of a manuscript by Akopyan et al. (2011) by Perrett and Zhou (2011) discusses data showing that this process may also occur by two steps – secretion to the bacterial surface followed by Type III secretion systems transport.

A manuscript by Knodler et al. (2010) reviewed here by Winter and Baumler (2011) describe the fate of some *S.* Typhimurium that find themselves within intestinal epithelial cells. In some instances, bacteria were surprisingly shown to escape the vacuole, replicate in the cytoplasm, and induce pyroptosis, which caused selective extrusion of these cells from the monolayer. This impacts both host clearance mechanisms and bacterial dissemination. Key to this inflammatory event is the activation of caspase-1, a topic that is reviewed by Miao and Rajan (2011). Upon bacterial sensing via membrane-bound toll-like receptors (TLRs) and intracellular NOD-like receptors (NLRs), pro-inflammatory pathways are activated which aid in clearance. Two of the NLR-containing complexes, NLRC4 and NLRP3 inflammasomes, and the activation of caspase-1 by these complexes, are key to this process, but *Salmonella* has also evolved mechanisms to subvert this response.

Increased details of *Salmonella* SPI-1 and SPI-2 function and how the factors that are secreted by these systems aid *Salmonella* in developing an intracellular niche are discussed by Malik-Kale et al. (2011). While the effectors for each pathogen are unique, the SPI-1 and SPI-2 type III secretion system translocated factors truly establish the intracellular niche for *Salmonella*, and their further understanding is key to a complete comprehension of host–pathogen interactions. They focus on the development of the unique *Salmonella*-containing vacuole and the role *Salmonella* plays in shaping its intracellular environment.

After progression of the bacterium through the epithelial barrier, Salmonellae encounter professional phagocytes. In the gut as well as following uptake/phagocytosis, Salmonellae are exposed to reactive oxygen (ROS) and nitrogen (RNS) intermediates. Henard and Vazquez-Torres (2011) describe the molecular targets of RNS (e.g., nitric oxide) as well as the ability of the bacterium to respond to this environment with mechanisms to both detoxify ROS and to repair damage- one of the important mechanisms

of intracellular survival. They also discuss the interesting finding that *Salmonella* can exploit ROS to enhance virulence.

The review from Guiney and Fierer (2011) discusses SpvR, SpvB, and SpvC – a positive transcriptional regulator and two SPI-2 secreted proteins, respectively. Interestingly, these loci are not found in S. Typhi but are strongly associated with non-typhoidal bacteremia causing strains. Though unclear concerning their role in virulence, they are an example of the species-specific virulence traits common in the Salmonellae.

All of the pathogenic events discussed in this *Salmonella* special topic rely upon effective environmental sensing and gene regulation. *Salmonella* possesses a complex regulatory network that has been studied in pieces, but not *in toto* where these regulatory network interconnections would be defined. Using high throughput genomic and proteomic assays and newly developed computational approaches for data integration, McDermott et al. (2011) show how these complex networks and inter-system regulatory pathways are being defined on a genome wide scale. This allows for a never before seen "30,000 foot view" of the interworkings of the virulence regulatory network in *Salmonella*.

ACKNOWLEDGMENTS

This work was supported by The National Institutes of Health grants AI066208 and AI043521.

REFERENCES

Ahmer, B. M., and Gunn, J. S. (2011). Interaction of *Salmonella* spp. with the intestinal microbiota. *Front. Microbiol.* 2:101. doi: 10.3389/fmicb.2011.00101

Akopyan, K., Edgren, T., Wang-Edgren, H., Rosqvist, R., Fahlgren, A., Wolf-Watz, H., and Fallman, M. (2011). Translocation of surface-localized effectors in type III secretion. *Proc. Natl. Acad. Sci. U.S.A.* 108, 1639–1644.

Andrews-Polymenis, H. L., Baumler, A. J., McCormick, B. A., and Fang, F. C. (2010). Taming the elephant: *Salmonella* biology, pathogenesis, and prevention. *Infect. Immun.* 78, 2356–2369.

Bhutta, Z. A., Naqvi, S. H., Razzaq, R. A., and Farooqui, B. J. (1991). Multidrug-resistant typhoid in children: presentation and clinical features. *Rev. Infect. Dis.* 13, 832–836.

Coburn, B., Grassl, G. A., and Finlay, B. B. (2007). *Salmonella*, the host and disease: a brief review. *Immunol. Cell Biol.* 85, 112–118.

Crump, J. A., Luby, S. P., and Mintz, E. D. (2004). The global burden of typhoid fever. *Bull. World Health Organ.* 82, 346–353.

Guiney, D. G., and Fierer, J. (2011). The role of the spv genes in *Salmonella* pathogenesis. *Front. Microbiol.* 2:129. doi: 10.3389/fmicb.2011.00129

Hallstrom, K., and McCormick, B. A. (2011). *Salmonella* interaction with and passage through the intestinal mucosa: through the lens of the organism. *Front. Microbiol.* 2:88. doi: 10.3389/fmicb.2011.00088

Henard, C. A., and Vazquez-Torres, A. (2011). Nitric oxide and *Salmonella* pathogenesis. *Front. Microbiol.* 2:84. doi: 10.3389/fmicb.2011.00084

Knodler, L. A., Vallance, B. A., Celli, J., Winfree, S., Hansen, B., Montero, M., and Steele-Mortimer, O. (2010). Dissemination of invasive *Salmonella* via bacterial-induced extrusion of mucosal epithelia. *Proc. Natl. Acad. Sci. U.S.A.* 107, 17733–17738.

Malik-Kale, P., Jolly, C. E., Lathrop, S., Winfree, S., Luterbach, C., and Steele-Mortimer, O. (2011). *Salmonella* – at home in the host cell. *Front. Microbiol.* 2:125. doi: 10.3389/fmicb.2011.00125

McDermott, J. E., Yoon, H., Nakayasu, E. S., Metz, T. O., Hyduke, D. R., Kidwai, A. S., Palsson, B. O., Adkins, J. N., and Heffron, F. (2011). Technologies and approaches to elucidate and model the virulence program of *Salmonella*. *Front. Microbiol.* 2:121. doi: 10.3389/fmicb.2011.00121

Miao, E. A., and Rajan, J. V. (2011). *Salmonella* and caspase-1: a complex interplay of detection and evasion. *Front. Microbiol.* 2:85. doi: 10.3389/fmicb.2011.00085

Perrett, C. A., and Zhou, D. (2011). Type three secretion system effector translocation: one step or two? *Front. Microbiol.* 2:50. doi: 10.3389/fmicb.2011.00050

Winter, S. E., and Baumler, A. J. (2011). *Salmonella* exploits suicidal behavior of epithelial cells. *Front. Microbiol.* 2:48. doi: 10.3389/fmicb.2011.00048

Received: 29 August 2011; accepted: 29 August 2011; published online: 19 September 2011.

Citation: Gunn JS (2011) Salmonella host–pathogen interactions: a special topic. Front. Microbio. 2:191. doi: 10.3389/fmicb.2011.00191

This article was submitted to Frontiers in Cellular and Infection Microbiology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 29 April 2011
doi: 10.3389/fmicb.2011.00088

Salmonella interaction with and passage through the intestinal mucosa: through the lens of the organism

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Salmonella enterica serotypes are invasive enteric pathogens spread through fecal contamination of food and water sources, and represent a constant public health threat around the world. The symptoms associated with salmonellosis and typhoid disease are largely due to the host response to invading *Salmonella*, and to the mechanisms these bacteria employ to survive in the presence of, and invade through the intestinal mucosal epithelia. Surmounting this barrier is required for survival within the host, as well as for further dissemination throughout the body, and subsequent systemic disease. In this review, we highlight some of the major hurdles *Salmonella* must overcome upon encountering the intestinal mucosal epithelial barrier, and examine how these bacteria surmount and exploit host defense mechanisms.

Keywords: *Salmonella*, intestinal mucosa, tight junctions

SALMONELLA BIOLOGY

Salmonella enterica are Gram (−) bacteria responsible for causing typhoid disease and gastroenteritis. *S. enterica* serovar Typhi (*S.* Typhi) is the primary cause of typhoid fever, while non-typhoidal *Salmonella* (NTS) strains can cause *Salmonella*-induced food poisoning called salmonellosis. While young children, the elderly, and immuno-compromised individuals are most at risk for complications, people at any age are susceptible to the diarrhea, intestinal cramping, and intestinal epithelial erosion associated with salmonellosis. The disease is primarily spread by the contamination of water and food items with fecal matter from infected hosts and is often self-limiting, but can cause prolonged complications (Graham et al., 2000).

Salmonella possess *Salmonella* pathogenicity islands (SPI), or collections of pathogenesis-related genes acquired horizontally. At least 21 SPIs have been identified in *S.* Typhimurium and *S.* Typhi combined (for review of functions, see Sabbagh et al., 2010). In *S.* Typhimurium and in *S.* Typhi, SPI-1 and SPI-2 contain genes for two type-three secretion systems (T3SS). Specific to Gram (−) bacteria, the T3SS likely evolved from the flagella basal body, and is composed of a motor, needle complex, and translocon through which secreted effectors are injected into host cells (Stebbins and Galan, 2003). The effectors provide various functions, including promoting bacterial entry, controlling inflammatory responses, and regulating bacterial survival within the cell (summarized in **Table 1**). The SPI-1 T3SS (T3SS1) is primarily associated with invasion (Galan, 1996). Effectors secreted through the SPI-2 T3SS (T3SS2) seem to primarily promote the intracellular survival of *Salmonella*, although they may not be absolutely required for *S.* Typhi survival within human macrophages (Forest et al., 2010). However, in *S.* Typhi infections of humanized non-obese–diabetic mice, the loss of some SPI-2 genes caused a competitive disadvantage (Libby et al., 2010). Also, SPI-2 genes are up-regulated during *S.* Typhi infection of macrophages *in vitro* (Faucher et al., 2006). While the functions of SPI-1 and SPI-2 effectors have traditionally been considered distinct from each other, there is increasing evidence suggesting overlap in the times at which effectors from each system are required (Lawley et al., 2006; Brawn et al., 2007). These data highlight the intricate level of coordination between SPI-1 and SPI-2 involved with *Salmonella* pathogenesis. Nonetheless, recent data challenges the dependence of T3SS1 in *Salmonella* invasion (Radtke et al., 2010). SPI-1 mutant strains of *Salmonella* were capable of invading HT-29 3D intestinal cells to a higher degree than HT-29 monolayers, although the level of invasion was less than that of the wild-type strains. Additionally, SPI-1 was required for wild-type levels of intracellular replication over a 24-h period. The additional loss of SPI-2 may enhance these phenotypes. These data suggest that various pathways are required for wild-type levels of invasion and intracellular replication. Other types of secretion systems that may promote pathogenicity have been identified in additional SPI loci (Sabbagh et al., 2010).

SALMONELLA INTERACTION WITH THE INTESTINAL MUCOSAL EPITHELIA

The gastrointestinal tract is the largest mucosal surface in the human body, with the epithelial monolayer surface area alone measuring 400 m^2 (MacDonald and Monteleone, 2005; Turner, 2009). Regulating the host microbiota, immune responses, and barrier functions is paramount to providing timely and controlled retaliation to pathogenic assaults, while simultaneously maintaining a healthy, balanced, intestinal environment (**Figure 1**).

GUT MICROBIOME

The human intestine is home to a plethora of mutualistic microorganisms, dominated mostly by *Lactobacillus*, *Bacteroides*, and *Firmicutes* (Backhed et al., 2005). Among other roles, these organisms assist immune system development and promote epithelial homeostasis (Rakoff-Nahoum et al., 2004; Mazmanian et al., 2005). Beyond

Table 1 | *Salmonella* effectors and their roles in pathogenesis.

Effector	Location	Function	Targets	T3SS apparatus
AvrA	SPI-1	Controls *Salmonella*-induced inflammation (Collier-Hyams et al., 2002; Ye et al., 2007).	IkBa, beta-catenin	1
SipA	SPI-1	Mediates invasion at apical surface by inducing actin bundling and promotes neutrophil migration to the apical surface. Also suspected to promote SCV formation. Undergoes cleavage by CASPASE-3 (Zhou et al., 1999; Brawn et al., 2007; Wall et al., 2007; Srikanth et al., 2010)	Actin	1
SipB	SPI-1	Component of T3SS1 translocon (Kaniga et al., 1995; Hayward et al., 2000)	Cholesterol	1
SipC	SPI-1	SPI-1 translocon component, induces actin bundling to promote invasion (Kaniga et al., 1995; McGhie et al., 2001)	Actin	1
SipD	SPI-1	Component of T3SS1 translocon (Lara-Tejero and Galan, 2009)		1
SopA		E3 ubiquitin ligase that may promote escape from SCV and promotes neutrophil migration. Also required during invasion (Wood et al., 2000; Raffatellu et al., 2005; Zhang et al., 2006)		1
SptP	SPI-1	Similar to GAPs, and is a tyrosine phosphatase. Reverses pro-inflammatory responses due to other *Salmonella* effectors (Stebbins and Galan, 2000)	Cdc42, Rac-1	1
SopB	SPI-5	Inositol polyphosphate phosphatase that promotes macropinocytosis, regulates SCV localization, and promotes fluid secretion (Norris et al., 1998; Hernandez et al., 2004)	Inositol phosphates	1
SpiC	SPI-2	Helps regulate T3SS2 secretion (Yu et al., 2002)	Hook 3, TassC	2
SseF	SPI-2	SCV regulation (Abrahams et al., 2006)	Microtubules	2
SseG	SPI-2	SCV positioning (Abrahams et al., 2006)	Microtubules	2
SopD		Promotes invasion and fluid secretion (Zhang et al., 2002; Raffatellu et al., 2005)		1/2
SopE	Bacteriophage	Promotes membrane ruffling and disrupts tight junctions (Hardt et al., 1998; Boyle et al., 2006)	Rac-1, Cdc42	2
SopE2		Promotes membrane ruffling and disrupts tight junctions (Stender et al., 2000; Boyle et al., 2006)	Cdc42	2
SspH1	Bacteriophage Gifsy-3	E3 ubiquitin ligase (Rytkonen and Holden, 2007)		1/2
SspH2	SPI-12	E3 ubiquitin ligase (Quezada et al., 2009)		2
PipB2		Promotes Sif extension (Knodler and Steele-Mortimer, 2005)	Kinesin-1	2
SifA		Sif formation and membrane integrity (Stein et al., 1996; Beuzon et al., 2000)	SKIP, Rab7	2
SopD2		Sif formation and promotes bacterial replication in mouse macrophages (Jiang et al., 2004)		2
SseJ		Negatively regulates Sifs and antagonizes SifA-mediated stability of SCV (Ruiz-Albert et al., 2002)	Cholesterol	2
SseL		Cysteine protease and has de-ubiquitinating activity. Helps attenuate *Salmonella* virulence (Rytkonen et al., 2007)		2
SteC		A kinase that promotes F-actin meshwork formation (Poh et al., 2008)		2
SpvB	pSLT (in *S.* Typhimurium)	Depolymerizes actin filaments *in vitro* (Lesnick et al., 2001)	Actin	2
SpvC	pSLT (in *S.* Typhimurium)	A phosphothreonine lyase required for complete virulence in murine models (Mazurkiewicz et al., 2008)		1/2

A summary of effectors whose functions in Salmonella pathogenesis have been identified.

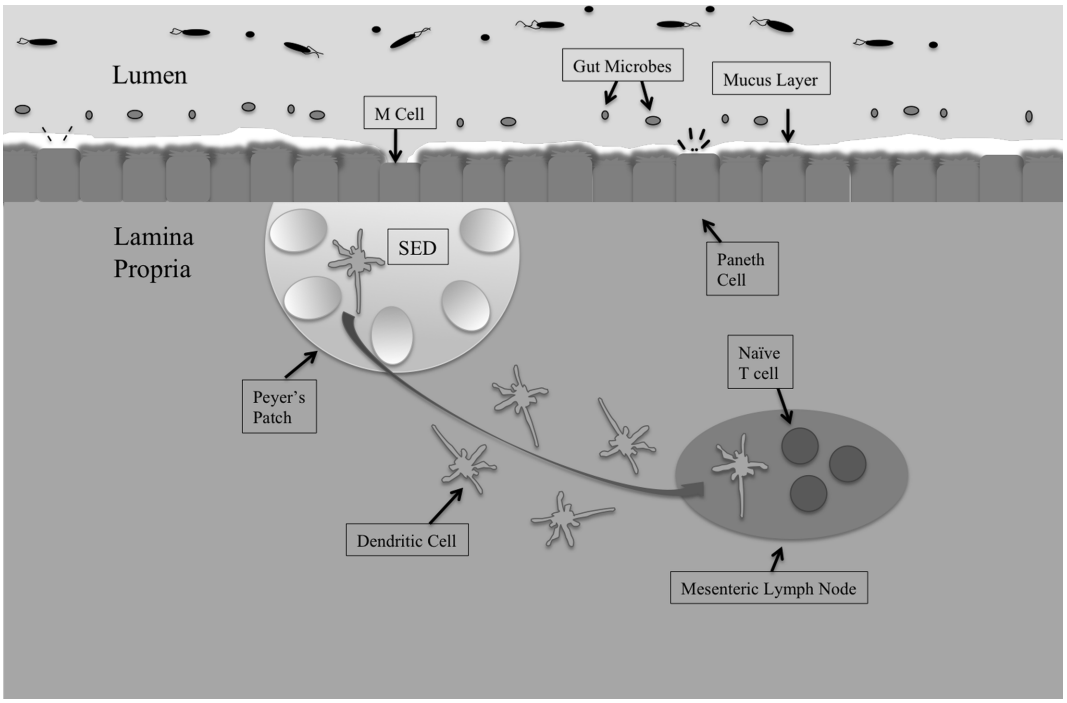

FIGURE 1 | The intestinal mucosal epithelium is home to various interacting cell types that come together to maintain intestinal homeostasis and protect against invading pathogens. The first line of defense is the host microbiota, populations of commensal organisms that compete with invading pathogens for nutrients and space. The mucus layer protects against *Salmonella* invasion of epithelial cells, and the bacteria must adhere to mucus components in order to remain in the intestines. The epithelial monolayer underlying the mucus layer contains distinct cell types with different roles. M cells sample intestinal antigens and are the preferred route of entry by *Salmonella*. Underlying the M cells is the subepithelial dome (SED) that houses Peyer's patches. Peyer's patches contain germinal centers and have associated dendritic cells. Dendritic cells take whole bacteria to the mesenteric lymph node (MLN), from which *Salmonella* can escape to promote systemic disease.

these regulatory roles, the microbiome also functions as a critical barrier to invading pathogens. The population of host microbes in the gut physically blocks pathogen access to the epithelial layer, and also outcompetes pathogens for nutrients, thus reducing the survival and invasiveness of intestinal pathogens. Nevertheless, *S.* Typhimurium is capable of maneuvering through the microbiome to reach intestinal epithelial cells. In mouse experiments with wild-type and avirulent *S.* Typhimurium, Stecher et al. demonstrate that an inflammatory state in the intestines not only permits wild-type *S.* Typhimurium to outcompete the microbiome during colonization, but also allows a normally avirulent strain to colonize the intestines in the presence of intestinal microbiota (Stecher et al., 2007). These data unveil an intriguing concept that *S.* Typhimurium utilizes inflammation to outcompete host microbiota in mouse models of salmonellosis.

Host microbiota may also regulate the degree of *S.* Typhimurium shedding by infected mice. Following infection with 10^8 CFU of bacteria, mice were classified into three groups corresponding to the level of fecal shedding: supershedders (>10^8 CFU/g feces), moderate shedders (10^4–10^8 CFU/g feces), and low shedders (<10^4 CFU/g feces). Treating low shedders with streptomycin converted these mice to supershedders (Lawley et al., 2008), suggesting that the absence of host microbes may have permitted *Salmonella* to colonize more heavily, thus elevating the level of shed bacteria. The significance of a gut microbiota barrier to *Salmonella* infection was also explored by Croswell et al. (2009). The authors found that treatment of mice with streptomycin, streptomycin–bacitracin, or ampicillin–vancomycin–neomycin–metronidazole (AVNM) reduced gut microflora populations, and that each treatment was subsequently associated with an increase in *Salmonella* colonization and *Salmonella*-induced inflammation compared to untreated mice. The authors further show that allowing 3 weeks between antibiotic treatment and infection still predisposed mice to greater levels of inflammation in response to *Salmonella* infection than was seen in mice that were never treated with antibiotics. These results suggest that antibiotics have long-lasting effects on gut microflora populations, and that the presence and composition of gut microfloral populations may play an important role in controlling *Salmonella* colonization.

Additionally, Barman et al. (2008) showed that salmonellosis may alter the microfloral population in FvB mice. Infected mice showed disrupted numbers of *Bacteroides, Lactobacillus/Enterococcus*, and *Eubacterium rectale/Clostridium coccoides* groups and of the *C. perfringens* group compared to uninfected mice. However, *Salmonella* did not replace these populations, and wild-type population numbers returned after *Salmonella* clearance, suggesting any effect salmonellosis has on the intestinal microbiota is not permanent.

MUCOSAL LAYER

Key to the mucosal epithelium is the formation of a mucus layer along the luminal lining of the gastrointestinal tract. Mucosal epithelial cells, specifically goblet cells, secrete glycosylated transmembrane

proteins called mucins at the cell surface. These proteins generate a layer of large complexes containing thread-like structures and oligosaccharides. The resulting gelatinous layer blocks contact between the underlying epithelial monolayer and large particles, including bacteria. The mucus layer also contains trefoil factors, which are peptides produced in tissues containing mucus-producing cells, such as the intestinal epithelia. Their various proposed functions include limiting intestinal inflammation (Playford et al., 1996) and regulation of immune system responses (Baus-Loncar et al., 2005). Adhering to the mucosal layer would be necessary for *Salmonella* to avoid being washed out of the intestines. Indeed, there is evidence of *Salmonella* binding to mucus, although increased adherence does not necessarily correlate with increased mucosal penetration (Nevola et al., 1987; McCormick et al., 1988; Vimal et al., 2000).

EPITHELIAL MONOLAYER: M CELLS AND THE ASSOCIATED PEYER'S PATCHES

The epithelial monolayer mediates interactions between triggers of immune responses, and the gut-associated lymphoid tissue (GALT), where immune responses originate (MacDonald and Monteleone, 2005; Turner, 2009). A follicle-associated epithelial (FAE) layer of columnar epithelial cells blankets lymphoid tissue within the intestinal wall. The intestinal mucosa contains a variety of cell types with unique functions including enterocytes, entero-endocrine cells, goblet cells, Paneth cells, and microfold (M) cells (**Table 2**). While all the unique epithelial cells found within the intestine are important for establishing the functional intestinal mucosal epithelium, in mice the M cells are a key route of invasion by *Salmonella*, and are thus discussed in greater detail here.

Microfold cells are specialized intestinal epithelial cells that are found in the FAE overlying mucosa-associated lymphoid tissue (MALT). Their primary role is to sample mucosal contents and transfer antigens from the lumen to underlying Peyer's patches (Tam et al., 2008), and thus act as sentinels of the intestinal epithelium. The overall structure of M cells differs in various ways from that of enterocytes. For example, the apical (mucosal) surface of M cells is not covered by the mucus layer observed over other cells in the intestines (Frey et al., 1996). Additionally, the apical brush border characteristic of enterocytes is absent from M cells, which instead contain microfolds. These features promote the sampling role of M cells, but also inadvertently provide opportunities for enteropathogen docking and invasion.

An additional trait of M cells that promotes both their role as intestinal sentinels and promotes enteropathogen invasion is their use of a transcytotic pathway to shuttle luminal contents to lymphoid tissue (Kraehenbuhl and Neutra, 2000). Following endocytosis of extra-cellular material (including invading pathogens), M cells transfer the endosomal contents to the basolateral (serosal) surface and to the underlying MALT. This action results in appropriate immune responses to infection. Likewise, bacteria engulfed at the apical surface can escape the M cell through this same pathway and disseminate to other areas.

M cells provide a key route of invasion by *S.* Typhimurium (Jensen et al., 1998). SPI-1 genes help regulate *Salmonella* invasion through M cells, although SPI-1-independent processes are also proposed to regulate M cell invasion (Clark et al., 1996). For example, *Salmonella* defective for *invA* are less able to invade M cells (Clark et al., 1998), but SPI-1 mutants are still capable of invading M cells (Martinez-Argudo and Jepson, 2008). These results suggest that while SPI-1 genes are needed for wild-type levels of invasion, SPI-1-independent mechanisms are also important for *Salmonella* invasion through M cells.

The lymphoid tissue underlying M cells contains Peyer's patches, which are large aggregates of B lymphocyte follicles that contain germinal centers, and can be found along the length of the intestine. Here, the antigens translocated by M cells are processed by local dendritic cells. Lysozyme-producing dendritic cells associated with Peyer's patches mediate uptake of *S.* Typhimurium (LeLouard et al., 2010). Peyer's patches also play a role in up-regulating intestinal IgA as a result of *Salmonella* infection (Hashizume et al., 2008).

EPITHELIAL MONOLAYER: STRUCTURE AND *SALMONELLA* ENTRY

The barrier function of the epithelial monolayer results from a variety of proteins that maintain close intercellular interactions. Desmosomes are composed of cadherins, which form an adhesive

Table 2 | Cells within the intestinal epithelial monolayer.

Cell type	General function	*Salmonella* infection impact
Goblet cell	Sustained and environmentally triggered mucin production (Deplancke and Gaskins, 2001)	Loss of goblet cells reported as a result of *S.* Typhimurium-mediated colitis in mice (Hapfelmeier et al., 2004, 2005), although this observation is not restricted only to colitis caused by *Salmonella*. Invasion of goblet cells reported in *Salmonella* infections of pig ileal loops (Meyerholz et al., 2002; Meyerholz and Stabel, 2003).
Paneth cell	Secrete the anti-microbial peptides lysozyme and alpha-defensin (Ayabe et al., 2000)	*S.* Typhimurium may modulate the production of anti-microbial peptides (Salzman et al., 2003)
M cells	Transfer antigens to Peyer's patches (Tam et al., 2008)	M cells are the preferred route of invasion by *Salmonella* (Jensen et al., 1998)
Enterocytes	*Absorptive/villus enterocytes*: Nutrient absorption *Crypt enterocytes*: Chloride and IgA secretion	*Salmonella* invasion of absorptive enterocytes reported in calf ileal loops (Frost et al., 1997), and in pig ileal loops (Meyerholz et al., 2002; Meyerholz and Stabel, 2003)
Entero-endocrine	Secretion of various hormone molecules, promote food digestion	*Salmonella*-specific responses undetermined

interface (Koch and Franke, 1994; Green and Simpson, 2007), and varying structural proteins, notably plakoglobin and desmoplakin (Hatsell and Cowin, 2001), all of which help disperse forces from physical stress. Meanwhile, the paracellular pathway, or area between adjacent epithelial cells, is kept closed by tight junctions and adherens junctions. Bacteria attempting to penetrate the epithelial monolayer must possess mechanisms for overcoming these strong interactions. As such, *Salmonella* possess several effectors that mediate disruption of the epithelial barrier and subsequent uptake into the non-phagocytic epithelial cells lining the intestinal lumen. These actions are carried out largely through the alteration of junctional protein localization and of Rho GTPase activity. The latter mechanism is especially important in the *Salmonella*-mediated modulation of tight junctions. Dis-regulating the Rho-GTPases leads to changes in organization of junctions and of actin at the cell membrane, which subsequently alters the membrane morphology in a manner referred to as "ruffling" (Finlay et al., 1991), and promotes *Salmonella* entry (**Figure 2**).

Interactions between classical (Ca^{2+} dependent) cadherins and catenin family proteins are the foundation of adherens junctions. E-cadherin (epithelial cadherin) is a single pass transmembrane protein and its ability to homodimerize with E-cadherins on neighboring cells makes it a key component of adherens junctions (Takeichi, 1991). E-cadherins form small, intercellular clusters that quickly associate with actin and, overtime, expand into larger bundles that strengthen cell–cell adhesion (Adams et al., 1998). p120-catenin stabilizes E-cadherin at the cell surface, and its loss induces down-regulation of E-cadherin (Davis et al., 2003). Further, beta-catenin, a transcription factor involved in cell proliferation and differentiation, can bind to the cytoplasmic domain of E-cadherin (Hartsock and Nelson, 2008), and direct the localization of E-cadherin from the ER to the plasma membrane (Chen et al., 1999). Phosphorylation of beta-catenin promotes its ubiquitination and subsequent degradation (Aberle et al., 1997). Duan et al. (2007) demonstrated that cultured epithelial cells infected with *S.* Typhimurium display increased phosphorylated beta-catenin. This increase in phosphorylated beta-catenin resulting from *Salmonella* infection could increase the level of beta-catenin degradation, and thus could limit E-cadherin translocation to the plasma membrane. Such an action would result in weakened adherens junctions and promote *Salmonella* invasion through the epithelial monolayer.

The tight junction is an important regulator of epithelial monolayer permeability (Martinez-Palomo and Erlij, 1975), and of cell polarity by preventing mixing of apical (mucosal) and basolateral (serosal) components. The "leak pathway" permits passage of larger solutes, such as bacterial peptides (but not whole bacteria), while the "small pore pathway" excludes solutes larger than 4 Å, and exhibits some charge selectivity (Forster, 2008; Turner, 2009). The core of tight junction complexes is composed of occludin, ZO, and claudin proteins. Occludin binds to several crucial tight junction proteins including ZO-1 (Furuse et al., 1994), ZO-2 (Itoh et al., 1999), and ZO-3 (Haskins et al., 1998) and its activity is regulated by PKC-mediated phosphorylation (Andreeva et al., 2001). ZO-1, ZO-2, and ZO-3 are members of the membrane-associated guanylate kinase (MAGUK) family (Gonzalez-Mariscal et al., 2000). ZO-1 and ZO-2 assist in the polymerization of claudins, which permits extension of tight junctions, and also recruit ZO-3 to the tight junction (Umeda et al., 2006; Tsukita et al., 2009). A specific role for ZO-3 has yet to be determined, although it is shown to not be required for tight junction formation (Adachi et al., 2006). Claudins, which are tetraspan transmembrane proteins, are key structural elements, as they recruit occludin (Furuse et al., 1998). Another class of tight junction proteins include junction-associated adhesion molecules (JAMs), or which are integral membrane proteins that belong to the immunoglobin superfamily.

Cytoskeletal regulators of tight junctions include myosin ATPase, AMP-activated protein kinases, and especially Rho-GTPases (Turner, 2009). Rho-GTPases regulate various cell functions and

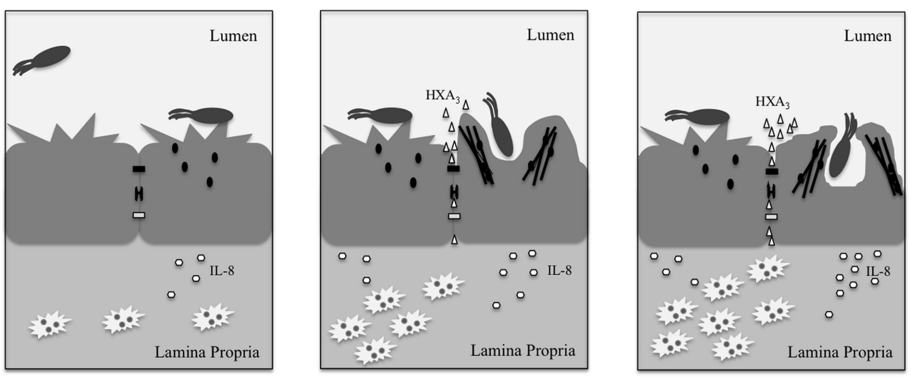

FIGURE 2 | Upon interacting with the epithelial cell, *Salmonella* secrete effectors (small black ovals) that promote release of the neutrophil chemoattractant, HXA_3 (small triangles), and membrane ruffling as a result of actin (black lines) re-organization. The release of HXA_3 from the apical (mucosal) surface creates a concentration gradient across tight junctions (small black rectangle), desmosomes (small black bracket), and adherens junctions (small white rectangle) and through the paracellular space. Neutrophils (jagged white ovals) are recruited via basolateral secretion of IL-8, and subsequently migrate from the basolateral surface to the apical surface to the point of infection due to the HXA_3 gradient. The actin re-organization alters the morphology of the cell membrane at the apical surface in a manner that promotes *Salmonella* uptake via macropinocytosis. Once inside the cell, *Salmonella* reside within a compartment termed the SCV that forms as a result of macropinocytosis.

are activated by guanine-nucleotide exchange factors (GEFs), which facilitate the active, GTP-bound state. GTPase activating protein (GAPs) inhibit Rho-GTPases by activating GTP hydrolysis, thereby inducing the inactive, GDP-bound state (Etienne-Manneville and Hall, 2002). Rho-GTPases are regulated by several proteins, the best-studied being Rho, Cdc42, and Rac. Rho is required for formation of focal adhesions and stress fibers, while constitutive Rac causes formation of plasma membrane extensions (Ridley and Hall, 1992; Ridley et al., 1992). Cdc42 activity promotes finger-like extrusions called filopodia (Nobes and Hall, 1995).

The tight junction is a key target of *Salmonella*, as infection with *S.* Typhimurium induces altered localization of ZO-2 and claudin-1, degradation of ZO-1, and promotes dephosphorylation of occludin in T84 cells (Kohler et al., 2007). *Salmonella* SPI-1 effectors SopB, SopE, SopE2, and SipA each play various roles in inducing ruffling and bacterial uptake by changing the localization and expression of ZO-1 and occludin, although SipA and SopB alone are not sufficient for this process (Boyle et al., 2006). These same effectors were also associated with altered epithelial cell polarity. Specifically, SopB (or SopB/SigD) is a phosphoinositide phosphatase that promotes formation of macropinosomes (Hernandez et al., 2004). This effector is capable of indirectly stimulating Cdc42-dependent cytoskeletal rearrangements (Zhou et al., 2001), which can lead to extension of the cell membrane around the bacterium. Similarly, SopE and SopE2 act like GEFs, and activate Cdc42 and Rac1 GTPases to induce cytoskeletal rearrangements that favor bacterial uptake (Stender et al., 2000). SipA and SipC associate with actin and promote bundling of actin filaments so as to facilitate macropinocytosis of *Salmonella* (McGhie et al., 2001).

As control mechanisms, *Salmonella* also secrete SptP and AvrA. SptP acts like a GAP, and essentially reverses the cytoskeletal rearrangements that occur during *Salmonella* uptake (Fu and Galan, 1999). AvrA, which stabilizes tight junctions (Liao et al., 2008) and inhibits NF-kB activation (Collier-Hyams et al., 2002), keeps the *Salmonella*-induced inflammation response in check.

IMMUNE RESPONSE

PAMP Receptors

Intestinal epithelial cells can recognize invading pathogens by capturing common viral or bacterial components, known as pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) are a well-studied family of PAMP receptors. Several types of intestinal epithelial cells have been shown to express TLRs (Elphick and Mahida, 2005; Tyrer et al., 2006; Palazzo et al., 2007; Gribar et al., 2008), and each type of receptor recognizes a unique PAMP. TLR4 recognizes lipopolysaccharide (LPS) from Gram (−) bacterial cell walls, TLR2 in concert with TLR1 or TLR6 recognizes triacyl or diacyl bacterial lipopeptide, respectively, and TLR5 recognizes flagellin protein from bacterial flagella. The binding of a TLR to its cognate antigen triggers activation of a signaling cascade that ultimately, via NF-kB activation, induces production of pro-inflammatory cytokines. TLR5-flagellin interaction during *S.* Typhimurium in mouse infections was shown to regulate the early stages of *Salmonella* infection and immune response. Peyer's patches and mesenteric lymph nodes (MLN) in TLR5-deficient mice accumulate more *Salmonella* than wild-type littermates, and aflagellate *Salmonella* accumulate more in Peyer's patches and MLN of wild-type mice than do wild-type *Salmonella* (Fournier et al., 2009). As *Salmonella* encounter Peyer's patches after traversing the epithelial monolayer, regulation at this stage would be key to preventing further bacterial dissemination.

NLRs, or nucleotide-binding and oligomerization domain (NOD)-like receptors, are another established family of PAMP receptors that recognize components of bacterial peptidoglycan. The NLR family includes Nod1 and Nod2, both of which are expressed intracellularly by intestinal epithelial cells (MacDonald and Monteleone, 2005). Nod1 recognizes – D-glutamyl-*meso*-diaminopimelic (DAP) acid, expressed predominantly by Gram (−) bacteria (Chamaillard et al., 2003), while Nod2 recognizes muramyl dipeptide (MDP), which is common to Gram (−) and Gram (+) bacteria (Girardin et al., 2003). Binding between Nod1 and Nod2 and their specific PAMPs is followed by association with the adaptor protein Rip2, which initiates production of NF-kB-dependent pro-inflammatory cytokines. A recent paper suggests the role of Rip2-mediated inflammatory responses is key during *Salmonella* infection only when SPI-2 effectors are present (Geddes et al., 2010). As SPI-2 effectors are associated predominantly with intracellular survival, Rip2, and thus Nod1 and Nod2, may be required for dampening the ability of *Salmonella* to survive after invading host cells.

NEUTROPHIL RECRUITMENT

The release of IL-8 resulting from pathogen-induced immune signaling stimulates the recruitment of neutrophils from blood vessels to the basolateral (serosal) surface (**Figure 2**). Neutrophils are phagocytic white blood cells that are among the first line of the innate immune defense in response to pathogens. Recruitment of neutrophils to the apical (mucosal) surface is mediated by release of hepoxilin A_3 (HXA_3), a potent neutrophil chemoattractant, and a metabolite of the arachidonic acid pathway (McCormick, 2007). SipA at the apical surface induces a lipid signal cascade that includes the activation of PKC, which causes the release of arachidonic acid from the plasma membrane (Wall et al., 2007). Arachidonic acid is then converted to HXA_3 by 12-lipoxygenase activity. Release of HXA_3 is facilitated by the ABC transporter, MRP2 (Pazos et al., 2008), and generates a concentration gradient across the tight junction and through the paracellular space. The recruitment of neutrophils superficially seems solely an act of host defense; however the rapid migration of these cells through the epithelial monolayer may actually loosen the epithelial cell–cell interactions, and thus create space through which *Salmonella* can invade, further facilitating PMN infiltration (Kohler et al., 2007). Indeed, PMN transmigration has been demonstrated to reduce epithelial monolayer resistance *in vitro* (Nash et al., 1987).

SALMONELLA RESIDE IN THE *SALMONELLA* CONTAINING VACUOLE

The *Salmonella* containing vacuole (SCV) is an intracellular vacuole that forms via macropinocytosis of *Salmonella*. The SopB effector helps direct the maturation of the SCV into a compartment

suitable for bacterial survival and replication (Bakowski et al., 2008), and may function to steer the compartment away from the endocytic pathway (Hernandez et al., 2004). After formation of the SCV, many T3SS2 effectors assist in formation of *Salmonella*-induced filaments (Sifs), localization of the SCV, and intravacuolar replication.

SifA, SseF, SseG, and SopD2 are involved with Sif formation, which results in the extension of tubules outward from the SCV after fusion with late endosomal compartments (Brumell et al., 2001). SifA is required for Sif formation (Stein et al., 1996), as its absence precludes formation of any Sif structures. SseF and SseG are also required for proper Sif formation, as mutants lacking either of these effectors only form Sif-like structures that differ in composition from fully formed Sifs (Kuhle et al., 2004). SopD2 has recently been shown to balance the effects of SifA, as its loss in SifA mutants restores SCV stability in the presence of other effectors (Schroeder et al., 2010). The role of Sifs in *Salmonella* survival and replication has yet to be determined. To date, these structures have not been observed *in vivo*. SseF and SseG also localize with, and promote bundling of, microtubules in the cytoplasm and help position the SCV near the microtubule organizing center (MTOC), which is close to the Golgi network (Kuhle et al., 2004; Ramsden et al., 2007). Proximity to the Golgi network appears necessary for efficient bacterial replication within the SCV, as disruption of the Golgi network and loss of SseG diminish bacterial growth (Salcedo and Holden, 2003).

Once *Salmonella* enter the host epithelial cell, they seem to form two populations with distinct doubling rates. One population, referred to as "hyper-replicating," doubles approximately every 20 min while the population as a whole doubles approximately every 95 min (Knodler et al., 2010). Interestingly, at least a third of the hyper-replicating population was found in the cytosol and express T3SS1 genes, while the slower replicating population was found in SCVs, and was expressing T3SS2 genes. These findings suggest that while *Salmonella* are capable of replicating within the SCV, cytosolic replication may be more efficient.

SALMONELLA INDUCES ILEAL SECRETION

One of the hallmarks of salmonellosis is a severe, watery diarrhea caused by high levels of fluid secretion into the intestines. Various T3SS1 effectors are proposed to mediate the level of fluid secretion into the gut, likely due to their ability to induce inflammation, including SopA, SopB, SopD, SopE2, and SipA (Zhang et al., 2002). Additionally, the loss of some genes encoded on SPI-5, including SopB, significantly reduced the level of *S. dublin*-induced fluid secretion from ligated ileal loops (Wood et al., 1998). One theory is that the rapid influx of neutrophils, in response to the inflammation caused by secreted effectors, impairs the epithelial barrier to an extent that results in leakage of extravascular fluids (Zhang et al., 2003). Neutrophil migration could also lead to chloride secretion by epithelial cells (Madara et al., 1993), an event that is compensated for by the subsequent secretion of water in an attempt by the host to restore ion balance.

MECHANISMS OF *SALMONELLA* ESCAPE FROM EPITHELIAL CELLS AND DISSEMINATION

Salmonella Typhi, using the same initial invasion mechanisms as *S.* Typhimurium can disseminate in humans to cause systemic disease through infection of the gallbladder, liver, spleen, and bone marrow (Gonzalez-Escobedo et al., 2011). In mice, *S.* Typhimurium is capable of invading and destroying M cells, allowing penetration of the intestinal epithelium (Jones et al., 1994), and subsequent phagocytosis by macrophages. *S.* Typhimurium survival within the macrophages is mediated by SPI-2 effectors (Cirillo et al., 1998) and is essential for dissemination.

The role of the MLN in bacterial dissemination was closely explored by Voedisch et al. Here, the authors show that dendritic cells are major suppliers of *S.* Typhimurium to the MLN, but they alone are not regulating dissemination. After permitting re-circulation of intestinal lymph following removal of the MLN (mesenteric adenectomy), the authors observed increased liver colonization by *S.* Typhimurium in wild-type mice, and increased colonization of liver and spleen in NRAMP1+ 129Sv mice. Wild-type levels of liver and spleen colonization were observed in Rag2-deficient mice, suggesting the observations in the adenectomized mice were not artifacts of a weak adaptive immune response (Voedisch et al., 2009). These results suggest that while dendritic cells supply the MLN with *S.* Typhimurium, the MLN is the final barrier to dissemination in this pathway, as its presence is needed to limit the circularization of infected dendritic cells.

Although most NTS infections are self-limiting, NTS strains are capable of inducing prolonged infection, particularly in those with weakened immune systems. To cause prolonged infection, *Salmonella* must have a mechanism in place to facilitate escape from infected host cells. A recent paper suggests *Salmonella* escape their intracellular niche by co-opting the epithelial cell shedding process, a host mechanism used to remove dying epithelial cells. During this process, called extrusion, cells adjacent to a dying cell contract and force the dying cell out into the lumen (Madara, 1990; Mayhew et al., 1999). The authors found that during *Salmonella* infection, extrusion rates were increased, and about 10% of infected cells underwent extrusion, followed by inflammatory cell death. In contrast, less than 1% of uninfected cells underwent extrusion, and those that were extruded did not exhibit inflammatory cell death (Knodler et al., 2010). Escape into the lumen can permit *Salmonella* to infect additional cells, or to exit the host completely as part of its transmission cycle.

CONCLUDING REMARKS

Salmonella employ various mechanisms to overcome host defense mechanisms. By evolving ways to subvert, mimic, antagonize, or exploit a defense strategy, *Salmonella* maintain their ability to infect vertebrate hosts. The present and future research endeavors aimed at better understanding the tools *Salmonella* use to invade and traverse the mucosal intestinal epithelia will provide invaluable knowledge that will help devise ways to better treat and prevent *Salmonella* infections.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health Grant DK56754 and the Crohn's and Colitis Foundation of America to Beth A. McCormick.

REFERENCES

Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997). β-catenin is a target for the ubiquitin–proteasome pathway. *EMBO J.* 16, 3793–3804.

Abrahams, G. L., Muller, P., and Hensel, M. (2006). Functional dissection of SseF, a Type III Effector protein involved in positioning the *Salmonella*-containing vacuole. *Traffic* 7, 950–965.

Adachi, M., Inoko, A., Hata, M., Furuse, K., Umeda, K., Itoh, M., and Tsukita, S. (2006). Normal establishment of epithelial tight junctions in mice and cultured cells lacking expression of ZO-3, a tight-junction MAGUK protein. *Mol. Cell. Biol.* 26, 9003–9015.

Adams, C. L., Chen, Y., Smith, S. J., and Nelson, W. J. (1998). Mechanisms of epithelial cell–cell adhesion and cell compaction revealed by high-resolution tracking of E-cadherin–green fluorescent protein. *J. Cell Biol.* 142, 1105–1119.

Andreeva, A. Y., Krause, E., Müller, E. C., Blasig, I. E., and Utepbergenov, D. I. (2001). Protein kinase C regulates the phosphorylation and cellular localization of occludin. *J. Biol. Chem.* 42, 38480–38486.

Ayabe, T., Satchell, D. P., Wilson, C. L., Parks, W. C., Selsted, M. E., and Ouellette, A. J. (2000). Secretion of microbicidal α-defensins by intestinal Paneth cells in response to bacteria. *Nat. Immunol.* 1, 113–118.

Backhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., and Gordon, J. I. (2005). Host-bacterial mutualism in the human intestine. *Science* 307, 1915–1920.

Bakowski, M. A., Braun, V., and Brumell, J. H. (2008). *Salmonella*-containing vacuoles: directing traffic and nesting to grow. *Traffic* 9, 2022–2031.

Barman, M., Unold, D., Shifley, K., Amir, E., Hung, K., Bos, N., and Salzman, N. (2008). Enteric salmonellosis disrupts the microbial ecology of the murine gastrointestinal tract. *Infect. Immun.* 76, 907–915.

Baus-Loncar, M., Schmid, J., Lalani, el-N., Rosewell, I., Goodlad, R. A., Stamp, G. W., Blin, N., and Kayademir, T. (2005). Trefoil factor 2 (Tff2) Deficiency in murine digestive track influences the immune system. *Cell Physiol. Biochem.* 16, 31–42.

Beuzon, C. R., Méresse, S., Unsworth, K. E. Ruíz-Albert, J., Garvis, S., Waterman, S. R. Ryder, T. A., Boucrot, E., and Holden, D. W. (2000). *Salmonella* maintains the integrity of its intracellular vacuole through the action of SifA. *EMBO J.* 19, 3235–3249.

Boyle, E. C., Brown, N. F., and Finlay, B. B. (2006). *Salmonella enterica* serovar Typhimurium effectors SopB, SopE, SopE2 and SipA disrupt tight junction structure and function. *Cell. Microbiol.* 8, 1946–1957.

Brawn, L. C., Hayward, R. D., and Koronakis, V. (2007). *Salmonella* SPI1 effector SipA persists after entry and cooperates with a SPI2 effector to regulate phagosome maturation and intracellular replication. *Cell Host Microbe* 1, 63–75.

Brumell, J. H., Tang, P., Mills, S. D., and Finlay, B. B. (2001). Characterization of *Salmonella*-induced filaments (Sifs) reveals a delayed interaction between *Salmonella*-containing vacuoles and late endocytic compartments. *Traffic* 2, 643–653.

Chamaillard, M., Hashimoto, M., Horie, Y., Masumoto, J., Qiu, S., Saab, L., Ogura, Y., Kawasaki, A., Fukase, K., Kusumoto, S., Valvano, M. A., Foster, S. J., Mak, T. W., Nuñez, G., and Inohara, N. (2003). An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat. Immunol.* 4, 702–707.

Chen, Y., Stewart, D. B., and Nelson, J. W. (1999). Coupling Assembly of the E-Cadherin/β-Catenin complex to efficient endoplasmic reticulum exit and basal-lateral membrane targeting of E-Cadherin in polarized MDCK cells. *J. Cell Biol.* 144, 687–699.

Cirillo, D. M., Valdivia, R. H., Monack, D. M., and Falkow, S. (1998). Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. *Mol. Microbiol.* 30, 175–188.

Clark, M. A., Hirst, B. H., and Jepson, M. A. (1998). Inoculum composition and *Salmonella* pathogenicity island 1 regulate M-cell invasion and epithelial destruction by *Salmonella typhimurium*. *Infect. Immun.* 66, 724–731.

Clark, M. A., Reed, K. A., Lodge, J., Stephen, J., Hirst, B. H., and Jepson, M. A. (1996). Invasion of murine intestinal M cells by *Salmonella typhimurium inv* mutants severely deficient for invasion of cultured cells. *Infect. Immun.* 64, 4363–4368.

Collier-Hyams, L. S., Zeng, H., Sun, J., Tomlinson, A. D., Bao, Z. Q., Chen, H., Madara, J. L., Orth, K., and Neish, A. S. (2002). Cutting edge: *Salmonella* AvrA effector inhibits the key proinflammatory, anti-apoptotic NF-kB pathway. *J. Immunol.* 169, 2846–2850.

Croswell, A., Amir, E., Teggatz, P., Barman, M., and Salzman, N. H. (2009). Prolonged impact of antibiotics on intestinal microbial ecology and susceptibility to enteric *Salmonella* infection. *Infect. Immun.* 77, 2741–2753.

Davis, M. A., Ireton, R. C., and Reynolds, A. B. (2003). A core function for p120-catenin in cadherin turnover. *J. Cell Biol.* 163, 525–534.

Deplancke, B., and Gaskins, H. R. (2001). Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. *Am. J. Clin. Nutr.* 73(Suppl.), 1131S–1141S.

Duan, Y., Liao, A. P., Kuppireddi, S., Ye, Z., Ciancio, M. J., and Sun, J. (2007). β-Catenin activity negatively regulates bacteria-induced inflammation. *Lab. Invest.* 87, 613–624.

Elphick, D. A., and Mahida, Y. R. (2005). Paneth cells: their role in innate immunity and inflammatory disease. *Gut* 54, 1802–1809.

Etienne-Manneville, S., and Hall, A. (2002). Rho GTPases in cell biology. *Nature* 420, 629–635.

Faucher, S. P., Porwollik, S., Dozois, C. M., McClelland, M., and Daigle, F. (2006). Transcriptome of *Salmonella enterica* serovar Typhi within macrophages revealed through the selective capture of transcribed sequences. *Proc. Natl. Acad. Sci. U.S.A.* 103, 1906–1911.

Finlay, B., Ruschkowski, S., and Dedhar, S. (1991). Cytoskeletal rearrangements accompanying *Salmonella* entry into epithelial cells. *J. Cell Sci.* 99, 283–296.

Forest, C. G., Ferraro, E., Sabbagh, S. C., and Daigle, F. (2010). Intracellular survival of *Salmonella enterica* serovar Typhi in human macrophages is SPI-2 independent. *Microbiology* 156(Pt 12), 3689–3698.

Forster, C. (2008). Tight junctions and the modulation of barrier function in disease. *Histochem. Cell Biol.* 130, 55–70.

Fournier, B., Williams, I. R., Gewirtz, A. T., and Neish, A. S. (2009). Toll-like receptor 5-dependent regulation of inflammation in systemic *Salmonella enterica serovar* Typhimurium infection. *Infect. Immun.* 77, 4121–4129.

Frey, A., Giannasca, K. T., Weltzin, R., Giannasca, P. J., Reggio, H., Lencer, W. I., and Neutra, M. R. (1996). Role of the glycocalyx in regulating access of microparticles to apical plasma membranes of intestinal epithelial cells: implications for microbial attachment and oral vaccine targeting. *J. Exp. Med.* 184, 1045–1059.

Frost, A. J., Bland, A. P., and Wallis, T. S. (1997). The early dynamic response of the calf ileal epithelium to *Salmonella typhimurium*. *Vet. Pathol.* 34, 369–386.

Fu, Y., and Galan, J. E. (1999). A *Salmonella* protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion. *Nature* 401, 293–297.

Furuse, M., Itoh, M., Hirase, T., Nagafuchi, A., Yonemura, S., Tsukita, S., and Tsukita, S. (1994). Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. *J. Cell Biol.* 127, 1617–1626.

Furuse, M., Sasaki, H., Fujimoto, K., and Tsukita, S. (1998). A single gene product, Claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. *J. Cell Biol.* 143, 391–401.

Galan, J. E. (1996). Molecular genetic bases of *Salmonella* entry into host cells. *Mol. Microbiol.* 20, 263–271.

Geddes, K., Rubino, S., Streutker, C., Cho, J. H., Magalhaes, J. G., Le Bourhis, L., Selvanantham, T., Girardin, S. E., and Philpott, D. J. (2010). Nod1 and Nod2 regulation of inflammation in the *Salmonella* colitis model. *Infect. Immun.* 78, 5107–5115.

Girardin, S. E., Boneca, I. G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., Philpott, D. J., and Sansonetti, P. J. (2003). Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J. Biol. Chem.* 278, 8869–8872.

Gonzalez-Escobedo, G., Marshall, J. M., and Gunn, J. S. (2011). Chronic and acute infection of the gall bladder by *Salmonella Typhi*: understanding the carrier state. *Nat. Rev. Microbiol.* 9, 9–14.

Gonzalez-Mariscal, L., Betanzos, A., and Avila-Flores, A. (2000). MAGUK proteins: structure and role in the tight junction. *Cell Dev. Biol.* 11, 315–324.

Graham, S. M., Molyneux, E. M., Walsh, A. L., Cheesbrough, J. S., Molyneux, M. E ., and Hart, C. A. (2000). Nontyphoidal *Salmonella* infections of children in tropical Africa. *Pediatr. Infect. Dis. J.* 19, 1189–1196.

Green, K. J., and Simpson, C. L. (2007). Desmosomes: new perspectives on a classic. *J. Investig. Dermatol.* 127, 2499–2515.

Gribar, S. C., Anand, R. J., Sodhi, C. P., and Hackam, D. J. (2008). The role of epithelial toll-like receptor signaling in the pathogenesis of intestinal inflammation. *J. Leukoc. Biol.* 83, 493–498.

Hapfelmeier, S., Ehrbar, K., Stecher, B., Barthel, M., Kremer, M., and Hardt, W. D. (2004). Role of the *Salmonella* pathogenicity island 1 effector proteins SipA, SopB, SopE, and SopE2 in *Salmonella enterica* subspecies 1 Serovar Typhimurium colitis in streptomycin-pretreated mice. *Infect. Immun.* 72, 795–809.

Hapfelmeier, S., Stecher, B., Barthel, M., Kremer, M., Müller, A. J., Heikenwalder, M., Stallmach, T., Hensel, M., Pfeffer, K., Akira, S., and Hardt, W. D. (2005). The *Salmonella* pathogenicity island (SPI)-2 and SPI-1 Type III secretion systems allow *Salmonella* Serovar *typhimurium* to trigger colitis via MyD88-dependent and MYD88-independent mechanisms. *J. Immunol.* 174, 1675–1685.

Hardt, W., Chen, L. M., Schuebel, K. E., Bustelo, X. R., and Galán, J. E. (1998). *S. typhimurium* encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. *Cell* 93, 815–826.

Hartsock, A., and Nelson, J. W. (2008). Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. *Biochim. Biophys. Acta* 1778, 660–669.

Hashizume, T., Togawa, A., Nochi, T., Igarashi, O., Kweon, M. N., Kiyono, H., and Yamamoto, M. (2008). Peyer's patches are required for intestinal immunoglobulin a responses to *Salmonella* spp. *Infect. Immun.* 76, 927–934.

Haskins, J., Gu, L., Wittchen, E. S., Hibbard, J., and Stevenson, B. R. (1998). ZO-3, a novel member of the MAGUK protein family found at the tight junction, interacts with ZO-1 and occludin. *J. Cell Biol.* 141, 199–208.

Hatsell, S., and Cowin, P. (2001). Deconstructing desmoplakin. *Nat. Cell Biol.* 3, E270–E272.

Hayward, R. D., McGhie, E. J., and Koronakis, V. (2000). Membrane fusion activity of purified SipB, a *Salmonella* surface protein essential for mammalian cell invasion. *Mol. Microbiol.* 37, 727–739.

Hernandez, L. D., Hueffer, K., Wenk, M. R., and Galan, J. E. (2004). *Salmonella* modulates vesicular traffic by altering phosphoinositide metabolism. *Science* 304, 1805–1807.

Itoh, M., Morita, K., and Tsukita, S. (1999). Characterization of ZO-2 as a MAGUK family member associated with tight as well as adherens junctions with a binding affinity to occludin and α-catenin. *J. Biochem. Chem.* 274, 5981–5986.

Jensen, V. B., Harty, J. T., and Jones, B. D. (1998). Interactions of the invasive pathogens *Salmonella Typhimurium, Listeria monocytogenes,* and *Shigella flexneri* with M cells and murine Peyer's patches. *Infect. Immun.* 66, 3758–3766.

Jiang, X., Rossanese, O. W., Brown, N. F., Kujat-Choy, S., Galán, J. E., Finlay, B. B., and Brumell, J. H. (2004). The related effector proteins SopD and SopD2 from *Salmonella enterica* serovar Typhimurium contribute to virulence during systemic infection of mice. *Mol. Microbiol.* 54, 1186–1198.

Jones, B. D., Ghori, N., and Falkow, S. (1994). *Salmonella typhimurium* initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. *J. Exp. Med.* 180, 15–23.

Kaniga, K., Tucker, S., Trollinger, D., and Galan, J. E. (1995). Homologs of the *Shigella* IpaB and IpaC invasins are required for *Salmonella Typhimurium* entry into cultured epithelial cells. *J. Bacteriol.* 177, 3965–3971.

Knodler, L. A., and Steele-Mortimer, O. (2005). The *Salmonella* effector PipB2 affects late endosome/lysosome distribution to mediate Sif extension. *Mol. Biol. Cell* 16, 4108–4123.

Knodler, L. A., Vallance, B. A., Celli, J., Winfree, S., Hansen, B., Montero, M., and Steele-Mortimer, O. (2010). Dissemination of invasive *Salmonella* via bacterial-induced extrusion of mucosal epithelia. *Proc. Natl. Acad. Sci. U.S.A.* 107, 17733–17738.

Koch, P. J., and Franke, W. W. (1994). Desmosomal cadherins: another growing multigene family of adhesion molecules. *Curr. Opin. Cell Biol.* 6, 682–687.

Kohler, H., Sakaguchi, T., Hurley, B. P., Kase, B. A., Reinecker, H. C., and McCormick, B. A. (2007). *Salmonella enterica* serovar Typhimurium regulates intercellular junction proteins and facilitates transepithelial neutrophil and bacterial passage. *Am. J. Physiol. Gastroinest. Liver Physiol.* 293, G178–G187.

Kraehenbuhl, J., and Neutra, M. R. (2000). Epithelial M cells: differentiation and function. *Annu. Rev. Cell Dev. Biol.* 16, 301–332.

Kuhle, V., Jackel, D., and Hensel, M. (2004). Effector proteins encoded by *Salmonella* pathogenicity island 2 interfere with the microtubule cytoskeleton after translocation into host cells. *Traffic* 5, 356–370.

Lara-Tejero, M., and Galan, J. E. (2009). *Salmonella enterica* Serovar Typhimurium pathogenicity island 1-encoded type III secretion system translocases mediate intimate attachment to nonphagocytic cells. *Infect. Immun.* 77, 2635–2642.

Lawley, T. D., Chan, K., Thompson, L. J., Kim, C. C., Govoni, G. R., and Monack, D. M. (2006). Genome-wide screen for *Salmonella* genes required for long-term systemic infection of the mouse. *PLoS Pathogens* 2, e11. doi: 10.1371/journal.ppat.0020011

Lawley, T. D., Bouley, D. M., Hoy, Y. E., Gerke, C., Relman, D. A., and Monack, D. M. (2008). Host transmission of *Salmonella enterica* Serovar typhimurium is controlled by virulence factors and indigenous intestinal microbiota. *Infect. Immun.* 76, 403–416.

LeLouard, H., Henri, S., De Bovis, B., Mugnier, B., Chollat-Namy, A., Malissen, B., Méresse, S., and Gorvel, J. P. (2010). Pathogenic bacteria and dead cells are internalized by a unique subset of Peyer's Patch dendritic cells that express lysozyme. *Gastroenterology* 138, 173–184.

Lesnick, M. L., Reiner, N. E., Fierer, J., and Guiney, D. G. (2001). The *Salmonella spvB* virulence gene encodes an enzyme that ADP-ribosylates actin and destabilizes the cytoskeleton of eukaryotic cells. *Mol. Microbiol.* 39, 1464–1470.

Liao, A. P., Petrof, E. O., Kuppireddi, S., Zhao, Y., Xia, Y., Claud, E. C., and Sun, J. (2008). *Salmonella* Type III effector AvrA stabilizes cell tight junctions to inhibit inflammation in intestinal epithelial cells. *PLOS ONE* 3, e2369. doi: 10.1371/journal.pone.0002369

Libby, S. J., Brehm, M. A., Greiner, D. L., Shultz, L. D., McClelland, M., Smith, K. D., Cookson, B. T., Karlinsey, J. E., Kinkel, T. L., Porwollik, S., Canals, R., Cummings, L. A., and Fang, F. C. (2010). Humanized nonobese diabetic-*scid IL2ry*^{null} mice are susceptible to lethal *Salmonella* Typhi infection. *Proc. Natl. Acad. Sci. U.S.A.* 107, 15589–15594.

MacDonald, T. T., and Monteleone, G. (2005). Immunity, inflammation, and allergy in the gut. *Science* 307, 1920–1925.

Madara, J. L. (1990). Maintenance of the macromolecular barrier at cell extrusion sites in intestinal epithelium: physiological rearrangement of tight junctions. *J. Membr. Biol.* 116, 177–184.

Madara, J. L., Patapoff, T. W., Gillece-Castro, B., Colgan, S. P., Parkos, C. A., Delp, C., Mrsny, R. J. (1993). 5'-Adenosine monophosphate is the neutrophil-derived paracrine factor that elicits chloride secretion from T84 intestinal epithelial cell monolayers. *J. Clin. Invest.* 91, 2320–2325.

Martinez-Argudo, I., and Jepson, M. A. (2008). *Salmonella* translocates across an *in vitro* M cell model independently of SPI-1 and SPI-2. *Microbiology* 154, 3887–3894.

Martinez-Palomo, A., and Erlij, D. (1975). Structure of tight junctions in epithelia with different permeability. *Proc. Natl. Acad. Sci. U.S.A.* 72, 4487–4491.

Mayhew, T. M., Myklebust, R., Whybrow, A., and Jenkins, R. (1999). Epithelial integrity, cell death and cell loss in mammalian small intestine. *Histol. Histolpathol.* 14, 257–267.

Mazmanian, S. K., Liu, C. H., Tzianabos, A. O., and Kasper, D. L. (2005). An immunodeficiency molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* 122, 107–118.

Mazurkiewicz, P., Thomas, J., Thompson, J. A., Liu, M., Arbibe, L., Sansonetti, P., and Holden, D. W. (2008). SpvC is a *Salmonella* effector with phosphothreonine lyase activity on host mitogen-activated protein kinases. *Mol. Microbiol.* 67, 1371–1383.

McCormick, B. A. (2007). Bacterial-induced hepoxilin A3 secretion as a pro-inflammatory mediator. *FEBS J.* 274, 3513–3518.

McCormick, B. A., Stocker, B. A. D., Laux, D. C., and Cohen, P. S. (1988). Roles of motility, chemotaxis, and penetration through and growth in intestinal mucus in the ability of an avirulent strain of *Salmonella* typhimurium to colonize the large intestine of streptomycin-treated mice. *Infect. Immun.* 56, 2209–2217.

McGhie, E. J., Hayward, R. D., and Koronakis, V. (2001). Cooperation between actin-binding proteins of invasive *Salmonella*: SipA potentiates SipC nucleation and bundling of actin. *EMBO J.* 20, 2131–2139.

Meyerholz, D. K., and Stabel, T. J. (2003). Comparison of early ileal invasion by *Salmonella enterica* serovars choleraesuis and typhimurium. *Vet. Pathol.* 40, 371–375.

Meyerholz, D. K., Stabel, T. J., Ackermann, M. R., Carlson, S. A., Jones, B. D., and Pohlenz, J. (2002). Early epithelial invasion by *Salmonella enterica* serovar typhimurium DT104 in the swine ileum. *Vet. Pathol.* 39, 712–720.

Nash, S., Stafford, J., and Madara, J. L. (1987). Effects of Polymorphonuclear leukocyte transmigration on the barrier function of cultured intestinal epithelial monolayers. *J. Clin. Invest.* 80, 1104–1113.

Nevola, J. J., Laux, D. C., and Cohen, P. S. (1987). *In vivo* colonization of the mouse large intestine and *in vitro* penetration of intestinal mucus by an avirulent smooth strain of *Salmonella* typhimurium and its lipopolysaccharide-deficient mutant. *Infect. Immun.* 55, 2884–2890.

Nobes, C. D., and Hall, A. (1995). Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 81, 53–62.

Norris, F. A., Wilson, M. P., Wallis, T. S., Galyov, E. E., and Majerus, P. W. (1998). SopB, a protein required for virulence of *Salmonella dublin*, is an inositol phosphate phosphatase. *Proc. Natl. Acad. Sci. U.S.A.* 95, 14057–14059.

Palazzo, M., Balsari, A., Rossini, A., Selleri, S., Calcaterra, C., Gariboldi, S., Zanobbio, L., Arnaboldi, F., Shirai, Y. F., Serrao, G., and Rumio, C. (2007). Activation of enteroendocrine cells via TLRs induces hormone, chemokine, and defensin secretion. *J. Immunology* 178, 4296–4303.

Pazos, M., Siccardi, D., Mumy, K. L., Bien, J. D., Louie, S., Shi, H. N., Gronert, K., Mrsny, R. J., and McCormick, B. A. (2008). Multidrug resistance-associ-

ated transporter 2 regulates mucosal inflammation by facilitating the synthesis of hepoxilin A3. *J. Immunol.* 181, 8044–8052.

Playford, R. J., Marchbank, T., Goodlad, R. A., Chinery, R. A., Poulsom, R., and Hanby, A. M. (1996). Transgenic mice that overexpress the human trefoil factor peptide pS2 have an increased resistance to intestinal damage. *Proc. Natl. Acad. Sci. U.S.A.* 93, 2137–2142.

Poh, J., Odendall, C., Spanos, A., Boyle, C., Liu, M., Freemont, P., and Holden, D. W. (2008). SteC is a *Salmonella* kinase required for SPI-2-dependent F-actin remodeling. *Cell. Microbiol.* 10, 20–30.

Quezada, C. M., Hicks, S. W., Galan, J. E., and Stebbins, C. E. (2009). A family of *Salmonella* virulence factors functions as a distinct class of autoregulated E3 ubiquitin ligases. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4864–4869.

Radtke, A., Wilson, J. W., Sarker, S., and Nickerson, C. A. (2010). Analysis of interactions of *Salmonella* type three secretion mutants with 3-D intestinal epithelial cells. *PLoS ONE* 5, e15750. doi: 10.1371/journal.pone.0015750

Raffatellu, M., Wilson, R. P., Chessa, D., Andrews-Polymenis, H., Tran, Q. T., Lawhon, S., Khare, S., Adams, L. G., and Bäumler, A. J. (2005). SipA, SopA, SopB, SopD, and SopE2 contribute to *Salmonella enterica* serotype typhimurium invasion of epithelial cells. *Infect. Immun.* 73, 146–154.

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S., and Medzhitov, R. (2004). Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 118, 229–241.

Ramsden, A. E., Mota, L. J., Münter, S., Shorte, S. L., and Holden, D. W. (2007). The SPI-2 type III secretion system restricts motility of *Salmonella*-containing vacuoles. *Cell. Microbiol.* 9, 2517–2529.

Ridley, A. J., and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* 70, 389–399.

Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., Hall, A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* 70, 401–410.

Ruiz-Albert, J., Yu, X. J., Beuzón, C. R., Blakey, A. N., Galyov, E. E., and Holden, D. W. (2002). Complementary activities of SseJ and SifA regulate dynamics of the *Salmonella typhimurium* vacuolar membrane. *Mol. Microbiol.* 44, 645–661.

Rytkonen, A., and Holden, D. W. (2007). Bacterial interference of ubiquitination and deubiquitination. *Cell Host Microbe* 1, 13–22.

Rytkonen, A., Poh, J., Garmendia, J., Boyle, C., Thompson, A., Liu, M., Freemont, P., Hinton, J. C. D., and Holden, D. W. (2007). SseL, a *Salmonella* deubiquitinase required for macrophage killing and virulence. *Proc. Natl. Acad. Sci. U.S.A.* 104, 3502–3507.

Sabbagh, S. C., Forest, C. G., Lepage, C., Leclerc, J. M., and Daigle, F. (2010). So similar, yet so different: uncovering distinctive features in the genomes of *Salmonella enterica* serovars Typhimurium and Typhi. *FEMS Microbiol.* 305, 1–13.

Salcedo, S. P., and Holden, D. W. (2003). SseG, a virulence protein that targets *Salmonella* to the golgi network. *EMBO J.* 22, 5003–5014.

Salzman, N. H., Chou, M. M., de Jong, H., Liu, L., Porter, E. M., and Paterson, Y. (2003). Enteric *Salmonella* infection inhibits paneth cell antimicrobial peptide expression. *Infect. Immun.* 71, 1109–1115.

Schroeder, N., Henry, T., de Chastellier, C., Zhao, W., Guilhon, A. A., Gorvel, J. P., and Méresse, S. (2010). The virulence protein SopD2 regulates membrane dynamics of *Salmonella*-containing vacuoles. *PLOS Pathogens* 6, e1001002. doi: 10.1371/journal.ppat.1001002

Srikanth, C. V., Wall, D. M., Maldonado-Contreras, A., Shi, H. N., Zhou, D., Demma, Z., Mumy, K. L., and McCormick, B. A. (2010). *Salmonella* pathogenesis and processing of secreted effectors by caspase-3. *Science* 330, 390–393.

Stebbins, C. E., and Galan, J. E. (2000). Modulation of host signaling by a bacterial mimic: structure of the *Salmonella* effector SptP bound to Rac1. *Mol. Cell* 6, 1449–1460.

Stebbins, C. E., and Galan, J. E. (2003). Priming virulence factors for delivery into the host. *Nat. Rev. Mol. Cell Biol.* 4, 738–744.

Stecher, B., Robbiani, R., Walker, A. W., Westendorf, A. M., Barthel, M., Kremer, M., Chaffron, S., Macpherson, A. J., Buer, J., Parkhill, J., Dougan, G., von Mering, C., and Hardt, W. D. (2007). *Salmonella enterica* serovar typhimurium exploits inflammation to compete with the intestinal microbiota. *PLOS Biol.* 5, 2177–2189. doi: 10.1371/journal.pbio.0050244

Stein, M., Leung, K. Y., Zwick, M., Garcia-del Portillo, F., and Finlay, B. B. (1996). Identification of a *Salmonella* virulence gene required for formation of filamentous structures containing lysosomal membrane glycoproteins within epithelial cells. *Mol. Microbiol.* 20, 151–164.

Stender, S., Friebel, A., Linder, S., Rohde, M., Mirold, S., and Hardt, W. D. (2000). Identification of SopE2 from *Salmonella typhimurium*, a conserved guanine nucleotide exchange factor for Cdc42 of the host cell. *Mol. Microbiol.* 36, 1206–1221.

Takeichi, M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* 251, 1451–1455.

Tam, M. A., Rydstrom, A., Sundquist, M., and Wick, M. J. (2008). Early cellular responses to *Salmonella* infection: dendritic cells, monocytes, and more. *Immunol. Rev.* 225, 140–162.

Tsukita, S., Katsuno, T., Yamazaki, Y., Umeda, K., Tamura, A., and Tsukita, S. (2009). Roles of ZO-1 and ZO-2 in establishment of the belt-like adherens and tight junctions with paracellular permselective barrier function. *Ann. N.Y. Acad. Sci.* 1165, 44–52.

Turner, J. R. (2009). Intestinal mucosal barrier function in health and disease. *Nat. Rev. Immunol.* 9, 799–809.

Tyrer, P., Foxwell, A. R., Cripps, A. W., Apicella, M. A., and Kyd, J. M. (2006). Microbial pattern recognition receptors mediate M-cell uptake of a gram-negative bacterium. *Infect. Immun.* 74, 625–631.

Umeda, K., Ikenouchi, J., Katahira-Tayama, S., Furuse, K., Sasaki, H., Nakayama, M., Matsui, T., Tsukita, S., Furuse, M., and Tsukita, S. (2006). ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. *Cell* 126, 741–754.

Vimal, D. B., Khullar, M., Gupta, S., and Ganguly, N. K. (2000). Intestinal mucins: the bindings sites for *Salmonella Typhimurium*. *Mol. Cell. Biochem.* 204, 107–117.

Voedisch, S., Koenecke, C., David, S., Herbrand, H., Förster, R., Rhen, M., and Pabst, O. (2009). Mesenteric lymph nodes confine DC-mediated dissemination of *Salmonella* and limit systemic disease in mice. *Infect. Immun.* 77, 3170–3180.

Wall, D. M., Nadeau, W. J., Pazos, M. A., Shi, H. N., Galyov, E. E., and McCormick, B. A. (2007). Identification of the *Salmonella enterica* serotype Typhimurium SipA domain responsible for inducing neutrophil recruitment across the intestinal epithelium. *Cell. Microbiol.* 9, 2299–2313.

Wood, M. W., Jones, M. A., Watson, P. R., Hedges, S., Wallis, T. S., and Galyov, E. E. (1998). Identification of a pathogenicity island required for *Salmonella* enteropathogenicity. *Mol. Microbiol.* 29, 883–891.

Wood, M. W., Jones, M. A., Watson, P. R., Siber, A. M., McCormick, B. A., Hedges, S., Rosqvist, R., Wallis, T. S., and Galyov, E. E. (2000). The secreted effector protein of *Salmonella dublin*, SopA, is translocated into eukaryotic cells and influences the induction of enteritis. *Cell. Microbiol.* 2, 293–303.

Ye, Z., Petrof, E. O., Boone, D., Claud, E. C., and Sun, J. (2007). *Salmonella* effector AvrA regulation of colonic epithelial cell inflammation by deubiquitination. *Am. J. Pathol.* 171, 882–892.

Yu, X., Ruiz-Albert, J., Unsworth, K. E., Garvis, S., Liu, M., Holden, D. W. (2002). SpiC is required for secretion of *Salmonella* pathogenicity island 2 type III secretion system proteins. *Cell. Microbiol.* 4, 531–540.

Zhang, S., Kingsley, R. A., Santos, R. L., Andrews-Polymenis, H., Raffatellu, M., Figueiredo, J., Nunes, J., Tsolis, R. M., Adams, L. G., and Bäumler, A. J. (2003). Molecular pathogenesis of *Salmonella enterica* serotype typhimurium-induced diarrhea. *Infect. Immun.* 71, 1–12.

Zhang, S., Santos, R. L., Tsolis, R. M., Stender, S., Hardt, W. D., Bäumler, A. J., and Adams, L. G. (2002). The *Salmonella enterica* serotype typhimurium effector proteins SipA, SopA, SopB, SopD, and SopE2 Act in concert to induce diarrhea in calves. *Infect. Immun.* 70, 3843–3855.

Zhang, Y., Higashide, W. M., McCormick, B. A., Chen, J., and Zhou, D. (2006). The inflammation-associated *Salmonella* SopA is a HECT-like E3 ubiquitin ligase. *Mol. Microbiol.* 62, 786–793.

Zhou, D., Chen, L. M., Hernandez, L., Shears, S. B., and Galán, J. E. (2001). A *Salmonella* inositol polyphosphatase acts in conjunction with other bacterial effectors to promote host cell actin cytoskeleton rearrangements and bacterial internalization. *Mol. Microbiol.* 39, 248–259.

Zhou, D., Mooseker, M. S., and Galán, J. E. (1999). Role of the *S. Typhimurium* actin-binding protein SipA in bacterial internalization. *Science* 283, 2092–2095.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 February 2011; accepted: 13 April 2011; published online: 29 April 2011. Citation: Hallstrom K and McCormick BA (2011) Salmonella interaction with and passage through the intestinal mucosa: through the lens of the organism. Front. Microbio. ***2****:88. doi: 10.3389/fmicb.2011.00088*

This article was submitted to Frontiers in Cellular and Infection Microbiology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 25 April 2011
doi: 10.3389/fmicb.2011.00085

Salmonella and Caspase-1: a complex interplay of detection and evasion

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Salmonellae are intracellular pathogens that replicate within epithelial cells and macrophages, and are a significant public health threat in both developed and developing countries. The innate immune system detects microbes through pattern recognition receptors, which are compartmentalized on the subcellular level to detect either extracellular (e.g., TLRs) or cytosolic (e.g., NLRs) perturbations. *Salmonella* infection is detected by the NLRC4 and NLRP3 inflammasomes, which activate Caspase-1, resulting in reduced bacterial burdens during infection. NLRC4 responds to the SPI1 type III secretion system via detection of inadvertently translocated flagellin and rod protein. The signals for NLRP3 detection during *Salmonella* infection remain undefined. *Salmonella* have evolved evasion strategies to attenuate Caspase-1 responses. We review recent findings describing the interplay between detection and evasion of *S. typhimurium* infection by the inflammasome. We discuss how the interplay between detection and evasion affects Caspase-1 effector functions mediated by IL-1β secretion, IL-18 secretion, and pyroptosis.

Keywords: *Salmonella*, Caspase-1, inflammasome, IL-1β, pyroptosis

INTRODUCTION

Salmonella typhi and *S. paratyphi* cause Typhoid fever, a continuing source of morbidity and mortality in developing countries. With 21 million cases and 200–600,000 deaths annually, antibiotic resistance is a growing concern (Parry et al., 2002; Bhutta and Threlfall, 2009). *S. typhimurium* and other non-typhoidal serotypes are food borne pathogens that cause self-limited gastroenteritis with periodic outbreaks from contaminated centralized food processing sites. The estimated burden of non-typhoidal Salmonellosis in the United States is 1.4 million cases annually (Voetsch et al., 2004). Invasive infections with non-typhoidal *Salmonella* have begun to emerge as the leading cause of community acquired bacteremia in sub-Saharan Africa and are associated with 21–47% mortality despite appropriate antibiotic therapy (Gordon et al., 2008; Kingsley et al., 2009). These isolates are predominantly *S. typhimurium* strains and are typically resistant to multiple antibiotics.

TYPE III SECRETION

Salmonellae manipulate host cellular physiology during infections by using type III secretion systems (T3SS). T3SS are very common virulence factors used by a wide array of Gram-negative pathogens, which function by facilitating the translocation of effector proteins into the cytosol of host cells (Hueck, 1998). Changing the complement of effectors that are translocated allows different bacteria to exert control over distinct host signaling pathways. For example, Salmonellae encode two virulence-associated T3SS within two distinct *Salmonella* pathogenicity islands (SPI), each resulting in the transfer of a distinct set of effectors (Ibarra and Steele-Mortimer, 2009). The SPI1 T3SS is expressed primarily in the gut lumen, and promotes epithelial cell invasion. It is critical for the induction of gastroenteritis. The SPI2 T3SS is expressed by bacteria within the vacuolar compartment of macrophages and epithelial cells. It is important for intracellular replication and causing systemic disease.

INNATE IMMUNE SENSORS

Innate immune system sensors can be classified by the cellular site of detection. Extracellular detectors, such as the Toll-like receptors (TLR) and C type Lectin receptors (CLR), respond to microbe associated molecular patterns (MAMPs) in the extracellular or vacuolar space. They tend to respond to molecules that are broadly conserved, such as LPS (TLR4) or flagellin (TLR5) and induce pro-inflammatory gene expression. Cytosolic detectors such as the Nod-like receptors (NLR), Rig-I-like receptors (RLR), and Aim2-like receptors (ALR) detect MAMPs in the cytosolic compartment or cytosolic perturbations caused by extracellular agonists. They induce either transcriptional or post-translational responses. Some of these sensors can respond to virulence properties of pathogens either directly or indirectly. For example, NLRC4 detects T3SS activity by sensing the inadvertent translocation of both flagellin and the T3SS rod protein into the cytosol of host cells (Miao et al., 2007; Miao and Warren, 2010). Interestingly, while flagellin in the extracellular space is a marker for any flagellated bacterium present within host tissues, in the cytosol it is instead a marker of a flagellated bacterium that possesses virulence factors that allow access to the cytosolic compartment. From the host's perspective, the threat posed by these two bacteria is quite different.

Toll-like receptors induce the expression of multiple cytokines and chemokines. While most of these factors are secreted upon stimulation, pro-IL-1β and pro-IL-18 are held in reserve, awaiting a second signal. Some cytosolic receptors in the NLR and ALR families form inflammasomes, multi-protein complexes which

serve as platforms for Caspase-1 activation. Activated Caspase-1 cleaves pro-IL-1β and pro-IL-18 to their mature secreted forms. Caspase-1 activation also induces a form of programmed cell death called pyroptosis (Bergsbaken et al., 2009).

INNATE IMMUNE SENSORS DETECTING *S. TYPHIMURIUM*

Several sensors can detect *S. typhimurium* infection. Both TLR4 and TLR5 play a role in the host response to *S. typhimurium* (**Figure 1**). *Tlr4*$^{-/-}$ mice show increased susceptibility to *S. typhimurium* infection, and *Tlr4-Tlr5*^{DKO} mice show an even more severe phenotype (Feuillet et al., 2006). *S. typhimurium* is also detected by NLRs, specifically NLRC4 (previously called Ipaf) and NLRP3 (previously called Nalp3 or cryopyrin), which also contribute to a reduced bacterial burden in the mouse model (Broz et al., 2010).

NLRC4 and NLRP3 both form inflammasomes and activate Caspase-1. Mice deficient in Caspase-1 have increased bacterial loads and succumb to *S. typhimurium* infection earlier than WT mice (Lara-Tejero et al., 2006; Raupach et al., 2006). *Nlrp3-Nlrc4*^{DKO} mice have a phenotype similar to *Casp1*$^{-/-}$ mice (Broz et al., 2010; **Figure 1**).

Caspase-1 reduces bacterial loads after *S. typhimurium* infection largely via the activities of IL-18. *Il18*$^{-/-}$ and *Casp1*$^{-/-}$ mice show similar susceptibilities to *S. typhimurium* infection while IL-1β$^{-/-}$ mice have a much more subtle phenotype (Raupach et al., 2006). IL-18 has numerous effects on the immune response, including the transcriptional induction of pro-inflammatory genes and the potentiation of IFN-γ secretion by T-cells and NK cells, both of which play significant roles in host defense against *S. typhimurium* (Eckmann and Kagnoff, 2001).

NLRP3 DETECTION

NLRP3 detects *S. typhimurium* within macrophages at late time-points. It responds to a diverse set of agonists, including ATP, pore forming toxins, crystals, viruses, bacteria, and fungi (Schroder and Tschopp, 2010; Rajan et al., 2011). These agonists are thought to trigger convergent signaling events that involve lysosomal destabilization, electrolyte imbalances, or mitochondrial dysfunction via ROS production (Hornung and Latz, 2010; Schroder and Tschopp, 2010; Zhou et al., 2011). Whether these pathways converge on a single terminal signal or not is not known. The kinetics of detection of different NLRP3 agonists is variable: ATP and nigericin are detected within 1 h, crystals typically within 4 h, while bacteria, viruses, and fungi are detected only after 6 or more hours. These differences likely reflect the length of time required to trigger a cytosolic event that is sensed by NLRP3.

Several bacteria can trigger NLRP3 activation *in vitro*, including *Listeria monocytogenes* (Mariathasan et al., 2006; Warren et al., 2008; Kim et al., 2010; Wu et al., 2010), *Staphylococcus aureus* (Mariathasan et al., 2006; Craven et al., 2009; Munoz-Planillo et al., 2009; Shimada et al., 2010), *Klebsiella pneumophila* (Willingham et al., 2009), *Porphyromonas gingivalis* (Huang et al., 2009), *Shigella flexneri* (Willingham et al., 2007), *Chlamydophila pneumoniae* (He et al., 2010), *Neisseria gonorrhoeae* (Duncan et al., 2009), *Mycobacterium*

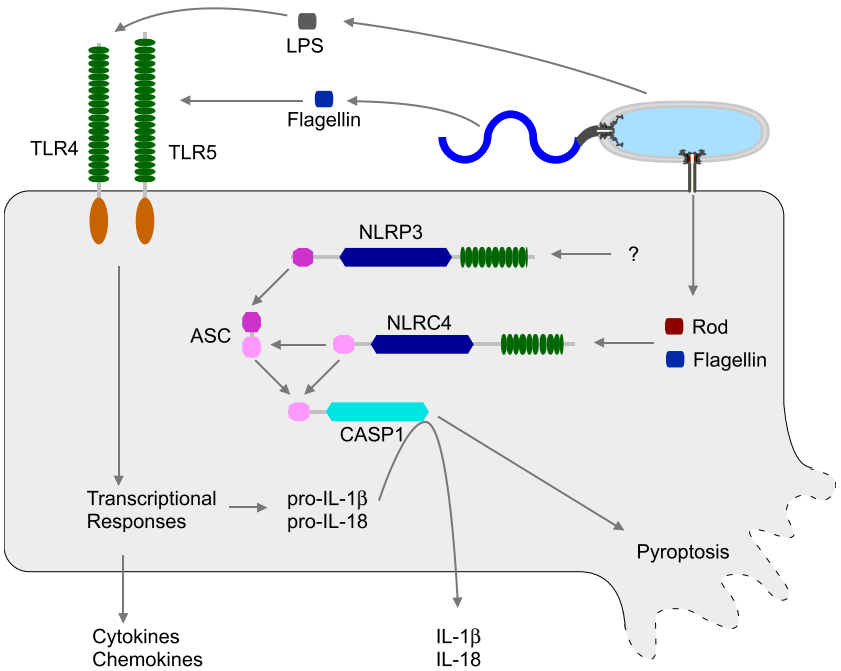

FIGURE 1 | Innate immune detection of *S. typhimurium*. *S. typhimurium* can be detected by both extracellular (TLR) and cytosolic (NLR) sensors. TLR4 responds to lipopolysaccharide (LPS), while TLR5 responds to extracellular flagellin monomers. TLRs induce transcriptional responses, including the synthesis and secretion of multiple cytokines and chemokines. TLRs also induce the synthesis of pro-IL-1β and pro-IL-18, but these are reserved in the cytosol awaiting processing by Caspase-1. Caspase-1 activity is controlled by inflammasomes. *S. typhimurium* is detected by two inflammasomes, NLRC4 and NLRP3. NLRC4 responds to cytosolic flagellin monomers or T3SS rod protein monomers, both delivered by the activity of a virulence-associated T3SS. NLRP3 detects *S. typhimurium* via unknown mechanisms.

tuberculosis (Koo et al., 2008), *Yersinia pseudotuberculosis* (Brodsky et al., 2010), and *S. typhimurium* (Broz et al., 2010). Although the terminal signaling events triggered by these pathogens are likely the same as those triggered by other NLRP3 agonists, the initial mechanisms are almost certainly divergent. For example *Y. pseudotuberculosis* triggers NLRP3 via the activity of T3SS effectors (Brodsky et al., 2010), but detection of *S. typhimurium* by NLRP3 does not require SPI1 or SPI2 T3SS (Broz et al., 2010). In contrast *L. monocytogenes* detection by NLRP3 requires lysis of the phagosome by listeriolysin O (Warren et al., 2008). *S. aureus* is also detected by NLRP3. Toxin-producing strains of *S aureus* are detected via the pore forming activities of their toxins (Craven et al., 2009; Munoz-Planillo et al., 2009) while non-toxin-producing strains are not detected, apparently evading NLRP3 detection by producing degradation resistant peptidoglycan structures. *S. aureus oatA* mutants that are defective in these cell wall modifications are detected via NLRP3 by a mechanism that involves peptidoglycan degradation in the phagosome (Shimada et al., 2010). Thus, even within a bacterial species different strains may be detected or may evade NLRP3.

NLRC4 DETECTION

NLRC4 detects the activity of T3SS and T4SS. It does so in part by responding to flagellin that is inadvertently translocated into the cytosolic compartment by T3/4SS (**Figure 2**; Molofsky et al., 2005, 2006; Amer et al., 2006; Franchi et al., 2006; Miao et al., 2006; Ren et al., 2006). T3SS efficiently select translocation substrates from the bacterial cytosol for injection into host cells. Because flagellin is targeted to the flagellar T3SS apparatus for secretion to form the flagellar filament (Minamino et al., 2008), it has features that likely predispose mistargeting to the virulence-associated T3SS. Specifically, it binds a secretion chaperone and has the physical properties required for export in an unfolded state through a T3SS secretion apparatus. The chaperone is required for efficient delivery and subsequent detection by NLRC4 (unpublished data). The mechanisms underlying accidental transfer by T4SS are less clear. As a cytosolic flagellin sensor, NLRC4 also detects flagellated bacteria that escape from the vacuolar compartment into the cytosol, such as *Listeria monocytogenes* (Warren et al., 2008).

NLRC4 can also detect T3SS activity in non-flagellated bacteria (Miao et al., 2006; Sutterwala et al., 2007; Suzuki et al., 2007), responding to inadvertent translocation of the T3SS rod protein (Miao and Warren, 2010; Miao et al., 2010b; **Figure 2**). The rod component of the T3SS apparatus forms the inner channel that spans the bacterial periplasm. Similar to flagellin, it is believed to polymerize into a hollow tube that permits the passage of secreted proteins (Marlovits et al., 2004), and there is sequence conservation at the carboxy-terminus of flagellin and the rod protein (Miao et al., 2010b). If rod proteins are inadvertently translocated into the macrophage cytosol, they are detected by NLRC4 (Miao et al., 2010b). This may occur if the T3SS exports excess rod monomers from the bacterial cytosol, or if rod monomers slough into the interior channel of the needle apparatus, resulting in translocation. In their correct location within the T3SS apparatus, rod proteins are not detected.

Macrophages detect *S. typhimurium* SPI1 T3SS via inadvertent translocation of flagellin and the SPI1 rod protein PrgJ. Because flagellin is one of the most highly produced proteins in the bacteria during growth in LB, which is thought to mimic the gut lumen, it is the dominant NLRC4 signal *in vitro*. Both flagellin and SPI1 are highly induced in the late logarithmic phase of growth in LB, and these bacteria are readily detected within 1 h by macrophages *in vitro*. However, if bacteria are grown to stationary phase, they no longer express SPI1 and NLRC4 does not detect *S. typhimurium* within the first several hours (Miao et al., 2010b). Thus the transcriptional profile of the bacterium is critical for detection by NLRC4.

EVASION OF NLRC4

Salmonella typhimurium have developed evasion strategies to prevent NLRC4 detection during intracellular replication in macrophages. Flagellin is repressed in the intracellular environment when SPI2 T3SS is active. In addition, the SPI2 T3SS rod protein, SsaI, has amino acid changes that prevent its detection by NLRC4 (Miao et al., 2010b; **Figure 2**). Elimination of these two evasion strategies by expressing PrgJ or flagellin from a SPI2 co-regulated promoter results in strong and persistent detection via NLRC4 and complete clearance of the bacteria *in vivo*, demonstrating that evasion of NLRC4 is essential for *S. typhimurium* virulence (Miao et al., 2010a,b).

These evasion strategies appear to be efficient, but not perfect. NLRC4 can detect *S. typhimurium* that are grown under SPI1/flagellin non-expressing conditions (stationary phase LB), but only at very late times post-infection (Broz et al., 2010). After infection of macrophages *in vitro*, NLRC4 does not detect the bacteria

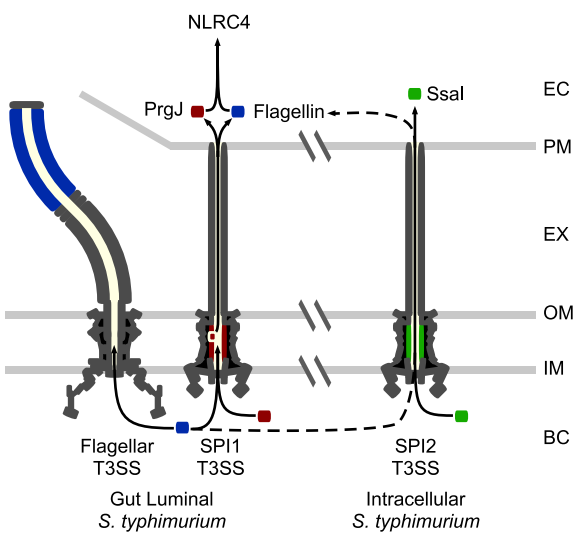

FIGURE 2 | NLRC4 detection of *S. typhimurium*. There are two major phases of *S. typhimurium* growth, extracellular in the gut lumen (modeled on the left), and intracellular within macrophages and epithelial cells (modeled on the right). While SPI1 and flagella are expressed in the gut lumen, SPI2 is expressed in the intracellular compartment. Inadvertent translocation of flagellin (blue) or the SPI1 T3SS rod protein, PrgJ (red), is detected by NLRC4, while the SPI2 rod, SsaI (green), is not detected. NLRC4 can detect intracellular adapted *S. typhimurium* via flagellin transfer through the SPI2 T3SS, albeit much less efficiently than gut adapted bacteria. EC: eukaryotic cytosol. PM: eukaryotic cell plasma membrane. EX: extracellular space. OM: bacterial outer membrane. IM: bacterial inner membranel. BC: bacterial cytosol.

during the early hours after infection. However, between 8- and 17-h post-infection, NLRC4 does detect *S. typhimurium* apparently via SPI2 T3SS translocation of flagellin (Broz et al., 2010; we have verified these results). Whether the detected flagellin arises from transcriptional re-induction at late time-points after infection of macrophages, or is due to residual protein left over from the prior growth in LB remains to be determined. In either case, this detection is sufficient to provide a benefit to the host *in vivo*, as NLRC4 dependent Caspase-1 activation results in reduced bacterial burden in infected mice, and a delay in the time of death (Broz et al., 2010). SPI2 detection is significantly delayed compared to SPI1 detection, and the amount of IL-1β secreted is considerably lower.

Thus, there is a fine interplay between detection and evasion of NLRC4 during *S. typhimurium* infection. NLRC4 does detect *S. typhimurium in vivo*, providing a benefit to the host. *S. typhimurium* expresses flagellin in the gut lumen, and represses it in the intracellular environment. We propose that NLRC4 only detects *S. typhimurium* which have recently emigrated from the gut to deeper tissues because these bacteria have residual flagellin present within their cytosol. Indeed, GFP expressed under the control of the *fliC* promoter is detectable in bacteria recovered from the Peyer's patches, but not from the draining lymph nodes or spleen (Cummings et al., 2006). Once the bacteria reprogram their gene expression for the intracellular environment, perhaps after one or more rounds of replication within host cells, we hypothesize that they subsequently evade NLRC4 completely by degrading residual flagellin in the bacterial cytosol and repressing its transcription.

PYROPTOSIS IS AN EFFECTIVE INNATE IMMUNE EFFECTOR MECHANISM *IN VIVO*

Caspase-1 activation results in both cytokine processing, and a form of cell death called pyroptosis (Bergsbaken et al., 2009), which is associated with stronger Caspase-1 activation than is required for cytokine processing. Like apoptosis, pyroptosis is a form of programmed cell death (Labbe and Saleh, 2008; Duprez et al., 2009). Both pyroptosis and apoptosis require the activation of specific signaling pathways triggered downstream of different caspase family proteases. This requirement contrasts with necrotic cell death (also called oncosis), which is an accidental form of cell death that does not have an absolute requirement for cellular signaling pathways. Pyroptosis, like necrosis, results in the lysis of the affected cell, releasing cytosolic contents after disruption of the plasma membrane, an inherently inflammatory event. In contrast, apoptotic blebs are cleared in an orderly fashion and are non-inflammatory manner (Labbe and Saleh, 2008; Duprez et al., 2009). Pyroptosis occurs rapidly, within 30–60 min *in vitro* and to date has only been characterized in macrophages and dendritic cells.

Pyroptosis was first observed in 1992 *in vitro* (Zychlinsky et al., 1992). Although there was some evidence that it occurred *in vivo* (Bergsbaken et al., 2009), the relevance of pyroptosis *in vivo* was largely undefined until recently. As mentioned above, we generated a *S. typhimurium* strain that persistently expresses flagellin (FliC^{ON}; Miao et al., 2010a). This strain was effectively detected and cleared by NLRC4 dependent Caspase-1 activation. Given that IL-18 secretion is the effector mechanism by which Caspase-1 clears WT *S. typhimurium* (Raupach et al., 2006), we expected that IL-18 would be required to clear FliC^{ON} *S. typhimurium*. However, mice deficient for both IL-1β and IL-18 retained their ability to clear these bacteria. Instead, pyroptosis released FliC^{ON} *S. typhimurium* into the extracellular space, where they are phagocytosed and killed by neutrophils (Miao et al., 2010a). We obtained similar results for *L. monocytogenes* strains engineered to persistently express and secrete flagellin or the *S. typhimurium* PrgJ rod protein; clearance of these strains could not be attributed to IL-1β and IL-18 secretion (unpublished data). Furthermore, after IP infection with *Legionella pneumophila* or *Burkholderia thailandensis*, *Casp1*$^{-/-}$ mice had increased bacterial burdens in the draining lymph nodes that could not be completely attributed to IL-1β or IL-18 secretion (Miao et al., 2010a). These results provide strong evidence that pyroptosis is an important innate immune effector mechanism against intracellular bacteria, including FliC^{ON} *S. typhimurium*.

SALMONELLA TYPHIMURIUM EFFECTIVELY EVADE PYROPTOSIS, BUT FAIL TO EVADE IL-18

Our work presents an apparent conundrum: both WT *S. typhimurium* and FliC^{ON} *S. typhimurium* are detected by NLRC4, but the former is affected by IL-18 while the latter is cleared through pyroptosis. We propose that this difference is attributable to two variables between these strains: the time to detection in a single infected cell, and the persistence of detection over the course of days.

At the single cell level under conditions that mimic systemic infection *S. typhimurium* does not trigger NLRC4 and NLRP3 until 17-h post-infection (under SPI1 repressing growth conditions; for example stationary phase LB cultures; Broz et al., 2010). In contrast, if FliC^{ON} *S. typhimurium* trigger NLRC4 within 6 h (Miao et al., 2010a). This is a significant difference. Under both conditions, Caspase-1 mediates cytokine secretion and pyroptosis. In the case of FliC^{ON} *S. typhimurium*, relatively early pyroptosis occurs before the bacteria can replicate in the macrophage, essentially short-circuiting the replicative cycle. Thus, FliC^{ON} are released and exposed to neutrophil killing before replication occurs. Some FliC^{ON} bacteria will also be taken up by macrophages, where they are again predicted to induce early pyroptosis before replication, exposing them to neutrophils before they can undergo a replicative cycle within the macrophage. This mechanism of clearance is so effective that any contribution of IL-1β and IL-18 is obscured in the mouse model. In contrast, WT *S. typhimurium* do not trigger NLRC4/NLRP3 until late times after infection of a single cell. At this time, pyroptosis should still occur, but only after the bacteria have replicated. Released bacteria are predicted to be exposed to neutrophils, but many will also infect new macrophages and continue the replicative cycle. Thus, we propose that WT *S. typhimurium* do not prevent pyroptosis from occurring, rather they void its effectiveness by delaying its onset.

Since *S. typhimurium* prevents the effectiveness of pyroptosis, one might be tempted to conclude that the role of pyroptosis in host defense against real world pathogens is negligible. However, we have evidence that during systemic infection with *Legionella pneumophila* or *Burkholderia thailandensis,* Caspase-1 reduces bacterial burdens independent of IL-1β and IL-18 (Miao et al., 2010a). These models require further research to accurately define the role of pyroptosis. Nevertheless, the fact that *S. typhimurium* actively constrains the effectiveness of pyroptosis attests to its potency in clearing intracellular pathogens that target macrophages or dendritic cells.

Unlike pyroptosis the effectiveness of IL-18 secretion is not avoided by WT *S. typhimurium*. In part, this may relate to the fact that pyroptosis is a cell intrinsic effector mechanism while IL-18 exerts its effects in a paracrine manner, inducing IFN-γ expression in NK cells and T cells. Therefore, although the effects of IL-18 can be delayed for several hours by delaying NLRC4/NLRP3 detection, it is likely that they cannot be circumvented, as is the case with pyroptosis.

COMPLEX INTERPLAY OF DETECTION AND EVASION

WT C57BL/6 mice succumb to *S. typhimurium* infection despite detection by TLR4, TLR5, NLRC4, and NLRP3, raising the question of the utility of these sensors to the host. However, it is crucial to note that this strain of mice is highly susceptible to *S. typhimurium* infection, at least in part due to a defect in *Nramp1* (also called *Slc11A1*), a metal ion transporter that depletes the phagosome of Fe^{2+} and Mn^{2+} (Gruenheid et al., 1997; Cellier et al., 2007). Even so, infection in C57BL/6 mice remains a powerful model to study innate immunity and bacterial pathogenesis because of the availability of knockout mice. The extension of results in this mouse strain to human disease should be interpreted appropriately. In humans, it is likely that detection by TLR4, TLR5, NLRC4, and NLRP3 will result in reduced bacterial burdens during both gastroenteritis and systemic disease, and we would predict that this will result in decreased symptom severity and earlier resolution of the infection. Further, IL-1β and IL-18 are expected to promote protective adaptive responses to reinfection (Sims and Smith, 2010).

FUTURE DIRECTIONS

Salmonella typhimurium is an important pathogen, and understanding how it interacts with the immune system during infection has implications for human disease caused by it and many other pathogens. In addition to the importance of studying *S. typhimurium* in its own right, it remains a particularly useful tool for dissecting the innate immune system because of the powerful genetic tools available. Studies using *S. typhimurium* have revealed the mechanisms of NLRC4 activation and the utility of pyroptosis. One of the crucial paradigms to emerge from the study of *S. typhimurium* detection is the complex interplay between detection and evasion, wherein innate immune detectors are able to respond to pathogens, but pathogens have evolved strategies that prevent maximal responses. Future studies examining the intricacies of detection and evasion of NLRC4 and NLRP3 will undoubtedly lead to novel insights into the mechanisms by which these sensors respond to *S. typhimurium* as well as many other bacterial pathogens.

REFERENCES

Amer, A., Franchi, L., Kanneganti, T. D., Body-Malapel, M., Ozoren, N., Brady, G., Meshinchi, S., Jagirdar, R., Gewirtz, A., Akira, S., and Nunez, G. (2006). Regulation of Legionella phagosome maturation and infection through flagellin and host Ipaf. *J. Biol. Chem.* 281, 35217–35223.

Bergsbaken, T., Fink, S. L., and Cookson, B. T. (2009). Pyroptosis: host cell death and inflammation. *Nat. Rev. Microbiol.* 7, 99–109.

Bhutta, Z. A., and Threlfall, J. (2009). Addressing the global disease burden of typhoid fever. *JAMA* 302, 898–899.

Brodsky, I. E., Palm, N. W., Sadanand, S., Ryndak, M. B., Sutterwala, F. S., Flavell, R. A., Bliska, J. B., and Medzhitov, R. (2010). A Yersinia effector protein promotes virulence by preventing inflammasome recognition of the type III secretion system. *Cell Host Microbe* 7, 376–387.

Broz, P., Newton, K., Lamkanfi, M., Mariathasan, S., Dixit, V. M., and Monack, D. M. (2010). Redundant roles for inflammasome receptors NLRP3 and NLRC4 in host defense against Salmonella. *J. Exp. Med.* 207, 1745–1755.

Cellier, M. F., Courville, P., and Campion, C. (2007). Nramp1 phagocyte intracellular metal withdrawal defense. *Microbes Infect.* 9, 1662–1670.

Craven, R. R., Gao, X., Allen, I. C., Gris, D., Bubeck Wardenburg, J., McElvania-Tekippe, E., Ting, J. P., and Duncan, J. A. (2009). *Staphylococcus aureus* alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells. *PLoS ONE* 4, e7446. doi: 10.1371/journal.pone.0007446

Cummings, L. A., Wilkerson, W. D., Bergsbaken, T., and Cookson, B. T. (2006). In vivo, fliC expression by *Salmonella enterica* serovar Typhimurium is heterogeneous, regulated by ClpX, and anatomically restricted. *Mol. Microbiol.* 61, 795–809.

Duncan, J. A., Gao, X., Huang, M. T., O'Connor, B. P., Thomas, C. E., Willingham, S. B., Bergstralh, D. T., Jarvis, G. A., Sparling, P. F., and Ting, J. P. (2009). *Neisseria gonorrhoeae* activates the proteinase cathepsin B to mediate the signaling activities of the NLRP3 and ASC-containing inflammasome. *J. Immunol.* 182, 6460–6469.

Duprez, L., Wirawan, E., Vanden T., Berghe, and Vandenabeele, P. (2009). Major cell death pathways at a glance. *Microbes Infect.* 11, 1050–1062.

Eckmann, L., and Kagnoff, M. F. (2001). Cytokines in host defense against Salmonella. *Microbes Infect.* 3, 1191–1200.

Feuillet, V., Medjane, S., Mondor, I., Demaria, O., Pagni, P. P., Galan, J. E., Flavell, R. A., and Alexopoulou, L. (2006). Involvement of toll-like receptor 5 in the recognition of flagellated bacteria. *Proc. Natl. Acad. Sci. U.S.A.* 103, 12487–12492.

Franchi, L., Amer, A., Body-Malapel, M., Kanneganti, T. D., Ozoren, N., Jagirdar, R., Inohara, N., Vandenabeele, P., Bertin, J., Coyle, A., Grant, E. P., and Nunez, G. (2006). Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages. *Nat. Immunol.* 7, 576–582.

Gordon, M. A., Graham, S. M., Walsh, A. L., Wilson, L., Phiri, A., Molyneux, E., Zijlstra, E. E., Heyderman, R. S., Hart, C. A., and Molyneux, M. E. (2008). Epidemics of invasive *Salmonella enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium infection associated with multidrug resistance among adults and children in Malawi. *Clin. Infect. Dis.* 46, 963–969.

Gruenheid, S., Pinner, E., Desjardins, M., and Gros, P. (1997). Natural resistance to infection with intracellular pathogens: the Nramp1 protein is recruited to the membrane of the phagosome. *J. Exp. Med.* 185, 717–730.

He, X., Mekasha, S., Mavrogiorgos, N., Fitzgerald, K. A., Lien, E., and Ingalls, R. R. (2010). Inflammation and fibrosis during *Chlamydia pneumoniae* infection is regulated by IL-1 and the NLRP3/ASC inflammasome. *J. Immunol.* 184, 5743–5754.

Hornung, V., and Latz, E. (2010). Critical functions of priming and lysosomal damage for NLRP3 activation. *Eur. J. Immunol.* 40, 620–623.

Huang, M. T., Taxman, D. J., Holley-Guthrie, E. A., Moore, C. B., Willingham, S. B., Madden, V., Parsons, R. K., Featherstone, G. L., Arnold, R. R., O'Connor, B. P., and Ting, J. P. (2009). Critical role of apoptotic speck protein containing a caspase recruitment domain (ASC) and NLRP3 in causing necrosis and ASC speck formation induced by *Porphyromonas gingivalis* in human cells. *J. Immunol.* 182, 2395–2404.

Hueck, C. J. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol. Mol. Biol. Rev.* 62, 379–433.

Ibarra, J. A., and Steele-Mortimer, O. (2009). Salmonella – the ultimate insider. Salmonella virulence factors that modulate intracellular survival. *Cell. Microbiol.* 11, 1579–1586.

Kim, S., Bauernfeind, F., Ablasser, A., Hartmann, G., Fitzgerald, K. A., Latz, E., and Hornung, V. (2010). *Listeria monocytogenes* is sensed by the NLRP3 and AIM2 inflammasome. *Eur. J. Immunol.* 40, 1545–1551.

Kingsley, R. A., Msefula, C. L., Thomson, N. R., Kariuki, S., Holt, K. E., Gordon, M. A., Harris, D., Clarke, L., Whitehead, S., Sangal, V., Marsh, K., Achtman, M., Molyneux, M. E., Cormican, M., Parkhill, J., MacLennan, C. A., Heyderman, R. S., and Dougan, G. (2009). Epidemic multiple drug resistant *Salmonella typhimurium* causing invasive disease in sub-Saharan Africa have a distinct genotype. *Genome Res.* 19, 2279–2287.

Koo, I. C., Wang, C., Raghavan, S., Morisaki, J. H., Cox, J. S., and Brown, E. J. (2008). ESX-1-dependent cytolysis in lysosome secretion and inflammasome activation during mycobacterial infection. *Cell. Microbiol.* 10, 1866–1878.

Labbe, K., and Saleh, M. (2008). Cell death in the host response to infection. *Cell Death Differ.* 15, 1339–1349.

Lara-Tejero, M., Sutterwala, F. S., Ogura, Y., Grant, E. P., Bertin, J., Coyle, A. J., Flavell, R. A., and Galan, J. E. (2006). Role of the caspase-1 inflammasome in *Salmonella typhimurium* pathogenesis. *J. Exp. Med.* 203, 1407–1412.

Mariathasan, S., Weiss, D. S., Newton, K., McBride, J., O'Rourke, K., Roose-Girma, M., Lee, W. P., Weinrauch, Y., Monack, D. M., and Dixit, V. M. (2006). Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440, 228–232.

Marlovits, T. C., Kubori, T., Sukhan, A., Thomas, D. R., Galan, J. E., and Unger, V. M. (2004). Structural insights into the assembly of the type III secretion needle complex. *Science* 306, 1040–1042.

Miao, E. A., Alpuche-Aranda, C. M., Dors, M., Clark, A. E., Bader, M. W., Miller, S. I., and Aderem, A. (2006). Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. *Nat. Immunol.* 7, 569–575.

Miao, E. A., Andersen-Nissen, E., Warren, S. E., and Aderem, A. (2007). TLR5 and Ipaf: dual sensors of bacterial flagellin in the innate immune system. *Semin. Immunopathol.* 29, 275–288.

Miao, E. A., Leaf, I. A., Treuting, P. M., Mao, D. P., Dors, M., Sarkar, A., Warren, S. E., Wewers, M. D., and Aderem, A. (2010a). Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. *Nat. Immunol.* 11, 1136–1142.

Miao, E. A., Mao, D. P., Yudkovsky, N., Bonneau, R., Lorang, C. G., Warren, S. E., Leaf, I. A., and Aderem, A. (2010b). Innate immune detection of the type III secretion apparatus through the NLRC4 inflammasome. *Proc. Natl. Acad. Sci. U.S.A.* 107, 3076–3080.

Miao, E. A., and Warren, S. E. (2010). Innate immune detection of bacterial virulence factors via the NLRC4 inflammasome. *J. Clin. Immunol.* 30, 502–506.

Minamino, T., Imada, K., and Namba, K. (2008). Mechanisms of type III protein export for bacterial flagellar assembly. *Mol. Biosyst.* 4, 1105–1115.

Molofsky, A. B., Byrne, B. G., Whitfield, N. N., Madigan, C. A., Fuse, E. T., Tateda, K., and Swanson, M. S. (2006). Cytosolic recognition of flagellin by mouse macrophages restricts *Legionella pneumophila* infection. *J. Exp. Med.* 203, 1093–1104.

Molofsky, A. B., Shetron-Rama, L. M., and Swanson, M. S. (2005). Components of the *Legionella pneumophila* flagellar regulon contribute to multiple virulence traits, including lysosome avoidance and macrophage death. *Infect. Immun.* 73, 5720–5734.

Munoz-Planillo, R., Franchi, L., Miller, L. S., and Nunez, G. (2009). A critical role for hemolysins and bacterial lipoproteins in *Staphylococcus aureus*-induced activation of the Nlrp3 inflammasome. *J. Immunol.* 183, 3942–3948.

Parry, C. M., Hien, T. T., Dougan, G., White, N. J., and Farrar, J. J. (2002). Typhoid fever. *N. Engl. J. Med.* 347, 1770–1782.

Rajan, J. V., Rodriguez, D., Miao, E. A., and Aderem, A. (2011). The NLRP3 inflammasome detects EMCV and VSV infection. *J. Virol.* 85, 4167–4172.

Raupach, B., Peuschel, S. K., Monack, D. M., and Zychlinsky, A. (2006). Caspase-1-mediated activation of interleukin-1beta (IL-1beta) and IL-18 contributes to innate immune defenses against *Salmonella enterica* serovar Typhimurium infection. *Infect. Immun.* 74, 4922–4926.

Ren, T., Zamboni, D. S., Roy, C. R., Dietrich, W. F., and Vance, R. E. (2006). Flagellin-deficient Legionella mutants evade caspase-1- and Naip5-mediated macrophage immunity. *PLoS Pathog.* 2, e18. doi: 10.1371/journal.ppat.0020018

Schroder, K., and Tschopp, J. (2010). The inflammasomes. *Cell* 140, 821–832.

Shimada, T., Park, B. G., Wolf, A. J., Brikos, C., Goodridge, H. S., Becker, C. A., Reyes, C. N., Miao, E. A., Aderem, A., Gotz, F., Liu, G. Y., and Underhill, D. M. (2010). *Staphylococcus aureus* evades lysozyme-based peptidoglycan digestion that links phagocytosis, inflammasome activation, and IL-1beta secretion. *Cell Host Microbe* 7, 38–49.

Sims, J. E., and Smith, D. E. (2010). The IL-1 family: regulators of immunity. *Nat. Rev. Immunol.* 10, 89–102.

Sutterwala, F. S., Mijares, L. A., Li, L., Ogura, Y., Kazmierczak, B. I., and Flavell, R. A. (2007). Immune recognition of *Pseudomonas aeruginosa* mediated by the IPAF/NLRC4 inflammasome. *J. Exp. Med.* 204, 3235–3245.

Suzuki, T., Franchi, L., Toma, C., Ashida, H., Ogawa, M., Yoshikawa, Y., Mimuro, H., Inohara, N., Sasakawa, C., and Nunez, G. (2007). Differential regulation of caspase-1 activation, pyroptosis, and autophagy via Ipaf and ASC in Shigella-infected macrophages. *PLoS Pathog.* 3, e111. doi: 10.1371/journal.ppat.0030111

Voetsch, A. C., Van T. J., Gilder, Angulo, F. J., Farley, M. M., Shallow, S., Marcus, R., Cieslak, P. R., Deneen, V. C., and Tauxe, R. V. (2004). FoodNet estimate of the burden of illness caused by nontyphoidal Salmonella infections in the United States. *Clin. Infect. Dis.* 38(Suppl. 3), S127–S134.

Warren, S. E., Mao, D. P., Rodriguez, A. E., Miao, E. A., and Aderem, A. (2008). Multiple Nod-like receptors activate caspase 1 during *Listeria monocytogenes* infection. *J. Immunol.* 180, 7558–7564.

Willingham, S. B., Allen, I. C., Bergstralh, D. T., Brickey, W. J., Huang, M. T., Taxman, D. J., Duncan, J. A., and Ting, J. P. (2009). NLRP3 (NALP3, Cryopyrin) facilitates in vivo caspase-1 activation, necrosis, and HMGB1 release via inflammasome-dependent and -independent pathways. *J. Immunol.* 183, 2008–2015.

Willingham, S. B., Bergstralh, D. T., O'Connor, W., Morrison, A. C., Taxman, D. J., Duncan, J. A., Barnoy, S., Venkatesan, M. M., Flavell, R. A., Deshmukh, M., Hoffman, H. M., and Ting, J. P. (2007). Microbial pathogen-induced necrotic cell death mediated by the inflammasome components CIAS1/cryopyrin/NLRP3 and ASC. *Cell Host Microbe* 2, 147–159.

Wu, J., Fernandes-Alnemri, T., and Alnemri, E. S. (2010). Involvement of the AIM2, NLRC4, and NLRP3 inflammasomes in caspase-1 activation by *Listeria monocytogenes*. *J. Clin. Immunol.* 30, 693–702.

Zhou, R., Yazdi, A. S., Menu, P., and Tschopp, J. (2011). A role for mitochondria in NLRP3 inflammasome activation. *Nature* 469, 221–225.

Zychlinsky, A., Prevost, M. C., and Sansonetti, P. J. (1992). *Shigella flexneri* induces apoptosis in infected macrophages. *Nature* 358, 167–169.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 March 2011; accepted: 11 April 2011; published online: 25 April 2011.
Citation: Miao EA and Rajan JV (2011) Salmonella and Caspase-1: a complex interplay of detection and evasion. Front. Microbio. ***2****:85. doi: 10.3389/fmicb.2011.00085*
This article was submitted to Frontiers in Cellular and Infection Microbiology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 06 May 2011
doi: 10.3389/fmicb.2011.00101

Interaction of *Salmonella* spp. with the intestinal microbiota

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Salmonella spp. are major cause of human morbidity and mortality worldwide. Upon entry into the human host, *Salmonella* spp. must overcome the resistance to colonization mediated by the gut microbiota and the innate immune system. They successfully accomplish this by inducing inflammation and mechanisms of innate immune defense. Many models have been developed to study *Salmonella* spp. interaction with the microbiota that have helped to identify factors necessary to overcome colonization resistance and to mediate disease. Here we review the current state of studies into this important pathogen/microbiota/host interaction in the mammalian gastrointestinal tract.

Keywords: *Salmonella*, microbiome, microbiota, colonization resistance

INTRODUCTION

Humans are colonized by trillions of bacteria that primarily reside on mucosal and epithelial surfaces (Costello et al., 2009). These microbes exist principally in a balanced symbiotic relationship with the host, thus resulting in little or no pathogenic outcome unless the host or the colonized mucosa becomes compromised (Lee and Mazmanian, 2010). The anatomic location for most of these microbes is the gut, which contains an estimated 10–100 trillion microbes representing at least 160 bacterial species per person (>1000 bacterial species can be found among different humans; Hooper and Gordon, 2001; Eckburg et al., 2005; Turnbaugh and Gordon, 2009; Lee and Mazmanian, 2010; Qin et al., 2010). The majority of these bacteria belong to one of two major phyla: Firmicutes and Bacteroidetes (Ley et al., 2006, 2008). These intestinal microbes provide many benefits for the host, including proper development of the immune system, the digestion of food and absorption of nutrients, the production of key vitamins (e.g., vitamin K and biotin), and protection against invading pathogenic organisms (Backhed et al., 2005; Lee and Mazmanian, 2010). Such protection against the colonization by pathogens has been called colonization resistance (van der Waaij et al., 1971; Stecher et al., 2008). In the literature, there is copious data to support the contention that the normal intestinal flora, or microbiota, protects against these invading microbes. For example, germ-free or abiotic mice possess increased susceptibility to enteric pathogens as well as abnormal intestinal mucosal immune system development (Bohnhoff et al., 1954; Miller and Bohnhoff, 1963; Gustafsson, 1982; Que and Hentges, 1985; Nardi et al., 1989; Lee and Mazmanian, 2010).

Salmonella species (spp.) are a significant group of intestinal pathogens with primary clinical manifestations of gastroenteritis and typhoid fever. Non-typhoidal *Salmonella* spp. (e.g., *S. enterica* serovar Typhimurium; *S.* Typhimurium) result in much of the food-borne disease diagnosed worldwide, while the primary cause of typhoid fever, the human specific pathogen (*S. enterica* serovar Typhi; *S.* Typhi) causes significant morbidity and mortality worldwide (Rabsch et al., 2001; Crump and Mintz, 2010; Graham, 2010). *Salmonella* spp. have been studied quite extensively with regard to their pathogenic properties including their ability to penetrate the intestinal barrier, and for typhoidal species, to replicate within host macrophages. However, only recently have studies begun to intensify with regard to the interaction of *Salmonella* spp. with the gastrointestinal microbiota. In this review, we will summarize the literature with regard to the role that the microbiota plays in colonization resistance against *Salmonella* spp., how salmonellae are able to overcome this colonization resistance, other factors that influence the survival of *Salmonella* spp. in the gut, and the methods that have been used to study *Salmonella*–microbiota interactions (**Figure 1**).

METHODS TO STUDY *SALMONELLA* SPP. INTERACTIONS WITH THE GUT MICROBIOTA

Several methods have been developed that could be used to study *Salmonella* spp. interactions with the gastrointestinal microbial community. We will briefly review methods to study *Salmonella* spp. gene expression and to screen for mutant phenotypes *in vivo*. Conversely, metagenomic and next generation sequencing methods can be used to study the effect of *Salmonella* spp. on the rest of the microbiota or the host.

The original genetic method to study gene expression *in vivo* was called *in vivo* expression technology (IVET; Mahan et al., 1993; Slauch et al., 1994). This is a promoter trapping strategy that can identify genes that are expressed *in vivo* but not *in vitro*. Essentially, a library of random *purA–lacZ* fusions is created in a *purA* deletion background. The *purA* gene is an essential metabolic gene so only those library members that contain fusions expressed *in vivo* can survive in the mouse. The survivors are recovered from the mouse and plated on

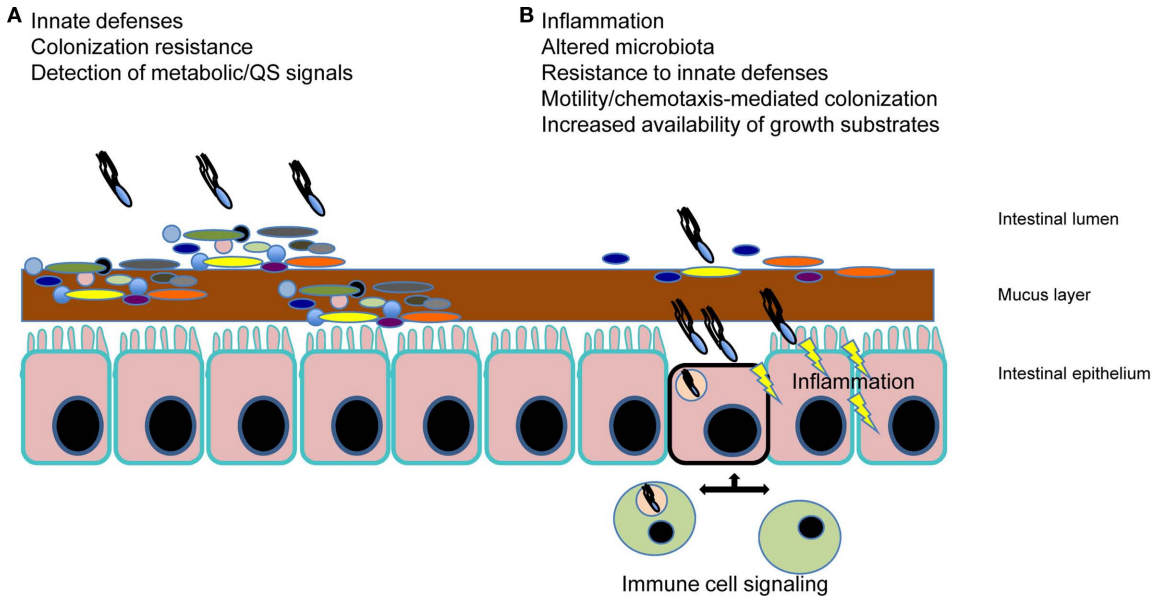

FIGURE 1 | *Salmonella* spp. interaction with the intestinal microbiota. **(A)** The gut microbiota (illustrated by the multiple colored bacteria) mediates "colonization resistance" by outcompeting invading pathogens such as *Salmonella*. In addition, innate immune defenses (e.g., antimicrobial molecules, mucus, sIgA, etc.) help to prevent pathogen colonization. At the time of this initial interaction of *Salmonella* with the microbiota, signals produced by the host and the gut microbes are being sensed by *Salmonella*, which influences the pathogenic outcome. **(B)** *Salmonella* spp. are able to overcome colonization resistance and initiate localized inflammation, which is key to disease manifestation. This inflammation is a result of motile *Salmonella* and SPI-1-mediated invasion of M-cells in Peyer's patches and enterocytes, as well as PAMP signaling. Phagocytic cell uptake and subsequent host cell signaling results in alteration of the microbiota, further induction of host innate resistance mechanisms (to which the bacteria respond with their own induced resistance), and the increased availability of growth substrates, some which specifically allow *Salmonella* to outcompete the microbiota. Perturbation of the microflora (e.g., antibiotic/probiotics) also creates an altered gut environment that can affect *Salmonella* spp. colonization.

X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), a colorimetric indicator of LacZ activity, to identify those fusions that are not expressed *in vitro*. This method was successful in identifying numerous metabolic and virulence genes that are expressed *in vivo* (Heithoff et al., 1997). One disadvantage of the method is that *purA* is essential throughout infection and may be too stringent. Other variations that used antibiotic resistance genes in place of *purA* were developed so that a pulse of antibiotic could be administered to the mouse to provide shorter periods of selection (Mahan et al., 1995; Young and Miller, 1997).

A variation of the IVET method is RIVET (Recombination-based IVET; Camilli et al., 1994; Camilli and Mekalanos, 1995), which utilizes *tnpR* fusions. The readout for *tnpR* is the site-specific recombination of a pair of target sites (*res* sites) placed elsewhere in the chromosome. DNA between the target sites is deleted, leaving a single *res* site. A variety of marker genes can be placed between these *res* sites. The first variant used a *res-tetRA-res* cassette so that the readout for *tnpR* expression is a change from tetracycline resistant to tetracycline sensitive. The advantage of RIVET is that the gene expression event is recorded permanently in the genome. RIVET has been used extensively to study *Vibrio cholerae* (Osorio et al., 2005; Lombardo et al., 2007). This method has also worked well for studying *S.* Typhimurium *phoPQ* and *pmrAB* expression in the gastrointestinal tract and for determining that the *S.* Typhimurium AHL detector, SdiA, becomes active in *Yersinia*-infected mice (Merighi et al., 2005; Smith et al., 2008; Dyszel et al., 2010; Noel et al., 2010).

GFP is another reporter that can be used to study single genes or entire libraries. A method termed differential fluorescence induction (DFI) can be used to sort bacteria that have high fluorescence *in vivo* from those that have low fluorescence *in vitro* (Valdivia and Falkow, 1996, 1997; Valdivia et al., 1996; Bumann and Valdivia, 2007). Though powerful, this method has not yet been used to study *Salmonella* spp. interactions specifically with the intestinal microbiota.

Phenotypic methods for studying *Salmonella* spp. interactions with the microbiota are advancing rapidly. For screening hundreds or thousands of *Salmonella* spp. mutants simultaneously there are now two different approaches. The first approach is to use microarrays to monitor the populations of individual mutants in a library, before and after a selective event, such as transit through an animal (Badarinarayana et al., 2001; Sassetti et al., 2001). All mutants that increase or decrease in proportion to the remainder of the library are readily identified. The microarray methodologies are primarily called transposon site hybridization (TraSH; Sassetti et al., 2001; Murry et al., 2008). Several TraSH variants have been successful in identifying *Salmonella* spp. genes required for host colonization (Lawley et al., 2006; Chaudhuri et al., 2009; Santiviago et al., 2009). The second approach uses next generation sequencing technology to measure the proportion of mutants in a library before and after the selective event. This method is primarily referred to as Tn-seq (van Opijnen et al., 2009; Opijnen and Camilli, 2010; Gallagher et al., 2011) or INSeq (Goodman et al., 2009). By barcoding each experiment, one can put up to 96 Tn-seq experiments

into a single sequencing run. This has been termed Bar-seq (Smith et al., 2010). Though clearly applicable and achievable, to date, no research groups have used any of these methods to study how *Salmonella* spp. interact specifically with other members of the intestinal microbiota.

Genomic DNA libraries, or cDNA libraries, of entire microbial communities can be constructed and screened for the presence of individual DNA sequences of interest, or the libraries can be sequenced in their entirety. Alternatively, the library can be screened for function in a heterologous host, typically *E. coli*. This is called metagenomics and has been used to identify the entire "metagenome" of gut microbial communities from several animal and human subjects (Handelsman, 2004). Newer deep sequencing methods are being used to sequence the entire gut community or "microbiome." This is a powerful technique for predicting the type and abundance of microbes present as well as metabolic pathways and function of the community as a whole (Booijink et al., 2007; Frank and Pace, 2008; Ventura et al., 2009; Qin et al., 2010; Vacharaksa and Finlay, 2010; Wang et al., 2010). The type and quantity of community variation can be characterized between healthy individuals, diseased individuals, and individuals after perturbations such as antibiotics or changes in diet (Turnbaugh and Gordon, 2009; Neu et al., 2010; Willing et al., 2010). In addition, quantitation of 16S rDNA has been used to study the effects of *Salmonella* and other pathogens on the gut microbial community (Lupp et al., 2007; Stecher et al., 2007; Barman et al., 2008; Sekirov et al., 2010). Using this technology, it was found that *Salmonella*-induced inflammation both decreased the population and altered the composition of the microbiota in a manner that was dependent upon *Salmonella* SPI1 and SPI2 and upon reactive oxygen and nitrogen species of the host (Stecher et al., 2007; Ackermann et al., 2008).

ANIMAL MODELS FOR STUDYING *SALMONELLA* SPP. INTERACTIONS WITH THE MICROBIOTA

CONVENTIONAL MICE

The most commonly used animal model for *S.* Typhimurium infection is the BALB/c or C57BL/6 mouse. These mice have a mutation in the *Slc11a1* gene (previously known as *Nramp1*) that leaves them susceptible to systemic infection by *S.* Typhimurium. The *Slc11A1* mutation causes a defect in ion transport in phagocytic vesicles allowing *S.* Typhimurium to survive in macrophages (Blackwell et al., 2001). This model serves as a surrogate for the infection of humans by the host-restricted serovar Typhi that causes Typhoid fever, though new humanized mouse models for *S.* Typhi have recently been developed (Firoz Mian et al., 2010; Libby et al., 2010; Song et al., 2010). Typhimurium is an extremely important human and animal pathogen in its own right, being one of the most common and most serious, causes of human food-borne gastroenteritis (Scallan et al., 2011). Additionally, in Africa, non-typhoidal serovars (NTS) including Typhimurium have become a major cause of systemic disease in immunocompromised patients (Kingsley et al., 2009; Gordon et al., 2010; Graham, 2010). One drawback of *Slc11A1* mouse strains is that they succumb to even low dose infection fairly rapidly, within 10 days. To study persistence, some researchers are using 129/SvJ or CBA mice that bear a functional *Slc11A1* allele (Lawley et al., 2006; Tsolis et al., 2011). *S.* Typhimurium persists in the GI tract for greater than 30 days in these mice and has been found to persist in the mesenteric lymph nodes and gallbladder as well (Monack et al., 2004; Lawley et al., 2006; Crawford et al., 2010).

STREPTOMYCIN-TREATED AND GNOTOBIOTIC MICE

While *S.* Typhimurium infection of susceptible mice has been used to model human typhoid fever, there are two problems with using conventional mice as a model for *S.* Typhimurium gastroenteritis. The first problem is that the mice do not get diarrhea as in the human infection. The second problem is that the normal microbiota causes bottlenecks in *Salmonella* spp. populations during phenotypic screening experiments. This is an issue for any method that requires large numbers of library members to undergo selection in the animal (IVET, TraSH, Tn-seq, etc.). Bottlenecks are the stochastic loss of library members, rendering the TraSH or Tn-seq results unreliable. Mice treated with antibiotics to disrupt their normal flora (most commonly streptomycin) do not cause *Salmonella* spp. populations to bottleneck (Hapfelmeier and Hardt, 2005; Stecher and Hardt, 2011). These mice also get symptoms that are closer to human gastroenteritis, allowing this human disease to be modeled. Gnotobiotic mice have the same advantages as streptomycin-treated mice but have the additional advantage that the composition of the microbial community can be controlled. Gnotobiotic simply means "known flora" and this can range from abiotic mice that have no flora (also known as germ-free or axenic), to mono-associated mice that are colonized with a single known microbial species, to poly-associated mice that are colonized with multiple species (Falk et al., 1998). The combination of gnotobiotic mice with TraSH, Tn-seq, and deep sequencing methods should revolutionize the study of how *Salmonella* spp. interact with the intestinal microbiota (Goodman et al., 2009; Faith et al., 2010).

ANTIMICROBIAL MECHANISMS OF THE HUMAN GUT AND GUT MICROFLORA

The human gut contains an arsenal of barriers to incoming pathogenic organisms. These barriers can come in many forms, including physical, chemical, enzymatic, or immune. *Salmonella* spp., which primarily colonize the distal ileum/cecum, first must overcome physical barriers in this environment. Although not always considered, a thick mucous layer covers the intestinal epithelium and presents a significant challenge to microbes that must traverse this layer to come into direct contact with the intestinal epithelium. Though the mucus provides an environment for attachment and nutrition, it both prevents epithelial cell engagement and harbors other antimicrobial substances such as IgA and cationic antimicrobial peptides (CAMPs; Lievin-Le Moal and Servin, 2006; Macpherson and Slack, 2007; Meyer-Hoffert et al., 2008). The CAMPs found in the gut are primarily either defensins or cathelicidins (Zasloff, 2002; Iimura et al., 2005; Ouellette, 2010; Salzman et al., 2010). CAMPs typically kill bacteria by forming pores in the membrane, but these peptides have also been shown to have immunomodulatory activities, primarily resulting in the recruitment of neutrophils to sites of infection (Yang et al., 2001; Bowdish et al., 2006; Hazlett and Wu, 2011). Mucins (e.g., MUC4), IgA, and antimicrobial peptides can be regulated by bacterial colonization

and thus represent an inducible mechanism of resistance (Salzman et al., 2003; Macpherson and Uhr, 2004; Raffatellu et al., 2009; Veldhuizen et al., 2009).

As well as traversing the mucous layer, colonizing pathogenic microbes must compete effectively with the existing microbiota. This microbiota provides a crucial line of defense as they can both compete for nutritional resources and for attachment sites to the intestinal epithelium. In addition, some microflora can produce bacteriocins, which are toxins that act in a similar manner to CAMPs but are produced by the microbiota instead of the host (Sanchez et al., 2010). If invading microbes are able to overcome the mucous layer and compete effectively against the microflora, other antimicrobial molecules must also be successfully overcome. Interleukin signaling from the mucosa results in the host production of lipocalin-2, which binds to the siderophore enterobactin/enterochelin in an attempt to withhold iron from bacteria (Raffatellu et al., 2009; Blaschitz and Raffatellu, 2010; Raffatellu and Bäumler, 2010). However, *Salmonella* also produces a second siderophore, salmochelin, which is not bound by lipocalin-2, thus enabling *Salmonella* spp. to compete for iron necessary for growth (Raffatellu et al., 2009). The Paneth cells found at the base of the intestinal crypts produce, in addition to the aforementioned CAMPs, antimicrobial products including angiogenins and the C-type lectin, RegIIIγ (regenerating gene; mouse)/HIP-PAP (hepatocarcinoma-intestine-pancreas/pancreas-associated protein; Human); Hooper et al., 2003; Cash et al., 2006). Although RegIIIγ is induced by mucosal damage and inflammatory stimuli, it is primarily effective against Gram-positive bacteria and thus would play a limited direct role against *Salmonella* spp. (Brandl et al., 2008; Godinez et al., 2009; Lehotzky et al., 2010).

The intestinal microbes themselves, in addition to producing bacteriocins, produce short chain fatty acids (SCFAs) as a consequence of their metabolic activity (Cummings and Macfarlane, 1991, 1997). Microbiota-produced butyrate and acetate can have dramatic effects on both the host and on *Salmonella* spp. during infection (Garner et al., 2009). Acetate and formate in the small intestine were found to have a positive effect on *Salmonella* Pathogenicity Island I-mediated invasion (Huang et al., 2008; Chavez et al., 2010) while propionate and butyrate, present in high concentrations in the cecum and colon, had the opposite effect (Gantois et al., 2006; Wong et al., 2006). These metabolic byproducts thus may represent environmental signals that direct *Salmonella* spp. to the distal ileum for invasion. Additionally, butyrate is known to induce expression of the human cathelicidin LL-37 (mouse: CRAMP), which could clearly affect invading pathogens (Raqib et al., 2006).

Given the antimicrobial environment of the human gut, how can pathogens such as *Salmonella* spp. overcome the intestinal flora and antimicrobial onslaught? It is likely a multifactorial response, both inherent and induced. *Salmonella* spp. have evolved numerous countermeasures to the antimicrobials present in the gut. Such mechanisms include resistance to both oxidative and non-oxidative host killing. Resistance to reactive oxygen and nitrogen compounds include such enzymes as superoxide dismutase and catalase (Vazquez-Torres and Fang, 2001; Prost et al., 2007; Ackermann et al., 2008; Kim et al., 2010). A well-studied resistance mechanism to non-oxidative killing includes the *in vivo* regulated modifications of lipopolysaccharide (LPS), a glycolipid that constitutes the majority of the outer leaflet of the outer membrane of *Salmonella* spp. (Guo et al., 1997; Gunn, 2008; Richards et al., 2010). These modifications, mediated primarily by environmental sensing via the two-component regulatory systems PhoP–PhoQ and PmrA–PmrB, result in resistance to CAMPs either by lack of CAMP binding to the bacterium or poor penetration of the CAMPs to the inner membrane, the site of lethal action (Guo et al., 1998; Richards et al., 2010). Thus, it can be hypothesized that upon *Salmonella* spp. entry into the intestinal environment and subsequent CAMP induction (CAMPs can activate the PhoP–PhoQ and PmrA–PmrB regulatory systems), these CAMPs may differentially affect the microbiota (reduce it) while allowing CAMP resistant *Salmonella* spp. to flourish.

INFLAMMATION AS A MECHANISM TO OVERCOME GUT COLONIZATION RESISTANCE

While the human intestine is always in a mild state of inflammation, invading pathogens trigger an induction of innate and adaptive immune responses. The inflammatory response of the host is triggered by effector molecules secreted by Type III secretion systems of *Salmonella* pathogenicity Islands I and II as well as extracellular and intracellular detection of pathogen associated molecular patterns (PAMPS) of the bacteria, which include LPS, peptidoglycan, and flagellin (Zhou and Galan, 2001; Abrahams and Hensel, 2006). LPS is detected through the combinatorial efforts of LPS-binding protein, CD14, MD-2, and toll-like receptor (TLR)-4 (Abreu, 2010). Peptidoglycan is detected by TLR-2 as well and intracellularly by proteins of the nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs; Abreu, 2010). Flagellin is detected by TLR-5 as well as NLRP3 (NLR family pyrin domain containing; previously NALP3) and NLRC4 (NLR family CARD domain containing; previously IPAF [IL-1β-converting enzyme protease activating factor]), both of which activate caspase-1 in response to *S.* Typhimurium (Grassl and Finlay, 2008; Broz et al., 2010). Such NLR signaling induces the expression of proinflammatory cytokines such as IL-1β and IL-18 (Tam et al., 2008). These cytokines, along with IL-23, result in an immune cascade of activation involving T-cell induced IL-17 and IFN-γ, ultimately resulting in the induction of host-derived intestinal defense mechanisms discussed earlier (Stecher et al., 2007; Santos et al., 2009).

Salmonella Typhimurium has been shown to be unable to colonize the mouse intestine in the absence of inflammation, as the normal flora in the non-inflamed state is able to effectively outcompete an avirulent (lacking inflammatory capacity) *Salmonella* intruder (Stecher et al., 2007; Santos et al., 2009; Winter et al., 2010). This phenomenon can be reversed by either mixing the avirulent *S.* Typhimurium with wildtype strains capable of inducing inflammation or in mice lacking the anti-inflammatory cytokine IL-10. Additionally, *Salmonella*-induced inflammation results in an altered microbiota, which may also favor growth of the pathogen (Lupp et al., 2007; Barman et al., 2008).

THE LINK BETWEEN THE HOST, MICROBIAL METABOLISM, AND *SALMONELLA* SPP. INTESTINAL COLONIZATION

As discussed above, nutrient availability can be increased upon *Salmonella* spp. induction of inflammation. Also discussed above, SCFA synthesis by the microbiota can be used both by

the host to induce defense mechanisms and by the bacterium to enhance invasion of the intestinal epithelium. Recently, direct links have arisen between *S.* Typhimurium, intestinal inflammation, and metabolic properties. Winter et al. (2010) demonstrated that reactive oxygen species released as a result of *Salmonella*-induced inflammation react with luminal thiosulfate to produce tetrathionate. Tetrathionate can then be used as a terminal electron acceptor for *Salmonella* spp. respiration, allowing for more efficient energy production relative to competing, fermenting microflora. Tetrathionate has been used since the early 1920s as an enrichment for *Salmonella* spp. in mixed microbial samples. Thus, the ability to overcome colonization resistance may reside in its ability to utilize inflammation-induced compounds to enhance growth.

In a similar vein, inflammation also releases or induces the expression of high-energy nutrients such as mucin and galactose-containing molecules found in the cecum and elsewhere in the gut (Stecher et al., 2008). It was shown that *S.* Typhimurium flagellar and chemotaxis mutants had reduced fitness in the inflamed but not the non-inflamed gut. It was reasoned that motility and the ability to chemotax allowed *S.* Typhimurium to utilize specific carbohydrates to both help continue the inflammation and to outgrow the microbiota. Thus, inflammation can result in the release of carbohydrates, which *Salmonella* spp. can use to aid growth if it possesses intact chemotactic and motility properties.

In a move away from the gut bacterial metabolic capabilities, a recent study has looked at host metabolic changes during *Salmonella* spp. intestinal infection (Antunes et al., 2011). Using sophisticated mass spectrometry methods, they determined that numerous metabolic pathways were affected, most prominently hormonal pathways such as those affecting steroid and eicosanoid synthesis. Such hormonal pathways have dramatic effects on the host, including wound healing, sugar metabolism, and immune system regulation. It is likely that some of these changes are a direct result of *Salmonella*-induced inflammation while others may be a result of the altered microbiota.

ANTIBIOTICS, NORMAL FLORA, AND *SALMONELLA* SPP. INFECTION

Antibiotics target certain classes of microbes, with many antibiotics having dramatic effects on the intestinal microbiota after administration to a host. Several studies (examples outlined below) have been completed examining the effect of antibiotics on the intestinal flora, and the effect that this flora disruption has on *Salmonella* spp. colonization and disease as well as the host intestinal metabolome (Antunes et al., 2011). Recently, clinically relevant doses of antibiotics were shown to affect the ratio of microbial phyla in the intestine but not the overall bacterial load (Lupp et al., 2007; Sekirov et al., 2008). Other studies have found that different antibiotics have variable effects on the total number and distribution of gut bacteria, but that each antibiotic tested enhanced *Salmonella*-induced epithelial cell invasion and inflammation (Croswell et al., 2009). After antibiotic removal and some recovery of the microbiota, mice were still susceptible to *Salmonella*-induced enteritis, suggesting that the correct balance of microbial diversity and numbers are required for effective colonization resistance. Furthermore, it was recently demonstrated that growth and dissemination of *S.* Typhimurium DT104 during antibiotic (fosfomycin) treatment could be abrogated by continuous feeding of some, but not all, *Lactobacillus* species (a probiotic approach; Asahara et al., 2011). This protective capacity was found to be associated primarily with increased organic acid production and maintenance of a decreased intestinal pH. Thus, alteration of the microbiota by the administration of antibiotics or probiotics can have dramatic effects on *Salmonella*-associated disease.

SALMONELLA SPP. CAN DETECT OTHER MICROBES TO AFFECT PATHOGENESIS

Salmonella Typhimurium has the ability to detect the *N*-acyl-L-homoserine lactone (AHL) signaling molecules of other microbes using SdiA, a LuxR homolog (Michael et al., 2001; Smith and Ahmer, 2003; Soares and Ahmer, 2011). *Salmonella* spp. cannot synthesize AHLs so this system is exclusively for detecting other microbes. Surprisingly, AHLs have not been found in the GI tract of healthy mammals, with the exception of the bovine rumen (Erickson et al., 2002; Smith et al., 2008; Edrington et al., 2009; Hughes et al., 2010). However, SdiA activity was detected in turtles colonized by *Aeromonas hydrophila* and in mice colonized by *Yersinia enterocolitica* (Smith et al., 2008; Dyszel et al., 2010). Both of these organisms are AHL-producing pathogens. In *Yersinia*-infected mice, the SdiA activity was primarily observed in the Peyer's patches (Dyszel et al., 2010). In competition with wildtype, an *sdiA* mutant had no apparent fitness defect, but this may have been due to the small percentage of *S.* Typhimurium detecting AHLs at any given time. When *S.* Typhimurium was engineered to produce AHLs, the wildtype was much more fit than the *sdiA* mutant. All members of the SdiA regulon were required for the fitness phenotype indicating that all of the regulon members are functional in mice (Dyszel et al., 2010). To date, the SdiA regulon consists of only two loci the *rck* operon, which contains six genes, and *srgE* (Ahmer et al., 1998; Smith and Ahmer, 2003). Rck is an outer membrane protein that confers resistance to complement killing, adhesion, and invasion of host cells (Ho et al., 2010; Rosselin et al., 2010). The SrgE protein is predicted to be a T3SS secreted effector protein with a coiled-coil domain (Samudrala et al., 2009). Why these genes are important to *Salmonella* spp. in the presence of AHLs is not known. It is also not known if this system is used to detect a specific AHL-producing pathogen, such as *Y. enterocolitica*, in a specific host, or if the system is more general and fitness benefits are achieved from detecting any of a number of AHL-producing pathogens in a variety of hosts.

As described above, the normal microbiota produces high concentrations of SCFAs as a byproduct of metabolism (Cummings et al., 1987). These SCFAs are a significant nutrient source for the host and other members of the microbiota and *S.* Typhimurium regulates virulence genes in response to these SCFAs (Lawhon et al., 2002; Huang et al., 2008; Sartor, 2008). It appears that the two-component regulatory system SirA/BarA may be responsible for the detection of SCFAs (Chavez et al., 2010). BarA is a histidine sensor kinase that phosphorylates the response regulator SirA (Pernestig et al., 2001). SirA then regulates the transcription of two small RNAs that function to bind and prevent the

function of the RNA binding protein CsrA (Suzuki et al., 2002). CsrA regulates numerous genes involved in metabolism, virulence, and biofilm formation (Babitzke and Romeo, 2007; Babitzke et al., 2009). SirA also directly regulates the *fim* operon that encodes Type 1 fimbriae (Teplitski et al., 2006). Because *S.* Typhimurium produces SCFAs during growth *in vitro*, the BarA/SirA system is active in pure culture in late exponential phase. The detection of SCFAs by *S.* Typhimurium *in vitro* could be considered quorum sensing while its detection of SCFAs in the GI tract could be considered interspecies signaling. However, acetate is also a metabolic byproduct and a carbon source, depending on the circumstances (Wolfe, 2005), so it is probably more accurate to think about the detection of SCFAs by *S.* Typhimurium as metabolic regulation rather than communication.

CONCLUDING THOUGHTS

As a GI pathogen, ingested *Salmonella* spp. must overcome a gauntlet of host defenses in order to colonize and result in human disease. The intestinal microbiota, by a variety of different means, is at the root of this colonization resistance. Future research directed at in depth studies of the *Salmonella*/microbiota/metabolome/innate defense interaction with cutting edge methods, as well as directing approaches to use the microbiota as tool to inhibit *Salmonella* spp. colonization, are keys to limiting salmonellosis and typhoid fever worldwide.

ACKNOWLEDGMENTS

The authors would like to acknowledge funding by the NIH to John S. Gunn (AI043521, AI066208) and Brian M. M. Ahmer (AI073971).

REFERENCES

Abrahams, G. L., and Hensel, M. (2006). Manipulating cellular transport and immune responses: dynamic interactions between intracellular *Salmonella enterica* and its host cells. *Cell. Microbiol.* 8, 728–737.

Abreu, M. T. (2010). Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nat. Rev. Immunol.* 10, 131–144.

Ackermann, M., Stecher, B., Freed, N. E., Songhet, P., Hardt, W. D., and Doebeli, M. (2008). Self-destructive cooperation mediated by phenotypic noise. *Nature* 454, 987–990.

Ahmer, B. M. M., Reeuwijk, J. V., Timmers, C. D., Valentine, P. J., and Heffron, F. (1998). *Salmonella typhimurium* encodes an SdiA homolog, a putative quorum sensor of the LuxR family, that regulates genes on the virulence plasmid. *J. Bacteriol.* 180, 1185–1193.

Antunes, L. C., Arena, E. T., Menendez, A., Han, J., Ferreira, R. B., Buckner, M. M., Lolic, P., Madilao, L. L., Bohlmann, J., Borchers, C. H., and Finlay, B. B. (2011). The impact of *Salmonella* infection on host hormone metabolism revealed by metabolomics. *Infect. Immun.* 79, 1759–1769.

Antunes, L. C., Han, J., Ferreira, R. B., Lolic, P., Borchers, C. H., and Finlay, B. B. (2011). The effect of antibiotic treatment on the intestinal metabolome. *Antimicrob. Agents Chemother.* 55, 1494–1503.

Asahara, T., Shimizu, K., Takada, T., Kado, S., Yuki, N., Morotomi, M., Tanaka, R., and Nomoto, K. (2011). Protective effect of *Lactobacillus casei* strain Shirota against lethal infection with multi-drug resistant *Salmonella enterica* serovar Typhimurium DT104 in mice. *J. Appl. Microbiol.* 110, 163–173.

Babitzke, P., Baker, C. S., and Romeo, T. (2009). Regulation of translation initiation by RNA binding proteins. *Annu. Rev. Microbiol.* 63, 27–44.

Babitzke, P., and Romeo, T. (2007). CsrB sRNA family: sequestration of RNA-binding regulatory proteins. *Curr. Opin. Microbiol.* 10, 156–163.

Backhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., and Gordon, J. I. (2005). Host-bacterial mutualism in the human intestine. *Science* 307, 1915–1920.

Badarinarayana, V., Estep, P. W. III, Shendure, J., Edwards, J., Tavazoie, S., Lam, F., and Church, G. M. (2001). Selection analyses of insertional mutants using subgenic-resolution arrays. *Nat. Biotechnol.* 19, 1060–1065.

Barman, M., Unold, D., Shifley, K., Amir, E., Hung, K., Bos, N., and Salzman, N. (2008). Enteric salmonellosis disrupts the microbial ecology of the murine gastrointestinal tract. *Infect. Immun.* 76, 907–915.

Blackwell, J. M., Goswami, T., Evans, C. A., Sibthorpe, D., Papo, N., White, J. K., Searle, S., Miller, E. N., Peacock, C. S., Mohammed, H., and Ibrahim, M. (2001). SLC11A1 (formerly NRAMP1) and disease resistance. *Cell. Microbiol.* 3, 773–784.

Blaschitz, C., and Raffatellu, M. (2010). Th17 cytokines and the gut mucosal barrier. *J. Clin. Immunol.* 30, 196–203.

Bohnhoff, M., Drake, B. L., and Miller, C. P. (1954). Effect of streptomycin on susceptibility of intestinal tract to experimental *Salmonella* infection. *Proc. Soc. Exp. Biol. Med.* 86, 132–139.

Booijink, C. C., Zoetendal, E. G., Kleerebezem, M., and de Vos, W. M. (2007). Microbial communities in the human small intestine: coupling diversity to metagenomics. *Future Microbiol.* 2, 285–295.

Bowdish, D. M., Davidson, D. J., and Hancock, R. E. (2006). Immunomodulatory properties of defensins and cathelicidins. *Curr. Top. Microbiol. Immunol.* 306, 27–66.

Brandl, K., Plitas, G., Mihu, C. N., Ubeda, C., Jia, T., Fleisher, M., Schnabl, B., DeMatteo, R. P., and Pamer, E. G. (2008). Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. *Nature* 455, 804–807.

Broz, P., Newton, K., Lamkanfi, M., Mariathasan, S., Dixit, V. M., and Monack, D. M. (2010). Redundant roles for inflammasome receptors NLRP3 and NLRC4 in host defense against *Salmonella*. *J. Exp. Med.* 207, 1745–1755.

Bumann, D., and Valdivia, R. H. (2007). Identification of host-induced pathogen genes by differential fluorescence induction reporter systems. *Nat. Protoc.* 2, 770–777.

Camilli, A., Beattie, D. T., and Mekalanos, J. J. (1994). Use of genetic recombination as a reporter of gene expression. *Proc. Natl. Acad. Sci. U.S.A.* 91, 2634–2638.

Camilli, A., and Mekalanos, J. J. (1995). Use of recombinase gene fusions to identify *Vibrio cholerae* genes induced during infection. *Mol. Microbiol.* 18, 671–683.

Cash, H. L., Whitham, C. V., Behrendt, C. L., and Hooper, L. V. (2006). Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science* 313, 1126–1130.

Chaudhuri, R. R., Peters, S. E., Pleasance, S. J., Northen, H., Willers, C., Paterson, G. K., Cone, D. B., Allen, A. G., Owen, P. J., Shalom, G., Stekel, D. J., Charles, I. G., and Maskell, D. J. (2009). Comprehensive identification of *Salmonella enterica* serovar typhimurium genes required for infection of BALB/c mice. *PLoS Pathog.* 5, e1000529. doi: 10.1371/journal.ppat.1000529

Chavez, R. G., Alvarez, A. F., Romeo, T., and Georgellis, D. (2010). The physiological stimulus for the BarA sensor kinase. *J. Bacteriol.* 192, 2009–2012.

Costello, E. K., Lauber, C. L., Hamady, M., Fierer, N., Gordon, J. I., and Knight, R. (2009). Bacterial community variation in human body habitats across space and time. *Science* 326, 1694–1697.

Crawford, R. W., Rosales-Reyes, R., Ramírez-Aguilar, M. de L., Chapa-Azuela, O., Alpuche-Aranda, C., and Gunn, J. S. (2010). Gallstones play a significant role in *Salmonella* spp. gallbladder colonization and carriage. *Proc. Natl. Acad. Sci. U.S.A.* 107, 4353–4358.

Croswell, A., Amir, E., Teggatz, P., Barman, M., and Salzman, N. H. (2009). Prolonged impact of antibiotics on intestinal microbial ecology and susceptibility to enteric *Salmonella* infection. *Infect. Immun.* 77, 2741–2753.

Crump, J. A., and Mintz, E. D. (2010). Global trends in typhoid and paratyphoid fever. *Clin. Infect. Dis.* 50, 241–246.

Cummings, J. H., and Macfarlane, G. T. (1991). The control and consequences of bacterial fermentation in the human colon. *J. Appl. Bacteriol.* 70, 443–459.

Cummings, J. H., and Macfarlane, G. T. (1997). Role of intestinal bacteria in nutrient metabolism. *JPEN J. Parenter. Enteral. Nutr.* 21, 357–365.

Cummings, J. H., Pomare, E. W., Branch, W. J., Naylor, C. P., and Macfarlane, G. T. (1987). Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* 28, 1221–1227.

Dyszel, J. L., Smith, J. N., Lucas, D. E., Soares, J. A., Swearingen, M. C., Vross, M. A., Young, G. M., and Ahmer, B. M. (2010). *Salmonella enterica* serovar Typhimurium can detect acyl homoserine lactone production by *Yersinia enterocolitica* in mice. *J. Bacteriol.* 192, 29–37.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E., and Relman, D. A. (2005). Diversity of the human intestinal microbial flora. *Science* 308, 1635–1638.

Edrington, T. S., Farrow, R. L., Sperandio, V., Hughes, D. T., Lawrence, T. E., Callaway, T. R., Anderson, R. C., and Nisbet, D. J. (2009). Acyl-homoserine-lactone autoinducer in the gastrointestinal [corrected] tract of feedlot cattle and correlation to season, *E. coli* O157:H7 prevalence, and diet. *Curr. Microbiol.* 58, 227–232.

Erickson, D. L., Nsereko, V. L., Morgavi, D. P., Selinger, L. B., Rode, L. M., and Beauchemin, K. A. (2002). Evidence of quorum sensing in the rumen ecosystem: detection of *N*-acyl homoserine lactone autoinducers in ruminal contents. *Can. J. Microbiol.* 48, 374–378.

Faith, J. J., Rey, F. E., O'Donnell, D., Karlsson, M., McNulty, N. P., Kallstrom, G., Goodman, A. L., and Gordon, J. I. (2010). Creating and characterizing communities of human gut microbes in gnotobiotic mice. *ISME J.* 4, 1094–1098.

Falk, P. G., Hooper, L. V., Midtvedt, T., and Gordon, J. I. (1998). Creating and maintaining the gastrointestinal ecosystem: what we know and need to know from gnotobiology. *Microbiol. Mol. Biol. Rev.* 62, 1157–1170.

Firoz Mian, M., Pek, E. A., Chenoweth, M. J., and Ashkar, A. A. (2010). Humanized mice are susceptible to *Salmonella typhi* infection. *Cell. Mol. Immunol.* 8, 83–87.

Frank, D. N., and Pace, N. R. (2008). Gastrointestinal microbiology enters the metagenomics era. *Curr. Opin. Gastroenterol.* 24, 4–10.

Gallagher, L. A., Shendure, J., and Manoil, C. (2011). Genome-scale identification of resistance functions in *Pseudomonas aeruginosa* using Tn-seq. *MBio* 2, e00315–e00310.

Gantois, I., Ducatelle, R., Pasmans, F., Haesebrouck, F., Hautefort, I., Thompson, A., Hinton, J. C., and Van Immerseel, F. (2006). Butyrate specifically down-regulates *Salmonella* pathogenicity island 1 gene expression. *Appl. Environ. Microbiol.* 72, 946–949.

Garner, C. D., Antonopoulos, D. A., Wagner, B., Duhamel, G. E., Keresztes, I., Ross, D. A., Young, V. B., and Altier, C. (2009). Perturbation of the small intestine microbial ecology by streptomycin alters pathology in a *Salmonella enterica* serovar typhimurium murine model of infection. *Infect. Immun.* 77, 2691–2702.

Godinez, I., Raffatellu, M., Chu, H., Paixao, T. A., Haneda, T., Santos, R. L., Bevins, C. L., Tsolis, R. M., and Baumler, A. J. (2009). Interleukin-23 orchestrates mucosal responses to *Salmonella enterica* serotype Typhimurium in the intestine. *Infect. Immun.* 77, 387–398.

Goodman, A. L., McNulty, N. P., Zhao, Y., Leip, D., Mitra, R. D., Lozupone, C. A., Knight, R., and Gordon, J. I. (2009). Identifying genetic determinants needed to establish a human gut symbiont in its habitat. *Cell Host Microbe* 6, 279–289.

Gordon, M. A., Kankwatira, A. M., Mwafulirwa, G., Walsh, A. L., Hopkins, M. J., Parry, C. M., Faragher, E. B., Zijlstra, E. E., Heyderman, R. S., and Molyneux, M. E. (2010). Invasive non-typhoid *Salmonellae* establish systemic intracellular infection in HIV-infected adults: an emerging disease pathogenesis. *Clin. Infect. Dis.* 50, 953–962.

Graham, S. M. (2010). Nontyphoidal salmonellosis in Africa. *Curr. Opin. Infect. Dis.* 23, 409–414.

Grassl, G. A., and Finlay, B. B. (2008). Pathogenesis of enteric *Salmonella* infections. *Curr. Opin. Gastroenterol.* 24, 22–26.

Gunn, J. S. (2008). The *Salmonella* PmrAB regulon: lipopolysaccharide modifications, antimicrobial peptide resistance and more. *Trends Microbiol.* 16, 284–290.

Guo, L., Lim, K. B., Gunn, J. S., Bainbridge, B., Darveau, R. P., Hackett, M., and Miller, S. I. (1997). Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes phoP-phoQ. *Science* 276, 250–253.

Guo, L., Lim, K. B., Poduje, C. M., Daniel, M., Gunn, J. S., Hackett, M., and Miller, S. I. (1998). Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. *Cell* 95, 189–198.

Gustafsson, B. E. (1982). The physiological importance of the colonic microflora. *Scand. J. Gastroenterol. Suppl.* 77, 117–131.

Handelsman, J. (2004). Metagenomics: application of genomics to uncultured microorganisms. *Microbiol. Mol. Biol. Rev.* 68, 669–685.

Hapfelmeier, S., and Hardt, W. D. (2005). A mouse model for *S. typhimurium*-induced enterocolitis. *Trends Microbiol.* 13, 497–503.

Hazlett, L., and Wu, M. (2011). Defensins in innate immunity. *Cell Tissue Res.* 343, 175–188.

Heithoff, D. M., Conner, C. P., Hanna, P. C., Julio, S. M., Hentschel, U., and Mahan, M. J. (1997). Bacterial infection as assessed by in vivo gene expression. *Proc. Natl. Acad. Sci. U.S.A.* 94, 934–939.

Ho, D. K., Jarva, H., and Meri, S. (2010). Human complement factor H binds to outer membrane protein Rck of *Salmonella*. *J. Immunol.* 185, 1763–1769.

Hooper, L. V., and Gordon, J. I. (2001). Commensal host-bacterial relationships in the gut. *Science* 292, 1115–1118.

Hooper, L. V., Stappenbeck, T. S., Hong, C. V., and Gordon, J. I. (2003). Angiogenins: a new class of microbicidal proteins involved in innate immunity. *Nat. Immunol.* 4, 269–273.

Huang, Y., Suyemoto, M., Garner, C. D., Cicconi, K. M., and Altier, C. (2008). Formate acts as a diffusible signal to induce *Salmonella* invasion. *J. Bacteriol.* 190, 4233–4241.

Hughes, D. T., Terekhova, D. A., Liou, L., Hovde, C. J., Sahl, J. W., Patankar, A. V., Gonzalez, J. E., Edrington, T. S., Rasko, D. A., and Sperandio, V. (2010). Chemical sensing in mammalian host-bacterial commensal associations. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9831–9836.

Iimura, M., Gallo, R. L., Hase, K., Miyamoto, Y., Eckmann, L., and Kagnoff, M. F. (2005). Cathelicidin mediates innate intestinal defense against colonization with epithelial adherent bacterial pathogens. *J. Immunol.* 174, 4901–4907.

Kim, B., Richards, S. M., Gunn, J. S., and Slauch, J. M. (2010). Protecting against antimicrobial effectors in the phagosome allows SodCII to contribute to virulence in *Salmonella enterica* serovar Typhimurium. *J. Bacteriol.* 192, 2140–2149.

Kingsley, R. A., Msefula, C. L., Thomson, N. R., Kariuki, S., Holt, K. E., Gordon, M. A., Harris, D., Clarke, L., Whitehead, S., Sangal, V., Marsh, K., Achtman, M., Molyneux, M. E., Cormican, M., Parkhill, J., MacLennan, C. A., Heyderman, R. S., and Dougan, G. (2009). Epidemic multiple drug resistant *Salmonella* Typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype. *Genome Res.* 19, 2279–2287.

Lawhon, S. D., Maurer, R., Suyemoto, M., and Altier, C. (2002). Intestinal short-chain fatty acids alter *Salmonella typhimurium* invasion gene expression and virulence through BarA/SirA. *Mol. Microbiol.* 46, 1451–1464.

Lawley, T. D., Chan, K., Thompson, L. J., Kim, C. C., Govoni, G. R., and Monack, D. M. (2006). Genome-wide screen for *Salmonella* genes required for long-term systemic infection of the mouse. *PLoS Pathog.* 2, e11. doi: 10.1371/journal.ppat.0020011.

Lee, Y. K., and Mazmanian, S. K. (2010). Has the microbiota played a critical role in the evolution of the adaptive immune system? *Science* 330, 1768–1773.

Lehotzky, R. E., Partch, C. L., Mukherjee, S., Cash, H. L., Goldman, W. E., Gardner, K. H., and Hooper, L. V. (2010). Molecular basis for peptidoglycan recognition by a bactericidal lectin. *Proc. Natl. Acad. Sci. U.S.A.* 107, 7722–7727.

Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R., and Gordon, J. I. (2008). Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat. Rev. Microbiol.* 6, 776–788.

Ley, R. E., Peterson, D. A., and Gordon, J. I. (2006). Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell* 124, 837–848.

Libby, S. J., Brehm, M. A., Greiner, D. L., Shultz, L. D., McClelland, M., Smith, K. D., Cookson, B. T., Karlinsey, J. E., Kinkel, T. L., Porwollik, S., Canals, R., Cummings, L. A., and Fang, F. C. (2010). Humanized nonobese diabetic-scid IL2rgammanull mice are susceptible to lethal *Salmonella* Typhi infection. *Proc. Natl. Acad. Sci. U.S.A.* 107, 15589–15594.

Lievin-Le Moal, V., and Servin, A. L. (2006). The front line of enteric host defense against unwelcome intrusion of harmful microorganisms: mucins, antimicrobial peptides, and microbiota. *Clin. Microbiol. Rev.* 19, 315–337.

Lombardo, M. J., Michalski, J., Martinez-Wilson, H., Morin, C., Hilton, T., Osorio, C. G., Nataro, J. P., Tacket, C. O., Camilli, A., and Kaper, J. B. (2007). An in vivo expression technology screen for *Vibrio cholerae* genes expressed in human volunteers. *Proc. Natl. Acad. Sci. U.S.A.* 104, 18229–18234.

Lupp, C., Robertson, M. L., Wickham, M. E., Sekirov, I., Champion, O. L., Gaynor, E. C., and Finlay, B. B. (2007). Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. *Cell Host Microbe* 2, 119–129.

Macpherson, A. J., and Slack, E. (2007). The functional interactions of commensal bacteria with intestinal secretory IgA. *Curr. Opin. Gastroenterol.* 23, 673–678.

Macpherson, A. J., and Uhr, T. (2004). Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science* 303, 1662–1665.

Mahan, M. J., Slauch, J. M., and Mekalanos, J. J. (1993). Selection of bacterial virulence genes that are specifically induced in host tissues. *Science* 259, 686–688.

Mahan, M. J., Tobias, J. W., Slauch, J. M., Hanna, P. C., Collier, R. J., and Mekalanos, J. J. (1995). Antibiotic-based selection for bacterial genes that are specifically induced during infection of a host. *Proc. Natl. Acad. Sci. U.S.A.* 92, 669–673.

Merighi, M., Ellermeier, C. D., Slauch, J. M., and Gunn, J. S. (2005). Resolvase – in vivo expression technology analysis of the *Salmonella enterica* serovar Typhimurium PhoP and PmrA regulons in BALB/c mice. *J. Bacteriol.* 187, 7407–7416.

Meyer-Hoffert, U., Hornef, M. W., Henriques-Normark, B., Axelsson,

L. G., Midtvedt, T., Putsep, K., and Andersson, M. (2008). Secreted enteric antimicrobial activity localises to the mucus surface layer. *Gut* 57, 764–771.

Michael, B., Smith, J. N., Swift, S., Heffron, F., and Ahmer, B. M. (2001). SdiA of *Salmonella enterica* is a LuxR homolog that detects mixed microbial communities. *J. Bacteriol.* 183, 5733–5742.

Miller, C. P., and Bohnhoff, M. (1963). Changes in the mouse's enteric microflora associated with enhanced susceptibility to *Salmonella* infection following streptomycin treatment. *J. Infect. Dis.* 113, 59–66.

Monack, D. M., Bouley, D. M., and Falkow, S. (2004). *Salmonella typhimurium* persists within macrophages in the mesenteric lymph nodes of chronically infected Nramp1+/+ mice and can be reactivated by IFNgamma neutralization. *J. Exp. Med.* 199, 231–241.

Murry, J. P., Sassetti, C. M., Lane, J. M., Xie, Z., and Rubin, E. J. (2008). Transposon site hybridization in *Mycobacterium tuberculosis*. *Methods Mol. Biol.* 416, 45–59.

Nardi, R. M., Silva, M. E., Vieira, E. C., Bambirra, E. A., and Nicoli, J. R. (1989). Intragastric infection of germfree and conventional mice with *Salmonella typhimurium*. *Braz. J. Med. Biol. Res.* 22, 1389–1392.

Neu, J., Lorca, G., Kingma, S. D., and Triplett, E. W. (2010). The intestinal microbiome: relationship to type 1 diabetes. *Endocrinol. Metab. Clin. North Am.* 39, 563–571.

Noel, J. T., Joy, J., Smith, J. N., Fatica, M., Schneider, K. R., Ahmer, B. M., and Teplitski, M. (2010). *Salmonella* SdiA recognizes *N*-acyl homoserine lactone signals from *Pectobacterium carotovorum* in vitro, but not in a bacterial soft rot. *Mol. Plant Microbe Interact.* 23, 273–282.

Opijnen, T., and Camilli, A. (2010). Genome-wide fitness and genetic interactions determined by Tn-seq, a high-throughput massively parallel sequencing method for microorganisms. *Curr. Protoc. Microbiol.* Chapter 1, Unit1E.3.

Osorio, C. G., Crawford, J. A., Michalski, J., Martinez-Wilson, H., Kaper, J. B., and Camilli, A. (2005). Second-generation recombination-based in vivo expression technology for large-scale screening for *Vibrio cholerae* genes induced during infection of the mouse small intestine. *Infect. Immun.* 73, 972–980.

Ouellette, A. J. (2010). Paneth cells and innate mucosal immunity. *Curr. Opin. Gastroenterol.* 26, 547–553.

Pernestig, A. K., Melefors, O., and Georgellis, D. (2001). Identification of UvrY as the cognate response regulator for the BarA sensor kinase in *Escherichia coli*. *J. Biol. Chem.* 276, 225–231.

Prost, L. R., Sanowar, S., and Miller, S. I. (2007). *Salmonella* sensing of anti-microbial mechanisms to promote survival within macrophages. *Immunol. Rev.* 219, 55–65.

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D. R., Li, J., Xu, J., Li, S., Li, D., Cao, J., Wang, B., Liang, H., Zheng, H., Xie, Y., Tap, J., Lepage, P., Bertalan, M., Batto, J. M., Hansen, T., Le Paslier, D., Linneberg, A., Nielsen, H. B., Pelletier, E., Renault, P., Sicheritz-Ponten, T., Turner, K., Zhu, H., Yu, C., Li, S., Jian, M., Zhou, Y., Li, Y., Zhang, X., Li, S., Qin, N., Yang, H., Wang, J., Brunak, S., Doré, J., Guarner, F., Kristiansen, K., Pedersen, O., Parkhill, J., Weissenbach, J., MetaHIT Consortium, Bork, P., Ehrlich, S. D., and Wang, J. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 464, 59–65.

Que, J. U., and Hentges, D. J. (1985). Effect of streptomycin administration on colonization resistance to *Salmonella typhimurium* in mice. *Infect. Immun.* 48, 169–174.

Rabsch, W., Tschape, H., and Baumler, A. J. (2001). Non-typhoidal salmonellosis: emerging problems. *Microbes Infect.* 3, 237–247.

Raffatellu, M., and Bäumler, A. J. (2010). *Salmonella*'s iron armor for battling the host and its microbiota. *Gut Microbes* 1, 70–72.

Raffatellu, M., George, M. D., Akiyama, Y., Hornsby, M. J., Nuccio, S. P., Paixao, T. A., Butler, B. P., Chu, H., Santos, R. L., Berger, T., Mak, T. W., Tsolis, R. M., Bevins, C. L., Solnick, J. V., Dandekar, S., and Bäumler, A. J. (2009). Lipocalin-2 resistance confers an advantage to *Salmonella enterica* serotype Typhimurium for growth and survival in the inflamed intestine. *Cell Host Microbe* 5, 476–486.

Raqib, R., Sarker, P., Bergman, P., Ara, G., Lindh, M., Sack, D. A., Nasirul Islam, K. M., Gudmundsson, G. H., Andersson, J., and Agerberth, B. (2006). Improved outcome in shigellosis associated with butyrate induction of an endogenous peptide antibiotic. *Proc. Natl. Acad. Sci. U.S.A.* 103, 9178–9183.

Richards, S. M., Strandberg, K. L., and Gunn, J. S. (2010). *Salmonella*-regulated lipopolysaccharide modifications. *Subcell. Biochem.* 53, 101–122.

Rosselin, M., Virlogeux-Payant, I., Roy, C., Bottreau, E., Sizaret, P. Y., Mijouin, L., Germon, P., Caron, E., Velge, P., and Wiedemann, A. (2010). Rck of *Salmonella enterica*, subspecies enterica serovar enteritidis, mediates zipper-like internalization. *Cell Res.* 20, 647–664.

Salzman, N. H., Chou, M. M., de Jong, H., Liu, L., Porter, E. M., and Paterson, Y. (2003). Enteric *Salmonella* infection inhibits Paneth cell antimicrobial peptide expression. *Infect. Immun.* 71, 1109–1115.

Salzman, N. H., Hung, K., Haribhai, D., Chu, H., Karlsson-Sjoberg, J., Amir, E., Teggatz, P., Barman, M., Hayward, M., Eastwood, D., Stoel, M., Zhou, Y., Sodergren, E., Weinstock, G. M., Bevins, C. L., Williams, C. B., and Bos, N. A. (2010). Enteric defensins are essential regulators of intestinal microbial ecology. *Nat. Immunol.* 11, 76–83.

Samudrala, R., Heffron, F., and McDermott, J. E. (2009). Accurate prediction of secreted substrates and identification of a conserved putative secretion signal for type III secretion systems. *PLoS Pathog.* 5, e1000375. doi: 10.1371/journal.ppat.1000375

Sanchez, B., Urdaci, M. C., and Margolles, A. (2010). Extracellular proteins secreted by probiotic bacteria as mediators of effects that promote mucosa-bacteria interactions. *Microbiology* 156, 3232–3242.

Santiviago, C. A., Reynolds, M. M., Porwollik, S., Choi, S. H., Long, F., Andrews-Polymenis, H. L., and McClelland, M. (2009). Analysis of pools of targeted *Salmonella* deletion mutants identifies novel genes affecting fitness during competitive infection in mice. *PLoS Pathog.* 5, e1000477. doi: 10.1371/journal.ppat.1000477

Santos, R. L., Raffatellu, M., Bevins, C. L., Adams, L. G., Tukel, C., Tsolis, R. M., and Baumler, A. J. (2009). Life in the inflamed intestine, *Salmonella* style. *Trends Microbiol.* 17, 498–506.

Sartor, R. B. (2008). Microbial influences in inflammatory bowel diseases. *Gastroenterology* 134, 577–594.

Sassetti, C. M., Boyd, D. H., and Rubin, E. J. (2001). Comprehensive identification of conditionally essential genes in mycobacteria. *Proc. Natl. Acad. Sci. U.S.A.* 98, 12712–12717.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., Jones, J. L., and Griffin, P. M. (2011). Foodborne illness acquired in the United States – major pathogens. *Emerg. Infect. Dis.* 17, 7–15.

Sekirov, I., Gill, N., Jogova, M., Tam, N., Robertson, M., de Llanos, R., Li, Y., and Finlay, B. B. (2010). *Salmonella* SPI-1-mediated neutrophil recruitment during enteric colitis is associated with reduction and alteration in intestinal microbiota. *Gut Microbes* 1, 30–41.

Sekirov, I., Tam, N. M., Jogova, M., Robertson, M. L., Li, Y., Lupp, C., and Finlay, B. B. (2008). Antibiotic-induced perturbations of the intestinal microbiota alter host susceptibility to enteric infection. *Infect. Immun.* 76, 4726–4736.

Slauch, J. M., Mahan, M. J., and Mekalanos, J. J. (1994). In vivo expression technology for selection of bacterial genes specifically induced in host tissues. *Meth. Enzymol.* 235, 481–492.

Smith, A. M., Heisler, L. E., St Onge, R. P., Farias-Hesson, E., Wallace, I. M., Bodeau, J., Harris, A. N., Perry, K. M., Giaever, G., Pourmand, N., and Nislow, C., (2010). Highly-multiplexed barcode sequencing: an efficient method for parallel analysis of pooled samples. *Nucleic Acids Res.* 38, e142.

Smith, J. N., and Ahmer, B. M. (2003). Detection of other microbial species by *Salmonella*: expression of the SdiA regulon. *J. Bacteriol.* 185, 1357–1366.

Smith, J. N., Dyszel, J. L., Soares, J. A., Ellermeier, C. D., Altier, C., Lawhon, S. D., Adams, L. G., Konjufca, V., Curtiss, R. III, Slauch, J. M., and Ahmer, B. M. (2008). SdiA, an *N*-acylhomoserine lactone receptor, becomes active during the transit of *Salmonella enterica* through the gastrointestinal tract of turtles. *PLoS ONE* 3, e2826. doi: 10.1371/journal.pone.0002826

Soares, J. A., and Ahmer, B. M. (2011). Detection of acyl-homoserine lactones by *Escherichia* and *Salmonella*. *Curr. Opin. Microbiol.* 14, 188–193.

Song, J., Willinger, T., Rongvaux, A., Eynon, E. E., Stevens, S., Manz, M. G., Flavell, R. A., and Galán, J. E. (2010). A mouse model for the human pathogen *Salmonella typhi*. *Cell Host Microbe* 8, 369–376.

Stecher, B., Barthel, M., Schlumberger, M. C., Haberli, L., Rabsch, W., Kremer, M., and Hardt, W. D. (2008). Motility allows *S.* Typhimurium to benefit from the mucosal defence. *Cell. Microbiol.* 10, 1166–1180.

Stecher, B., and Hardt, W. D. (2011). Mechanisms controlling pathogen colonization of the gut. *Curr. Opin. Microbiol.* 14, 82–91.

Stecher, B., Robbiani, R., Walker, A. W., Westendorf, A. M., Barthel, M., Kremer, M., Chaffron, S., Macpherson, A. J., Buer, J., Parkhill, J., Dougan, G., von Mering, C., Hardt, W. D. (2007). *Salmonella enterica* serovar typhimurium exploits inflammation to compete with the intestinal microbiota. *PLoS Biol.* 5, 2177–2189. doi: 10.1371/journal.pbio.0050244

Suzuki, K., Wang, X., Weilbacher, T., Pernestig, A. K., Melefors, O., Georgellis, D., Babitzke, P., and Romeo, T. (2002). Regulatory circuitry of the

CsrA/CsrB and BarA/UvrY systems of *Escherichia coli*. *J. Bacteriol.* 184, 5130–5140.

Tam, M. A., Rydstrom, A., Sundquist, M., and Wick, M. J. (2008). Early cellular responses to *Salmonella* infection: dendritic cells, monocytes, and more. *Immunol. Rev.* 225, 140–162.

Teplitski, M., Al-Agely, A., and Ahmer, B. M. (2006). Contribution of the SirA regulon to biofilm formation in *Salmonella enterica* serovar Typhimurium. *Microbiology* 152, 3411–3424.

Tsolis, R. M., Xavier, M. N., Santos, R. L., and Bäumler, A. J. (2011). How to become a top model: The impact of animal experimentation on human *Salmonella* disease research. *Infect. Immun.* 79, 1806–1814.

Turnbaugh, P. J., and Gordon, J. I. (2009). The core gut microbiome, energy balance and obesity. *J. Physiol. (Lond.)* 587, 4153–4158.

Vacharaksa, A., and Finlay, B. B. (2010). Gut microbiota: metagenomics to study complex ecology. *Curr. Biol.* 20, R569–R571.

Valdivia, R. H., and Falkow, S. (1996). Bacterial genetics by flow cytometry: rapid isolation of *Salmonella typhimurium* acid-inducible promoters by differential fluorescence induction. *Mol. Microbiol.* 22, 367–378.

Valdivia, R. H., and Falkow, S. (1997). Fluorescence-based isolation of bacterial genes expressed within host cells. *Science* 277, 2007–2011.

Valdivia, R. H., Hromockyj, A. E., Monack, D., Ramakrishnan, L., and Falkow, S. (1996). Applications for green fluorescent protein (GFP) in the study of host-pathogen interactions. *Gene,* 173, 47–52.

van der Waaij, D., Berghuis-de Vries, J. M., and Lekkerkerk, L.-V. (1971). Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. *J. Hyg. (Lond.)* 69, 405–411.

van Opijnen, T., Bodi, K. L., and Camilli, A. (2009). Tn-seq: high-throughput parallel sequencing for fitness and genetic interaction studies in microorganisms. *Nat. Methods* 6, 767–772.

Vazquez-Torres, A., and Fang, F. C. (2001). *Salmonella* evasion of the NADPH phagocyte oxidase. *Microbes Infect.* 3, 1313–1320.

Veldhuizen, E. J., Koomen, I., Ultee, T., van Dijk, A., and Haagsman, H. P. (2009). *Salmonella* serovar specific upregulation of porcine defensins 1 and 2 in a jejunal epithelial cell line. *Vet. Microbiol.* 136, 69–75.

Ventura, M., Turroni, F., Canchaya, C., Vaughan, E. E., O'Toole, P. W., and van Sinderen, D. (2009). Microbial diversity in the human intestine and novel insights from metagenomics. *Front. Biosci.* 14, 3214–3221.

Wang, Y., Antonopoulos, D. A., Zhu, X., Harrell, L., Hanan, I., Alverdy, J. C., Meyer, F., Musch, M. W., Young, V. B., and Chang, E. B. (2010). Laser capture microdissection and metagenomic analysis of intact mucosa-associated microbial communities of human colon. *Appl. Microbiol. Biotechnol.* 88, 1333–1342.

Willing, B. P., Dicksved, J., Halfvarson, J., Andersson, A. F., Lucio, M., Zheng, Z., Järnerot, G., Tysk, C., Jansson, J. K., and Engstrand, L. (2010). A pyrosequencing study in twins shows that gastrointestinal microbial profiles vary with inflammatory bowel disease phenotypes. *Gastroenterology* 139, 1844. e1–1854.e1.

Winter, S. E., Thiennimitr, P., Winter, M. G., Butler, B. P., Huseby, D. L., Crawford, R. W., Russell, J. M., Bevins, C. L., Adams, L. G., Tsolis, R. M., Roth, J. R., and Bäumler, A. J. (2010). Gut inflammation provides a respiratory electron acceptor for *Salmonella*. *Nature* 467, 426–429.

Wolfe, A. J. (2005). The acetate switch. *Microbiol. Mol. Biol. Rev.* 69, 12–50.

Wong, J. M., de Souza, R., Kendall, C. W., Emam, A., and Jenkins, D. J. (2006). Colonic health: fermentation and short chain fatty acids. *J. Clin. Gastroenterol.* 40, 235–243.

Yang, D., Chertov, O., and Oppenheim, J. J. (2001). Participation of mammalian defensins and cathelicidins in anti-microbial immunity: receptors and activities of human defensins and cathelicidin (LL-37). *J. Leukoc. Biol.* 69, 691–697.

Young, G. M., and Miller, V. L. (1997). Identification of novel chromosomal loci affecting *Yersinia enterocolitica* pathogenesis. *Mol. Microbiol.* 25, 319–328.

Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature* 415, 389–395.

Zhou, D., and Galan, J. (2001). *Salmonella* entry into host cells: the work in concert of type III secreted effector proteins. *Microbes Infect.* 3, 1293–1298.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 16 March 2011; paper pending published: 04 April 2011; accepted: 25 April 2011; published online: 06 May 2011.

Citation: Ahmer BMM and Gunn JS (2011) Interaction of Salmonella spp. with the intestinal microbiota. Front. Microbio. ***2****:101. doi: 10.3389/fmicb.2011.00101*

This article was submitted to Frontiers in Cellular and Infection Microbiology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 03 June 2011
doi: 10.3389/fmicb.2011.00125

Salmonella – at home in the host cell

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The Gram-negative bacterium *Salmonella enterica* has developed an array of sophisticated tools to manipulate the host cell and establish an intracellular niche, for successful propagation as a facultative intracellular pathogen. While *Salmonella* exerts diverse effects on its host cell, only the cell biology of the classic "trigger"-mediated invasion process and the subsequent development of the *Salmonella*-containing vacuole have been investigated extensively. These processes are dependent on cohorts of effector proteins translocated into host cells by two type III secretion systems (T3SS), although T3SS-independent mechanisms of entry may be important for invasion of certain host cell types. Recent studies into the intracellular lifestyle of *Salmonella* have provided new insights into the mechanisms used by this pathogen to modulate its intracellular environment. Here we discuss current knowledge of *Salmonella*-host interactions including invasion and establishment of an intracellular niche within the host.

Keywords: effectors, invasion, membrane tubules, phagosome, type III secretion system, vacuole

INTRODUCTION

Salmonella enterica are facultative intracellular pathogens that are found in the gastrointestinal tract of mammalian, avian, and reptilian hosts. These Gram-negative bacteria are highly versatile and can adapt to a wide range of conditions both in the natural environment and within host organisms. While there are more than 2,500 *S. enterica* serovars only a few are commonly associated with disease in mammals. In humans, *Salmonella* are primarily associated with either localized intestinal infection or severe systemic disease. *Salmonella* gastroenteritis is usually self-resolving in healthy adults. It is one of the most common causes of foodborne disease, possibly affecting over 90 million people globally each year (Majowicz et al., 2010), and can be caused by many serovars although the most common are serovars Typhimurium and Enteritidis. Systemic disease in healthy humans (typhoid) is caused by serovar Typhi and a handful of other serovars that are strictly adapted to humans and higher primates. Immunocompromised individuals, such as those with AIDS or cancer, often develop systemic salmonellosis when infected with non-typhoidal *Salmonella* serovars (Gordon, 2008).

The interplay between *Salmonella* and its vertebrate hosts is complex and involves a variety of virulence factors, although two of the most important are the type III secretion systems 1 and 2 (T3SS1 and T3SS2). Together these are used to inject over 30 effector proteins into the cytoplasm of host cells where they act on a variety of pathways. In epithelial cells, T3SS effectors are essential for both invasion and the subsequent establishment of the intracellular niche by *Salmonella* (**Figure 1**). The intracellular niche is a modified phagosome, known as the *Salmonella*-containing vacuole (SCV), which undergoes extensive T3SS effector-dependent membrane remodeling. This review focuses on how *Salmonella* establish their intracellular niche in epithelial cells with particular emphasis on invasion and SCV biogenesis.

SALMONELLA TYPE III SECRETION SYSTEMS

Type III secretion systems are sophisticated contact-dependent delivery systems used by many Gram-negative bacterial pathogens to inject bacterial effector proteins into host cells. These nanoinjection systems consist of 20–30 proteins, many of which have homology to proteins in the flagellar export apparatus [for review (Marlovits and Stebbins, 2010)]. While all T3SSs are structurally similar, the effectors secreted by these delivery systems are extremely diverse (Samudrala et al., 2009). T3SS1 and T3SS2 are encoded on different regions of the chromosome, known as *Salmonella* pathogenicity islands 1 and 2 (SPI1 and SPI2) respectively, and are functionally and temporally distinct. The SPI1-encoded T3SS1 translocates a cohort of effectors that drive "trigger"-mediated invasion of host cells whereas the SPI2-encoded T3SS2 is induced after invasion and is required for modulation of the intracellular environment. Nevertheless, it is now apparent that some overlap exists and effectors from both systems mediate biogenesis of the SCV (Hernandez et al., 2004; Drecktrah et al., 2005; Lawley et al., 2006; Brawn et al., 2007).

SALMONELLA ENTRY

In vivo, *Salmonella* can be found in a variety of phagocytic and non-phagocytic cells, including macrophages, dendritic cells, neutrophils, M cells, and enterocytes (Wallis et al., 1986; Jones et al., 1994; Richter-Dahlfors et al., 1997; Rescigno et al., 2001; Salcedo et al., 2001; Meyerholz et al., 2002; Geddes et al., 2007). Bacterial internalization, whether by phagocytosis or *Salmonella*-mediated invasion, involves actin remodeling at its core, which results in formation of plasma membrane extensions and ingestion of the target particle into the membrane bound phagosome. Common features of this process are the involvement of Rho family GTPases and phosphoinositides, which are instrumental in actin remodeling, membrane trafficking and signal transduction. The Rho

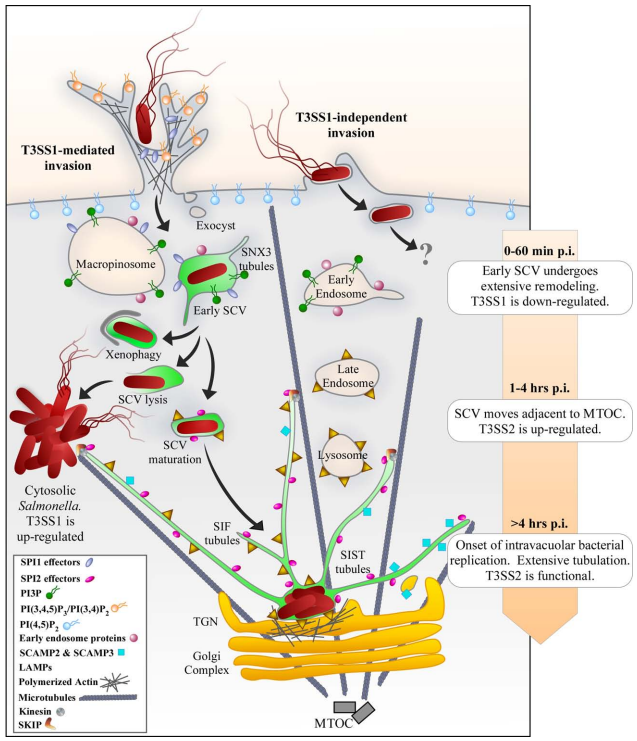

FIGURE 1 | Biogenesis of the SCV. Invasive *Salmonella* use T3SS1 to translocate effector proteins into host cells. Several of these effectors drive actin-mediated ruffling and internalization of the bacteria into a modified phagosome or SCV. T3SS1 effectors are also present on the SCV membrane and are important for rapid remodeling of the membranes as well as more sustained effects. The early SCV has many characteristics of early endosomes, including the phospholipid PI(3)P and proteins that interact with it such as Rab5 and SNX1. Dynamic tubular networks containing SNX1 or SNX3 are involved in membrane remodeling during this early stage in SCV biogenesis. During this initial phase of infection the majority of *Salmonella* down-regulate T3SS1 and induce T3SS2, which is required for subsequent steps in SCV biogenesis. The majority of SCVs relocate to a juxtanuclear location within 1–2 h and become enriched in proteins, such as Lamp1, Rab7, and vacuolar ATPase, that are normally found in late endosomes and lysosomes. However, some SCVs do not undergo this maturation process and instead either lyse and release the bacteria into the cytosol or are targeted by the autophagy system. In the mature SCV replication is initiated 4–6 h post invasion and is accompanied by the formation of a dynamic tubular network that extends from the surface of the SCV. Tubules enriched in Lamp1 are known as Sifs although another population of Lamp1-ve tubules (SISTs) has recently been described. In epithelial cells cytosolic *Salmonella* replicate to high numbers, compared to bacteria inside SCVs, and become re-induced for T3SS1 and flagella. *Salmonella* can also invade cells via T3SS1-independent mechanisms (right side), although biogenesis of the SCV under these conditions has not been well studied.

GTPases, Cdc42, and Rac1, are required for the initiation of actin polymerization during Fc gamma receptor-mediated phagocytosis as well as for *Salmonella* invasion (Zhou and Galan, 2001; Hoppe and Swanson, 2004; Scott et al., 2005). Remodeling of F-actin during phagocytosis is also dependent on phosphatidylinositol (4,5) bisphosphate [PI(4,5)P_2] a phospholipid with multiple roles in the nucleation, elongation, and bundling of actin filaments (Hilpela et al., 2004; Scott et al., 2005). Local changes in membrane phospholipid composition can also affect the net charge on the cytoplasmic surface of membranes resulting in the selective recruitment of proteins such as members of the Rho family of GTPases (Magalhaes and Glogauer, 2010).

Bacterial internalization is accompanied by changes in host cell signaling pathways, affecting a number of vital cellular processes, including membrane trafficking, cell division, apoptosis, microbial killing, cytokine production, and antigen presentation. The ultimate fate of intracellular *Salmonella* is determined by a complex interplay of both host and bacterial factors. Here we focus on the entry methods employed by *Salmonella* invasion of non-phagocytic epithelial cells.

T3SS1-DEPENDENT INVASION

Type III secretion systems 1-mediated invasion of non-phagocytic cells by *Salmonella* has been extensively studied. Morphologically, this "trigger"-mediated invasion process is characterized by the extremely rapid appearance of membrane ruffles on the surface of the host cell, and subsequent formation of spacious phagosomes/vacuoles, or macropinosomes, which may or may not contain bacteria. These events are mediated by a subset of T3SS1 effectors (SipA, SipC, SopB, SopE, SopE2) that act in concert to induce massive localized rearrangements of actin and the plasma membrane as well as activating signaling pathways [for review (McGhie et al., 2009; Dunn and Valdivia, 2010)]. While SipA and SipC directly bind actin (McGhie et al., 2001; Hayward and Koronakis, 2002), SopB, SopE, and SopE2 indirectly modulate actin activity by stimulating Rho family GTPases, which are common targets of bacterial T3SS effectors.

SipA stabilizes actin filaments by enhancing the localized actin-bundling activity of T-plastin (Zhou et al., 1999) while also inhibiting the filament-severing activities of cofilin and gelsolin (McGhie et al., 2004). SipC can independently cause actin rearrangements via its distinct actin-bundling and actin-nucleating domains (Hayward and Koronakis, 1999; Myeni and Zhou, 2010). In addition to modulating actin, SipC interacts directly with Exo70, a component of the exocyst complex, which mediates docking and fusion of exocytic vesicles with the plasma membrane (Nichols and Casanova, 2010). SopB (also known as SigD) is a phosphoinositide phosphatase, which can hydrolyse a number of phosphoinositides and inositol phosphates *in vitro* and has diverse effects on host cells (Norris et al., 1998; Steele-Mortimer et al., 2000; Knodler et al., 2005; Dukes et al., 2006; Patel and Galan, 2006; Dai et al., 2007; Bujny et al., 2008; Mallo et al., 2008; Patel et al., 2009; Bakowski et al., 2010; Braun et al., 2010). During invasion, SopB participates in actin remodeling by activating RhoG (Patel and Galan, 2006). In its active state RhoG recruits the ELMO–Dock180 complex to the membrane to promote Rac activation and has been implicated in membrane ruffling and engulfment (Gauthier-Rouviere et al., 1998; Katoh and Negishi, 2003). SopB does not directly activate RhoG but instead targets a guanine-nucleotide exchange factor (GEF), known as SH3-containing GEF (SGEF) that promotes the exchange of GDP for GTP on RhoG (Patel and Galan, 2006). Although SopB phosphatase activity is required for SGEF activation the mechanism remains undefined (Raffatellu et al., 2005; Patel and Galan, 2006).

Whereas SopB activates a GEF, SopE and SopE2 act by mimicking mammalian GEFs. These effectors are members of a family of bacterial GEFs, which bind to Rho GTPases including Rac1 and Cdc42, and activate them by catalyzing exchange of GDP for GTP (Hardt et al., 1998; Stevens et al., 2003). SopE and SopE2 are about 70% identical (Stender et al., 2000), and have different specificities and distribution amongst *S. enterica* serovars. SopE2 appears to be specific for Cdc42 whereas SopE acts on Cdc42 and Rac1. Interestingly, the requirement for Cdc42, but not Rac1, is dispensable for *Salmonella* invasion in some model systems (Criss et al., 2001; Patel and Galan, 2006).

Recent work suggests that SopE can also activate RalA, a GTPase that is required for assembly of the exocyst (Nichols and Casanova, 2010) and has been implicated in Fc gamma receptor-mediated phagocytosis (Corrotte et al., 2010). Thus SopE and SipC together appear to direct fusion of exocytic vesicles with the plasma membrane at the site of entry, presumably as a source of membrane for the expanding ruffle or phagocytic cup (Nichols and Casanova, 2010). SopB may also stimulate membrane fusion at the site of entry by increasing local levels of PI(3)P resulting in the plasma membrane recruitment of VAMP8, a member of the endosomal synaptobrevin/VAMP family that mediates homotypic fusion of early endosomes (EE; Pryor et al., 2004; Dai et al., 2007). Thus, *Salmonella* T3SS1-mediated invasion may involve fusion of several intracellular sources of membrane at the site of entry.

In the context of this review, it is worth mentioning several non-receptor kinases implicated in T3SS1-induced actin reorganization and/or downstream signaling. These include Abl, Ack, and the focal adhesin kinase FAK (Murli et al., 2001; Shi and Casanova, 2006; Ly and Casanova, 2009). The signal from these kinases is further mediated to the actin cytoskeleton by scaffold proteins such as p130Cas/BCAR1 (Shi and Casanova, 2006), Shank3 (Huett et al., 2009), and IQGAP1 (Brown et al., 2007), during *Salmonella* invasion. p130Cas/BCAR1 is a member of the Crk-associated substrate (Cas) scaffolding protein family that interacts with FAK (Petch et al., 1995; Schuetz et al., 2004) and is involved in membrane ruffling (Sharma and Mayer, 2008). Intriguingly, IQGAP1 also interacts with Exo70 and it has been implicated in exocyst localization and exocytosis (Rittmeyer et al., 2008).

T3SS1-INDEPENDENT INVASION

Type III secretion systems-independent mechanisms may be important for invasion of specific cell types or under certain conditions (Heffernan et al., 1992b; Aiastui et al., 2010; Radtke et al., 2010; Rosselin et al., 2010). In the absence of T3SS1 at least two non-fimbrial outer membrane proteins (Omps), Rck and PagN, can mediate zipper-like invasion of non-phagocytic cells. Both of these Omps, when expressed in non-invasive *E. coli* strains, confer the ability to bind to and invade fibroblastic, epithelial, and endothelial cells, although the efficiency of invasion is cell type dependent (Heffernan et al., 1992b; Lambert and Smith, 2008, 2009; Rosselin et al., 2010). Rck is a 19 kDa member of a family of virulence-associated Omps expressed in Gram-negative bacteria, which promote internalization in epithelial cells and internalization and survival in macrophages (Heffernan et al., 1992a; Cirillo et al., 1996). Rck-mediated entry is dependent upon the Arp2/3 complex and Rac1 and Cdc42 which mobilize actin rearrangements resulting in membrane ruffling and bacterial uptake (Unsworth et al., 2004; Rosselin et al., 2010). PagN is a 26 kDa protein with similarity to the known invasins/adhesins, Hek and Tia, of pathogenic *E. coli* (Lambert and Smith, 2008). The binding of PagN to cell surface heparin sulfate proteoglycans mediates both adhesion and invasion of *Salmonella* (Lambert and Smith, 2009).

Each *Salmonella* serovar has over a dozen adhesin gene clusters many of which are implicated in pathogenesis (Edwards et al., 2002; Clayton et al., 2008). Included in this group are distinct types of fimbriae (type I fimbriae, plasmid-encoded fimbriae, long polar fimbriae, thin aggregative fimbriae) and non-fimbrial adhesins (ShdA and SiiE). While type I fimbriae can mediate binding to epithelial cells, they also help induce actin-dependent uptake in the absence of T3SS1 (Misselwitz et al., 2010). SiiE is a giant adhesin that is required for efficient T3SS1-mediated invasion into polarized epithelial cells but not into non-polarized epithelial cells or fibroblasts (Gerlach et al., 2008).

Although T3SS1-mediated invasion has been the center of attention for over a decade the recent use of alternative *in vitro* model systems have made it evident that *Salmonella* can use multiple ways to invade non-phagocytic host cells. It is not clear why *Salmonella* has developed alternate mechanisms for cellular invasion, but it is hypothesized that these Omps and/or adhesins when used in conjunction with T3SS1-mediated invasion may help provide cell and host specificity. While none of these mechanisms rival T3SS1-mediated invasion in complexity and efficiency they may certainly play an important roles in *Salmonella* pathogenesis.

LIFE IN A VACUOLE

Following internalization into the host cell *Salmonella* are located in the SCV, a vacuolar niche modified and adapted by the bacteria via the activities of both T3SS1 and T3SS2 effectors (**Figure 1**). The newly formed SCV is enriched in EE proteins, such as EEA1, which are subsequently replaced by late endosomal/lysosomal (LE/lys) markers, such as the lysosomal glycoprotein Lamp1 (Garcia-del Portillo and Finlay, 1995; Steele-Mortimer et al., 1999). In cultured epithelial cells, the SCV migrates from the cell periphery to a juxtanuclear position within 1–2 h following invasion (Abrahams et al., 2006; Ramsden et al., 2007). Bacterial replication is initiated approximately 4–6 h post invasion and is accompanied by the extension of Lamp1 enriched membrane tubules (Sifs) from the surface of the SCV (Garcia-del Portillo et al., 1993). T3SS1 effectors play an important role, not only in invasion but also in early stages of SCV biogenesis. In contrast T3SS2, which is induced intracellularly following invasion, is required for later events in SCV biogenesis including the onset of bacterial replication and Sif formation (Waterman and Holden, 2003). Recent studies, regarding the roles of T3SS effectors and host proteins in SCV biogenesis, have provided new insights into the mechanisms used by *Salmonella* to establish its intracellular niche. Here we highlight some of these findings and discuss their significance. For convenience, SCV biogenesis is separated into three stages: early, intermediate, and late.

EARLY VACUOLE DEVELOPMENT – T3SS1 EFFECTORS, SPACIOUS VACUOLES, AND TUBULES

Immediately after formation, the SCV undergoes rapid membrane remodeling (**Figure 1**) predominantly driven by the activities of the T3SS1 effector SopB, which is translocated during entry and then persists in the host cell for several hours following invasion (Kubori and Galan, 2003; Drecktrah et al., 2005). During initial contact with the host plasma membrane, the phosphoinositide phosphatase activity of SopB manipulates plasma membrane phosphoinositides to activate Akt via $PI(3,4)P_2$ and/or $PI(3,4,5)P_3$ (Steele-Mortimer et al., 2000) and also drives macropinosomes/phagosome formation via removal of $PI(4,5)P_2$ (Terebiznik et al., 2002). Thereafter, SopB located on the cytosolic face of the SCV directs an increase in PI(3)P, a phosphoinositide normally associated with EE, and a decrease in $PI(4,5)P_2$ (Hernandez et al., 2004; Mallo et al., 2008; Bakowski et al., 2010). SopB-dependent acquisition of PI(3)P is not directly driven by SopB phosphatase activity but rather a result of SopB-dependent recruitment of the small GTPase Rab5 to the SCV membrane (Hernandez et al., 2004; Mallo et al., 2008). As a consequence the Rab5 interacting protein Vps34 is also recruited, and it is the activity of this type III PI3K that generates PI(3)P in the SCV membrane (Mallo et al., 2008). SopB activity is also required for reducing levels of negatively charged $PI(4,5)P_2$ and phosphatidylserine from the SCV, which results in the dissociation of several Rab proteins from the SCV and may serve to delay SCV-lysosome fusion (Hernandez et al., 2004; Mallo et al., 2008; Bakowski et al., 2010).

Phosphoinositide binding proteins, such as the sorting nexins (SNX), are important regulators of membrane trafficking. The SNX protein family comprises over 30 members, each of which contains a Phox homology (SNX-PX) domain that binds to membrane phosphoinositides (Worby and Dixon, 2002). Two SNX proteins, SNX1 and SNX3, participate in early SCV biogenesis in a SopB-dependent manner (Bujny et al., 2008; Braun et al., 2010). Many SNX proteins, including SNX1, also contain a BAR (Bin-amphiphysin-Rvs) domain, which can sense or induce membrane curvature (Van Weering et al., 2010). In SNX1 the PX and BAR domains, co-operatively target the protein to specific parts of the EE, enriched in PI(3)P and $PI(3,5)P_2$, where they promote tubule formation (Carlton et al., 2004). SNX1 is a member of the retromer sorting complex that mediates retrieval of receptors from the endolysosomal pathway to the TGN, a process that is believed to be mediated by membrane tubules (Carlton et al., 2004). During *Salmonella* invasion of epithelial cells SNX1 first accumulates in the proximity of membrane ruffles and then, within 15 min post invasion, localizes to tubules that emanate from the SCV and are associated with vacuolar shrinking (Bujny et al., 2008). In SNX1 depleted cells, movement of the SCV to the juxtanuclear region and onset of replication are delayed and the cation-independent mannose-6-phosphate receptor (CI M6PR) accumulates on SCVs (Bujny et al., 2008). CI M6PR is used for delivery of soluble lysosomal enzymes to lysosomes and is normally excluded from SCVs, this has been interpreted as evidence for lack of lysosomal fusion with the SCV (Garcia-del Portillo and Finlay, 1995). An alternative explanation for the exclusion of CI M6PR from the SCV is that CI-MPR is recruited to the nascent SCV but is then efficiently removed in a SNX1-dependent manner. Together with studies showing delivery of lysosomal content markers to the SCV this supports a model in which SCVs can fuse with lysosomes during biogenesis (Oh et al., 1996; Drecktrah et al., 2007).

Unlike SNX1, SNX3 does not contain a BAR domain or interact with retromer. However, it also localizes to the endocytic pathway and has been shown to function in sorting and membrane invagination within multivesicular bodies (Pons et al., 2008; Xu and Hensel, 2010). In *Salmonella*-infected cells, SNX3 transiently localizes to SCVs at early times post invasion (10 min) and thereafter to membrane tubules formed 30–60 min post invasion (Braun et al., 2010). In SNX3 depleted cells, delivery of Rab7 and Lamp1 to the SCV is impeded, indicating a requirement for this SNX in SCV maturation (Braun et al., 2010). Since SopB is required for formation of both SNX1 and SNX3 tubules (Bujny et al., 2008; Braun et al., 2010), modulation of phosphoinositides in the SCV membrane by this T3SS1 effector appears to be a prerequisite for SCV biogenesis.

Another T3SS1 protein implicated in early SCV biogenesis is SptP, a bifunctional protein that functions both as a GTPase activating protein (GAP) and as a tyrosine phosphatase (Fu and Galan, 1999; Kubori and Galan, 2003; Patel and Galan, 2006; Humphreys et al., 2009). The GAP activity down-regulates Cdc42 and Rac1 and is required for termination of membrane ruffling (Fu and Galan, 1999; Kubori and Galan, 2003; Patel and Galan, 2006). Subsequently, SptP dephosphorylates valosin-containing protein (VCP), a member of the AAA protein family that functions in a variety of physiological processes (Humphreys et al., 2009). However, while VCP and its adaptors (p47 and Ufd1) are required for efficient SCV maturation their functions during this process have not been elucidated.

INTERMEDIATE VACUOLE DEVELOPMENT – A QUESTION OF POSITION

In common with many other intracellular pathogens, including *Chlamydia* and a variety of viruses, *Salmonella* use dynein-mediated transport along microtubules to reach a juxtanuclear position adjacent to the microtubule organizing center (MTOC). It is hypothesized that this location is important for access to a supply of cellular and/or pathogen components delivered by dynein motors traveling along microtubules (Wileman, 2007). Movement of the SCV to the MTOC, and maintenance at that position, are multi-factorial processes involving at least three T3SS2 effectors (SseF, SseG, and SifA) as well as two T3SS1 effectors (SipA and SopB; Salcedo and Holden, 2003; Abrahams et al., 2006; Deiwick et al., 2006; Brawn et al., 2007; Wasylnka et al., 2008). *Salmonella* strains deficient in *sseF* or *sseG* display a predominantly scattered distribution and recruitment of the dynein motor complex to the SCV requires SseF (Abrahams et al., 2006). Although the mechanism of action of these two effectors remains undetermined, they form a structural and functional interaction that is required for maintenance of the juxtanuclear position (Deiwick et al., 2006). Intriguingly, the actin based motor, non-muscle myosin II, is also implicated in SCV positioning (Wasylnka et al., 2008). Again this process is not well understood, however, myosin II activity may be modulated locally on the SCV via a process involving SopB phosphatase activity and activation of the Rho kinase (ROCK; Wasylnka et al., 2008). How the myosin-actin and

dynein-microtubule driven processes complement or overlap with one another in SCV relocation has not been addressed.

LATE VACUOLE DEVELOPMENT – HOLDING STILL AND EXTENDING

During the later stages of infection (>6 h post invasion) SCVs are maintained at the MTOC/juxtanuclear region through the actions of effectors that modulate both microtubule and actin based transport (Abrahams et al., 2006; Deiwick et al., 2006). At the same time membrane tubules formed on the cytosolic surface of the SCV extend rapidly along microtubules toward the cell periphery (Drecktrah et al., 2008). To maintain the position of the SCV while simultaneously generating a dynamic tubular network, the bacteria uses a set of effector proteins that exhibit both co-operative and antagonistic activities.

Undoubtedly the best understood T3SS2 effector is SifA, which is required for the formation of Sif tubules (Stein et al., 1996), the most prominent tubular structures induced by *Salmonella*. Sif formation involves at least two host cell proteins; the plus end directed microtubule motor kinesin-1 and the kinesin-binding protein SKIP (SifA and kinesin-interacting protein; Boucrot et al., 2005; Dumont et al., 2010). Kinesin recruitment to the SCV and its subsequent activation are critical steps in late SCV maturation [for review see (Leone and Meresse, 2011)]. Recruitment of kinesin does not require SifA, but is instead driven by the T3SS2 effector PipB2, although subsequently, SifA modulates the level of kinesin on the SCV via interaction with SKIP (Henry et al., 2006). In the absence of SifA or SKIP, SCVs accumulate excess kinesin and the membrane becomes compromised resulting in release of the bacteria into the cytoplasm (Beuzon et al., 2000; Dumont et al., 2010). In uninfected mammalian cells SKIP binds to the GTP-bound active form of Rab9, a small GTPase localized to endosomes, and is involved in positioning of LE/Lys (Dumont et al., 2010). In infected cells, SifA prevents the interaction of SKIP with Rab9 and, since SKIP specifically interacts with active GTP-bound Rab9, may antagonize Rab9 activity (Jackson et al., 2008). Alternatively, by binding SKIP, SifA may increase the amount of GTP-Rab9 that is available to interact with an alternate target. Rab9 normally regulates retrograde transport from late endosomes to the TGN but it has also been implicated in the formation of lysosome related organelles (LRO), such as melanosomes and platelet dense bodies (Kloer et al., 2010). During LRO formation GTP-Rab9 interacts with a protein complex (BLOC-3; Kloer et al., 2010), and perhaps a similar mechanism is involved in the formation of Sifs, which could also be considered a form of LRO.

Another effector that co-operates with SifA in late SCV biogenesis is the T3SS1 effector SipA. In cells infected with *Salmonella* lacking SipA, SifA is mislocalized and SCVs are redistributed to the cell periphery (Brawn et al., 2007). The SCV positioning activity of SipA is located in the N-terminal domain of the protein and when expressed exogenously in epithelial cells this domain can associate with LE/Lys and promote clustering in the juxtanuclear region (Brawn et al., 2007). In addition, the actin binding activity of SipA, which is located in the C-terminal domain, also has a role in late SCV biogenesis, being required for accumulation of F-actin around the SCV (Meresse et al., 2001; Guignot et al., 2004). This SipA-dependent actin accumulation is likely a result of its ability to inhibit actin disassembly (McGhie et al., 2004). Indeed, depolymerization of the actin cytoskeleton results in redistribution of SCVs toward the cell periphery, although the exact role of actin accumulation around the SCV has not been well studied (Wasylnka et al., 2008).

Another T3SS2 effector protein involved in SCV/Sif biogenesis and stability is SseJ, a protein with some homology to glycerophospholipid:cholesterol acyltransferases (GCATs; Lossi et al., 2008). These enzymes are involved in cholesterol esterification and lipid body formation. SseJ can esterify cholesterol *in vitro*, in cells and during infection and SCVs and Sifs are enriched in cholesterol (Nawabi et al., 2008). Activation of SseJ depends upon binding GTP-bound RhoA and, although many bacterial effectors are known to manipulate GTPases, this is the first instance of an effector being regulated by the activity of a host GTPase (Christen et al., 2009). Intriguingly, SifA can interact specifically with GDP-bound RhoA, acting as a GEF, and it is possible that SifA, SKIP, SseJ, and RhoA co-operatively promote host membrane tubulation (Ohlson et al., 2008; Vinh et al., 2010). In *S. enterica* serovar Typhi, the causative agent of human typhoid fever, *sseJ* is a pseudogene. Surprisingly, when Typhi is trans-complemented with the functional Typhimurium *sseJ* gene there is a significant decrease in the level of toxicity of the organism to cultured epithelial cells (Trombert et al., 2010). This finding suggests that perhaps loss of SseJ activity is one factor that has contributed to the development of Typhi as an organism associated with systemic disease.

Live-cell imaging studies at later time points (>6 h post invasion) have revealed that formation of *Salmonella*-induced tubules may involve interactions with the secretory as well as endolysosomal pathways. Thus, although the majority of tubules are highly enriched in Lamp1, a subpopulation is characterized by the presence of the secretory carrier membrane proteins SCAMP2 and SCAMP3 (Mota et al., 2009). These large tetraspan proteins are found predominantly on post-Golgi endocytic and exocytic membranes, but can also be found on PI(3)P enriched endosomes and perhaps function in recycling of endocytosed receptors (Singleton et al., 1997; Castle and Castle, 2005; Aoh et al., 2009). *Salmonella*-induced SCAMP3 tubules (SISTs) are observed in close association with the Lamp1 positive Sifs and similarly contain multiple T3SS2 effectors (PipB, PipB2, SifA, SseG, SseJ; Mota et al., 2009). Also described recently are LAMP1 negative tubules (LNTs) that may be precursors to Sifs and SISTs and contribute to membrane stability of the SCV (Schroeder et al., 2010). LNTs have been shown in only low numbers of epithelial cells infected with WT *Salmonella* and it is possible that the T3SS2 effector SopD limits their production under these conditions (Schroeder et al., 2010). The function of all of the *Salmonella*-induced tubules remains unknown, and indeed it is unclear whether any of them are even formed *in vivo*. One possibility is that *Salmonella* might use them to intercept host cell trafficking for nutritional or membrane requirements (Mota et al., 2009).

ESCAPE FROM THE VACUOLE AND THE HOST CELL?

Although the SCV is considered the primary intracellular niche for *Salmonella* the bacteria can also be found in the cytoplasm.

In some cells, such as macrophages, the cytosol is a lethal environment for *Salmonella*, however, in epithelial cells the cytoplasm supports robust growth (Beuzon et al., 2002; Brumell et al., 2002; Knodler et al., 2010). Intriguingly, this cytosolic stage may serve as a critical transition step that precedes egress into the extracellular environment (Knodler et al., 2010), although this vital stage of the host-pathogen relationship is virtually unstudied for *Salmonella*. We have recently addressed this question using a polarized epithelial cell model and found that *Salmonella* take advantage of a host mechanism known as extrusion, which is normally used to remove dead or dying cells during the turnover of the intestinal epithelium (Knodler et al., 2010). The first step in this process seems to be escape from the SCV into the cytoplasm, where bacterial replication far exceeds that occurring within SCVs. The cytoplasmic bacteria also express different virulence genes compared to vacuolar bacteria. Specifically, instead of expressing genes encoding for T3SS2 these bacteria are induced for T3SS1 and flagellar motility. The significance of this is apparent when the infected cell is extruded from the monolayer, and in the process releases a large number of invasive (T3SS1 induced and motile) bacteria. *In vivo* these extruded bacteria may either infect adjacent cells, in order to repeat the intracellular cycle, or be shed into the environment. Thus, although the SCV is an important intracellular niche for *Salmonella*, there may be times when escape from the SCV and growth in the cytosol is just as important for the infectious cycle.

AUTOPHAGY

Autophagy (to eat oneself) is a tightly regulated process that is crucial for normal cell homeostasis and is the major mechanism by which cells reallocate nutrients from non-essential processes to essential processes during starvation. In essence it involves degradation of intracellular components via the lysosome. Eukaryotic cells also use a modified form of autophagy, known as xenophagy (to eat foreign matter), to control and/or degrade intracellular bacteria and viruses. In the case of *Salmonella* autophagy appears to have an important role in controlling escape from the SCV. In infected HeLa cells microtubule-associated protein light chain 3 (LC3), a protein often used to monitor autophagy, as well as the ubiquitin binding adaptor proteins p62 and NDP52 are present on approximately 20% of SCVs 1 h p.i. (Birmingham and Brumell, 2006; Birmingham et al., 2006; Cemma et al., 2011). Subsequently, bacteria are associated with ubiquitinated proteins (Birmingham and Brumell, 2006; Birmingham et al., 2006). The recruitment of LC3 to the SCV seems to involve similar mechanisms as involved in macroautophagy, and is dependent on lipidation of LC3. The lipidation step requires Atg7, Atg3, and Atg5 enzymes and in cells lacking these LC3 recruitment is defective and there is increased cytosolic replication of *Salmonella* (Birmingham et al., 2006; Kageyama et al., 2011). LC3 recruitment is preceeded by the association of an Atg9L1-positive autophagosome-like double membrane structure around the still-intact SCV as early as 30 min after infection (Kageyama et al., 2011). It has been proposed that this isolation membrane, which ultimately becomes the xenophagosome, is formed through an Atg9L1-dependent mechanism, and potentially requires PI3-kinase and the ULK1 complex (Kageyama et al., 2011). The LC3 lipidation machinery is apparently recruited independently of these proteins via an unknown targeting mechanism. According to this model LC3 is then involved in closing the isolation membrane thus forming the xenophagosome (Kageyama et al., 2011). How the autophagy pathway recognizes the SCV is not completely understood although it is dependent on the T3SS1, and it appears that membrane damage caused by the secretion system is an important trigger (Birmingham and Brumell, 2006).

CONCLUSION

The co-evolution of *Salmonella* serovars and their vertebrate hosts has resulted in the development of a tightly interwoven relationship as illustrated by the complexity of host-pathogen interactions seen at a single cell level. The mechanisms by which *Salmonella* invade non-phagocytic vertebrate cells and subsequently establish a replicative environment have been extensively investigated. Many of the molecular interactions between host and pathogen have been elucidated in recent years. Nevertheless, many important questions remain unanswered. How do *Salmonella* avoid host cell defenses such as phagolysosomal fusion? How do *Salmonella* complete the intracellular cycle and ensure release and/or transmission? What determines whether the bacteria escape into the cytosol or get targeted for xenophagy? Most of the emphasis in the last 10–15 years has been on T3SS1-mediated invasion and subsequent SCV biogenesis, however, it is now clear that *Salmonella* can use a variety of mechanisms to invade non-phagocytic cells and that the SCV is not the only intracellular niche. In order to address these questions, and advance our understanding of this fascinating pathogen, we may have to turn to alternate model systems, such as polarized epithelial cultures, and consider T3SS1-independent mechanisms of invasion as well as the canonical T3SS1-mediated invasion.

ACKNOWLEDGMENTS

Work in the author's laboratory is funded by the Intramural Research Program (DIR) of the National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIAID/NIH).

REFERENCES

Abrahams, G. L., Muller, P., and Hensel, M. (2006). Functional dissection of SseF, a type III effector protein involved in positioning the salmonella-containing vacuole. *Traffic* 7, 950–965.

Aiastui, A., Pucciarelli, M. G., and Garcia-Del Portillo, F. (2010). Salmonella enterica serovar typhimurium invades fibroblasts by multiple routes differing from the entry into epithelial cells. *Infect. Immun.* 78, 2700–2713.

Aoh, Q. L., Castle, A. M., Hubbard, C. H., Katsumata, O., and Castle, J. D. (2009). SCAMP3 negatively regulates epidermal growth factor receptor degradation and promotes receptor recycling. *Mol. Biol. Cell* 20, 1816–1832.

Bakowski, M. A., Braun, V., Lam, G. Y., Yeung, T., Heo, W. D., Meyer, T., Finlay, B. B., Grinstein, S., and Brumell, J. H. (2010). The phosphoinositide phosphatase SopB manipulates membrane surface charge and trafficking of the Salmonella-containing vacuole. *Cell Host Microbe* 7, 453–462.

Beuzon, C. R., Meresse, S., Unsworth, K. E., Ruiz-Albert, J., Garvis, S., Waterman, S. R., Ryder, T. A., Boucrot, E., and Holden, D. W. (2000). Salmonella maintains the integrity of its intracellular vacuole through the action of SifA. *EMBO J.* 19, 3235–3249.

Beuzon, C. R., Salcedo, S. P., and Holden, D. W. (2002). Growth and killing of a Salmonella enterica serovar Typhimurium sifA mutant strain in the cytosol of different host cell lines. *Microbiology* 148, 2705–2715.

Birmingham, C. L., and Brumell, J. H. (2006). Autophagy recognizes intracellular Salmonella enterica serovar

Typhimurium in damaged vacuoles. *Autophagy* 2, 156–158.

Birmingham, C. L., Smith, A. C., Bakowski, M. A., Yoshimori, T., and Brumell, J. H. (2006). Autophagy controls Salmonella infection in response to damage to the Salmonella-containing vacuole. *J. Biol. Chem.* 281, 11374–11383.

Boucrot, E., Henry, T., Borg, J. P., Gorvel, J. P., and Meresse, S. (2005). The intracellular fate of Salmonella depends on the recruitment of kinesin. *Science* 308, 1174–1178.

Braun, V., Wong, A., Landekic, M., Hong, W. J., Grinstein, S., and Brumell, J. H. (2010). Sorting nexin 3 (SNX3) is a component of a tubular endosomal network induced by Salmonella and involved in maturation of the Salmonella-containing vacuole. *Cell. Microbiol.* 12, 1352–1367.

Brawn, L. C., Hayward, R. D., and Koronakis, V. (2007). Salmonella SPI1 effector SipA persists after entry and cooperates with a SPI2 effector to regulate phagosome maturation and intracellular replication. *Cell Host Microbe* 1, 63–75.

Brown, M. D., Bry, L., Li, Z., and Sacks, D. B. (2007). IQGAP1 regulates Salmonella invasion through interactions with actin, Rac1, and Cdc42. *J. Biol. Chem.* 282, 30265–30272.

Brumell, J. H., Tang, P., Zaharik, M. L., and Finlay, B. B. (2002). Disruption of the Salmonella-containing vacuole leads to increased replication of Salmonella enterica serovar typhimurium in the cytosol of epithelial cells. *Infect. Immun.* 70, 3264–3270.

Bujny, M. V., Ewels, P. A., Humphrey, S., Attar, N., Jepson, M. A., and Cullen, P. J. (2008). Sorting nexin-1 defines an early phase of Salmonella-containing vacuole-remodeling during Salmonella infection. *J. Cell. Sci.* 121, 2027–2036.

Carlton, J., Bujny, M., Peter, B. J., Oorschot, V. M., Rutherford, A., Mellor, H., Klumperman, J., Mcmahon, H. T., and Cullen, P. J. (2004). Sorting nexin-1 mediates tubular endosome-to-TGN transport through coincidence sensing of high- curvature membranes and 3-phosphoinositides. *Curr. Biol.* 14, 1791–1800.

Castle, A., and Castle, D. (2005). Ubiquitously expressed secretory carrier membrane proteins (SCAMPs) 1-4 mark different pathways and exhibit limited constitutive trafficking to and from the cell surface. *J. Cell. Sci.* 118, 3769–3780.

Cemma, M., Kim, P. K., and Brumell, J. H. (2011). The ubiquitin-binding adaptor proteins p62/SQSTM1 and NDP52 are recruited independently to bacteria-associated microdomains to target Salmonella to the autophagy pathway. *Autophagy* 7, 341–345.

Christen, M., Coye, L. H., Hontz, J. S., Larock, D. L., Pfuetzner, R. A., Megha,, and Miller, S. I. (2009). Activation of a bacterial virulence protein by the GTPase RhoA. *Sci. Signal.* 2, ra71.

Cirillo, D. M., Heffernan, E. J., Wu, L., Harwood, J., Fierer, J., and Guiney, D. G. (1996). Identification of a domain in Rck, a product of the Salmonella typhimurium virulence plasmid, required for both serum resistance and cell invasion. *Infect. Immun.* 64, 2019–2023.

Clayton, D. J., Bowen, A. J., Hulme, S. D., Buckley, A. M., Deacon, V. L., Thomson, N. R., Barrow, P. A., Morgan, E., Jones, M. A., Watson, M., and Stevens, M. P. (2008). Analysis of the role of 13 major fimbrial subunits in colonisation of the chicken intestines by Salmonella enterica serovar Enteritidis reveals a role for a novel locus. *BMC Microbiol.* 8, 228.

Corrotte, M., Nyguyen, A. P., Harlay, M. L., Vitale, N., Bader, M. F., and Grant, N. J. (2010). Ral isoforms are implicated in Fc gamma R-mediated phagocytosis: activation of phospholipase D by RalA. *J. Immunol.* 185, 2942–2950.

Criss, A. K., Ahlgren, D. M., Jou, T. S., Mccormick, B. A., and Casanova, J. E. (2001). The GTPase Rac1 selectively regulates Salmonella invasion at the apical plasma membrane of polarized epithelial cells. *J. Cell. Sci.* 114, 1331–1341.

Dai, S., Zhang, Y., Weimbs, T., Yaffe, M. B., and Zhou, D. (2007). Bacteria-generated PtdIns(3)P recruits VAMP8 to facilitate phagocytosis. *Traffic* 8, 1365–1374.

Deiwick, J., Salcedo, S. P., Boucrot, E., Gilliland, S. M., Henry, T., Petermann, N., Waterman, S. R., Gorvel, J. P., Holden, D. W., and Meresse, S. (2006). The translocated Salmonella effector proteins SseF and SseG interact and are required to establish an intracellular replication niche. *Infect. Immun.* 74, 6965–6972.

Drecktrah, D., Knodler, L. A., Galbraith, K., and Steele-Mortimer, O. (2005). The Salmonella SPI1 effector SopB stimulates nitric oxide production long after invasion. *Cell. Microbiol.* 7, 105–113.

Drecktrah, D., Knodler, L. A., Howe, D., and Steele-Mortimer, O. (2007). Salmonella trafficking is defined by continuous dynamic interactions with the endolysosomal system. *Traffic* 8, 212–225.

Drecktrah, D., Levine-Wilkinson, S., Dam, T., Winfree, S., Knodler, L. A., Schroer, T. A., and Steele-Mortimer, O. (2008). Dynamic behavior of Salmonella-induced membrane tubules in epithelial cells. *Traffic* 9, 2117–2129.

Dukes, J. D., Lee, H., Hagen, R., Reaves, B. J., Layton, A. N., Galyov, E. E., and Whitley, P. (2006). The secreted Salmonella dublin phosphoinositide phosphatase, SopB, localizes to PtdIns(3)P-containing endosomes and perturbs normal endosome to lysosome trafficking. *Biochem. J.* 395, 239–247.

Dumont, A., Boucrot, E., Drevensek, S., Daire, V., Gorvel, J. P., Pous, C., Holden, D. W., and Meresse, S. (2010). SKIP, the host target of the Salmonella virulence factor SifA, promotes kinesin-1-dependent vacuolar membrane exchanges. *Traffic* 11, 899–911.

Dunn, J. D., and Valdivia, R. H. (2010). Uncivil engineers: Chlamydia, Salmonella and Shigella alter cytoskeleton architecture to invade epithelial cells. *Future Microbiol.* 5, 1219–1232.

Edwards, R. A., Olsen, G. J., and Maloy, S. R. (2002). Comparative genomics of closely related salmonellae. *Trends Microbiol.* 10, 94–99.

Fu, Y., and Galan, J. E. (1999). A salmonella protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion. *Nature* 401, 293–297.

Garcia-del Portillo, F., and Finlay, B. B. (1995). Targeting of Salmonella typhimurium to vesicles containing lysosomal membrane glycoproteins bypasses compartments with mannose 6-phosphate receptors. *J. Cell Biol.* 129, 81–97.

Garcia-del Portillo, F., Zwick, M. B., Leung, K. Y., and Finlay, B. B. (1993). Salmonella induces the formation of filamentous structures containing lysosomal membrane glycoproteins in epithelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 90, 10544–10548.

Gauthier-Rouviere, C., Vignal, E., Meriane, M., Roux, P., Montcourier, P., and Fort, P. (1998). RhoG GTPase controls a pathway that independently activates Rac1 and Cdc42Hs. *Mol. Biol. Cell* 9, 1379–1394.

Geddes, K., Cruz, F., and Heffron, F. (2007). Analysis of cells targeted by Salmonella type III secretion *in vivo*. *PLoS Pathog.* 3, e196. doi: 10.1371/journal.ppat.0030196

Gerlach, R. G., Claudio, N., Rohde, M., Jackel, D., Wagner, C., and Hensel, M. (2008). Cooperation of Salmonella pathogenicity islands 1 and 4 is required to breach epithelial barriers. *Cell. Microbiol.* 10, 2364–2376.

Gordon, M. A. (2008). Salmonella infections in immunocompromised adults. *J. Infect.* 56, 413–422.

Guignot, J., Caron, E., Beuzon, C., Bucci, C., Kagan, J., Roy, C., and Holden, D. W. (2004). Microtubule motors control membrane dynamics of Salmonella-containing vacuoles. *J. Cell. Sci.* 117, 1033–1045.

Hardt, W. D., Chen, L. M., Schuebel, K. E., Bustelo, X. R., and Galan, J. E. (1998). S. typhimurium encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. *Cell* 93, 815–826.

Hayward, R. D., and Koronakis, V. (1999). Direct nucleation and bundling of actin by the SipC protein of invasive Salmonella. *EMBO J.* 18, 4926–4934.

Hayward, R. D., and Koronakis, V. (2002). Direct modulation of the host cell cytoskeleton by Salmonella actin-binding proteins. *Trends Cell Biol.* 12, 15–20.

Heffernan, E. J., Harwood, J., Fierer, J., and Guiney, D. (1992a). The Salmonella typhimurium virulence plasmid complement resistance gene rck is homologous to a family of virulence-related outer membrane protein genes, including pagC and ail. *J. Bacteriol.* 174, 84–91.

Heffernan, E. J., Reed, S., Hackett, J., Fierer, J., Roudier, C., and Guiney, D. (1992b). Mechanism of resistance to complement-mediated killing of bacteria encoded by the Salmonella typhimurium virulence plasmid gene rck. *J. Clin. Invest.* 90, 953–964.

Henry, T., Couillault, C., Rockenfeller, P., Boucrot, E., Dumont, A., Schroeder, N., Hermant, A., Knodler, L. A., Lecine, P., Steele-Mortimer, O., Borg, J. P., Gorvel, J. P., and Meresse, S. (2006). The Salmonella effector protein PipB2 is a linker for kinesin-1. *Proc. Natl. Acad. Sci. U.S.A.* 103, 13497–13502.

Hernandez, L. D., Hueffer, K., Wenk, M. R., and Galan, J. E. (2004). Salmonella modulates vesicular traffic by altering phosphoinositide metabolism. *Science* 304, 1805–1807.

Hilpela, P., Vartiainen, M. K., and Lappalainen, P. (2004). Regulation of the actin cytoskeleton by PI(4,5)P2 and PI(3,4,5)P3. *Curr. Top. Microbiol. Immunol.* 282, 117–163.

Hoppe, A. D., and Swanson, J. A. (2004). Cdc42, Rac1, and Rac2 display distinct patterns of activation during phagocytosis. *Mol. Biol. Cell* 15, 3509–3519.

Huett, A., Leong, J. M., Podolsky, D. K., and Xavier, R. J. (2009). The cytoskeletal scaffold Shank3 is recruited to pathogen-induced actin rearrangements. *Exp. Cell Res.* 315, 2001–2011.

Humphreys, D., Hume, P. J., and Koronakis, V. (2009). The Salmonella effector SptP dephosphorylates host AAA+ ATPase VCP to promote development of its intracellular replicative niche. *Cell Host Microbe* 5, 225–233.

Jackson, L. K., Nawabi, P., Hentea, C., Roark, E. A., and Haldar, K. (2008). The Salmonella virulence protein SifA is a G protein antagonist. *Proc. Natl. Acad. Sci. U.S.A.* 105, 14141–14146.

Jones, B. D., Ghori, N., and Falkow, S. (1994). Salmonella typhimurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. *J. Exp. Med.* 180, 15–23.

Kageyama, S., Omori, H., Saitoh, T., Sone, T., Guan, J. L., Akira, S., Imamoto, F., Noda, T., and Yoshimori, T. (2011). The LC3 recruitment mechanism is separate from Atg9L1-dependent membrane formation in the autophagic response against Salmonella. *Mol. Biol. Cell.* doi:10.1091/mbc.E10-11-0893. [Epub ahead of print].

Katoh, H., and Negishi, M. (2003). RhoG activates Rac1 by direct interaction with the Dock180-binding protein Elmo. *Nature* 424, 461–464.

Kloer, D. P., Rojas, R., Ivan, V., Moriyama, K., Van Vlijmen, T., Murthy, N., Ghirlando, R., Van Der Sluijs, P., Hurley, J. H., and Bonifacino, J. S. (2010). Assembly of the biogenesis of lysosome-related organelles complex-3 (BLOC-3) and its interaction with Rab9. *J. Biol. Chem.* 285, 7794–7804.

Knodler, L. A., Finlay, B. B., and Steele-Mortimer, O. (2005). The Salmonella effector protein SopB protects epithelial cells from apoptosis by sustained activation of Akt. *J. Biol. Chem.* 280, 9058–9064.

Knodler, L. A., Vallance, B. A., Celli, J., Winfree, S., Hansen, B., Montero, M., and Steele-Mortimer, O. (2010). Dissemination of invasive Salmonella via bacterial-induced extrusion of mucosal epithelia. *Proc. Natl. Acad. Sci. U.S.A.* 107, 17733–17738.

Kubori, T., and Galan, J. E. (2003). Temporal regulation of salmonella virulence effector function by proteasome-dependent protein degradation. *Cell* 115, 333–342.

Lambert, M. A., and Smith, S. G. (2008). The PagN protein of Salmonella enterica serovar Typhimurium is an adhesin and invasin. *BMC Microbiol.* 8, 142.

Lambert, M. A., and Smith, S. G. (2009). The PagN protein mediates invasion via interaction with proteoglycan. *FEMS Microbiol. Lett.* 297, 209–216.

Lawley, T. D., Chan, K., Thompson, L. J., Kim, C. C., Govoni, G. R., and Monack, D. M. (2006). Genome-wide screen for Salmonella genes required for long-term systemic infection of the mouse. *PLoS Pathog.* 2, e11. doi: 10.1371/journal.ppat.0020011

Leone, P., and Meresse, S. (2011). Kinesin regulation by Salmonella. *Virulence* 2, 63–66.

Lossi, N. S., Rolhion, N., Magee, A. I., Boyle, C., and Holden, D. W. (2008). The Salmonella SPI-2 effector SseJ exhibits eukaryotic activator-dependent phospholipase A and glycerophospholipid: cholesterol acyltransferase activity. *Microbiology* 154, 2680–2688.

Ly, K. T., and Casanova, J. E. (2009). Abelson tyrosine kinase facilitates Salmonella enterica serovar Typhimurium entry into epithelial cells. *Infect. Immun.* 77, 60–69.

Magalhaes, M. A., and Glogauer, M. (2010). Pivotal Advance: Phospholipids determine net membrane surface charge resulting in differential localization of active Rac1 and Rac2. *J. Leukoc. Biol.* 87, 545–555.

Majowicz, S. E., Musto, J., Scallan, E., Angulo, F. J., Kirk, M., O'brien, S. J., Jones, T. F., Fazil, A., and Hoekstra, R. M. (2010). The global burden of nontyphoidal Salmonella gastroenteritis. *Clin. Infect. Dis.* 50, 882–889.

Mallo, G. V., Espina, M., Smith, A. C., Terebiznik, M. R., Aleman, A., Finlay, B. B., Rameh, L. E., Grinstein, S., and Brumell, J. H. (2008). SopB promotes phosphatidylinositol 3-phosphate formation on Salmonella vacuoles by recruiting Rab5 and Vps34. *J. Cell Biol.* 182, 741–752.

Marlovits, T. C., and Stebbins, C. E. (2010). Type III secretion systems shape up as they ship out. *Curr. Opin. Microbiol.* 13, 47–52.

McGhie, E. J., Brawn, L. C., Hume, P. J., Humphreys, D., and Koronakis, V. (2009). Salmonella takes control: effector-driven manipulation of the host. *Curr. Opin. Microbiol.* 12, 117–124.

McGhie, E. J., Hayward, R. D., and Koronakis, V. (2001). Cooperation between actin-binding proteins of invasive Salmonella: SipA potentiates SipC nucleation and bundling of actin. *EMBO J.* 20, 2131–2139.

McGhie, E. J., Hayward, R. D., and Koronakis, V. (2004). Control of actin turnover by a salmonella invasion protein. *Mol. Cell* 13, 497–510.

Meresse, S., Unsworth, K. E., Habermann, A., Griffiths, G., Fang, F., Martinez-Lorenzo, M. J., Waterman, S. R., Gorvel, J. P., and Holden, D. W. (2001). Remodelling of the actin cytoskeleton is essential for replication of intravacuolar Salmonella. *Cell. Microbiol.* 3, 567–577.

Meyerholz, D. K., Stabel, T. J., Ackermann, M. R., Carlson, S. A., Jones, B. D., and Pohlenz, J. (2002). Early epithelial invasion by Salmonella enterica serovar Typhimurium DT104 in the swine ileum. *Vet. Pathol.* 39, 712–720.

Misselwitz, B., Kreibich, S. K., Rout, S., Stecher, B., Periaswamy, B., and Hardt, W. D. (2010). Salmonella enterica serovar Typhimurium binds to HeLa cells via Fim-mediated reversible adhesion and irreversible type three secretion system 1-mediated docking. *Infect. Immun.* 79, 330–341.

Mota, L. J., Ramsden, A. E., Liu, M., Castle, J. D., and Holden, D. W. (2009). SCAMP3 is a component of the Salmonella-induced tubular network and reveals an interaction between bacterial effectors and post-Golgi trafficking. *Cell. Microbiol.* 11, 1236–1253.

Murli, S., Watson, R. O., and Galan, J. E. (2001). Role of tyrosine kinases and the tyrosine phosphatase SptP in the interaction of Salmonella with host cells. *Cell. Microbiol.* 3, 795–810.

Myeni, S. K., and Zhou, D. (2010). The C terminus of SipC binds and bundles F-actin to promote Salmonella invasion. *J. Biol. Chem.* 285, 13357–13363.

Nawabi, P., Catron, D. M., and Haldar, K. (2008). Esterification of cholesterol by a type III secretion effector during intracellular Salmonella infection. *Mol. Microbiol.* 68, 173–185.

Nichols, C. D., and Casanova, J. E. (2010). Salmonella-directed recruitment of new membrane to invasion foci via the host exocyst complex. *Curr. Biol.* 20, 1316–1320.

Norris, F. A., Wilson, M. P., Wallis, T. S., Galyov, E. E., and Majerus, P. W. (1998). SopB, a protein required for virulence of Salmonella dublin, is an inositol phosphate phosphatase. *Proc. Natl. Acad. Sci. U.S.A.* 95, 14057–14059.

Oh, Y. K., Alpuche-Aranda, C., Berthiaume, E., Jinks, T., Miller, S. I., and Swanson, J. A. (1996). Rapid and complete fusion of macrophage lysosomes with phagosomes containing Salmonella typhimurium. *Infect. Immun.* 64, 3877–3883.

Ohlson, M. B., Huang, Z., Alto, N. M., Blanc, M. P., Dixon, J. E., Chai, J., and Miller, S. I. (2008). Structure and function of Salmonella SifA indicate that its interactions with SKIP, SseJ, and RhoA family GTPases induce endosomal tubulation. *Cell Host Microbe* 4, 434–446.

Patel, J. C., and Galan, J. E. (2006). Differential activation and function of Rho GTPases during Salmonella-host cell interactions. *J. Cell Biol.* 175, 453–463.

Patel, J. C., Hueffer, K., Lam, T. T., and Galan, J. E. (2009). Diversification of a Salmonella virulence protein function by ubiquitin-dependent differential localization. *Cell* 137, 283–294.

Petch, L. A., Bockholt, S. M., Bouton, A., Parsons, J. T., and Burridge, K. (1995). Adhesion-induced tyrosine phosphorylation of the p130 src substrate. *J. Cell. Sci.* 108 (Pt 4), 1371–1379.

Pons, V., Luyet, P. P., Morel, E., Abrami, L., Van Der Goot, F. G., Parton, R. G., and Gruenberg, J. (2008). Hrs and SNX3 functions in sorting and membrane invagination within multivesicular bodies. *PLoS Biol.* 6, e214. doi: 10.1371/journal.pbio.0060214

Pryor, P. R., Mullock, B. M., Bright, N. A., Lindsay, M. R., Gray, S. R., Richardson, S. C., Stewart, A., James, D. E., Piper, R. C., and Luzio, J. P. (2004). Combinatorial SNARE complexes with VAMP7 or VAMP8 define different late endocytic fusion events. *EMBO Rep.* 5, 590–595.

Radtke, A. L., Wilson, J. W., Sarker, S., and Nickerson, C. A. (2010). Analysis of interactions of Salmonella type three secretion mutants with 3-D intestinal epithelial cells. *PLoS One* 5, e15750. doi: 10.1371/journal.pone.0015750

Raffatellu, M., Wilson, R. P., Chessa, D., Andrews-Polymenis, H., Tran, Q. T., Lawhon, S., Khare, S., Adams, L. G., and Baumler, A. J. (2005). SipA, SopA, SopB, SopD, and SopE2 contribute to Salmonella enterica serotype typhimurium invasion of epithelial cells. *Infect. Immun.* 73, 146–154.

Ramsden, A. E., Holden, D. W., and Mota, L. J. (2007). Membrane dynamics and spatial distribution of Salmonella-containing vacuoles. *Trends Microbiol.* 15, 516–524.

Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., Granucci, F., Kraehenbuhl, J. P., and Ricciardi-Castagnoli, P. (2001). Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat. Immunol.* 2, 361–367.

Richter-Dahlfors, A., Buchan, A. M., and Finlay, B. B. (1997). Murine salmonellosis studied by confocal microscopy: Salmonella typhimurium resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes *in vivo*. *J. Exp. Med.* 186, 569–580.

Rittmeyer, E. N., Daniel, S., Hsu, S. C., and Osman, M. A. (2008). A dual role for IQGAP1 in regulating exocytosis. *J. Cell. Sci.* 121, 391–403.

Rosselin, M., Abed, N., Virlogeux-Payant, I., Bottreau, E., Sizaret, P. Y., Velge, P., and Wiedemann, A. (2010). Heterogeneity of T3SS-1-independent entry mechanisms used by Salmonella Enteritidis to invade different cell types. *Microbiology* 157, 839–847.

Salcedo, S. P., and Holden, D. W. (2003). SseG, a virulence protein that targets Salmonella to the Golgi network. *EMBO J.* 22, 5003–5014.

Salcedo, S. P., Noursadeghi, M., Cohen, J., and Holden, D. W. (2001). Intracellular replication of Salmonella typhimurium strains in specific subsets of splenic macrophages *in vivo*. *Cell. Microbiol.* 3, 587–597.

Samudrala, R., Heffron, F., and Mcdermott, J. E. (2009). Accurate prediction of secreted substrates and identification of a conserved putative secretion signal for type III secretion systems. *PLoS Pathog.* 5, e1000375. doi: 10.1371/journal.ppat.1000375

Schroeder, N., Henry, T., De Chastellier, C., Zhao, W., Guilhon, A. A., Gorvel, J. P., and Meresse, S. (2010). The virulence protein SopD2 regulates membrane dynamics of Salmonella-containing vacuoles. *PLoS Pathog.* 6, e1001002. doi: 10.1371/journal.ppat.1001002

Schuetz, G., Rosario, M., Grimm, J., Boeckers, T. M., Gundelfinger, E. D., and Birchmeier, W. (2004). The neuronal scaffold protein Shank3 mediates signaling and biological function of the receptor tyrosine kinase Ret in epithelial cells. *J. Cell Biol.* 167, 945–952.

Scott, C. C., Dobson, W., Botelho, R. J., Coady-Osberg, N., Chavrier, P., Knecht, D. A., Heath, C., Stahl, P., and Grinstein, S. (2005). Phosphatidylinositol-4,5-bisphosphate hydrolysis directs actin remodeling during phagocytosis. *J. Cell Biol.* 169, 139–149.

Sharma, A., and Mayer, B. J. (2008). Phosphorylation of p130Cas initiates Rac activation and membrane ruffling. *BMC Cell Biol.* 9, 50. doi: 10.1186/1471-2121-9-50

Shi, J., and Casanova, J. E. (2006). Invasion of host cells by Salmonella typhimurium requires focal adhesion kinase and p130Cas. *Mol. Biol. Cell* 17, 4698–4708.

Singleton, D. R., Wu, T. T., and Castle, J. D. (1997). Three mammalian SCAMPs (secretory carrier membrane proteins) are highly related products of distinct genes having similar subcellular distributions. *J. Cell. Sci.* 110, 2099–2107.

Steele-Mortimer, O., Knodler, L. A., Marcus, S. L., Scheid, M. P., Goh, B., Pfeifer, C. G., Duronio, V., and Finlay, B. B. (2000). Activation of Akt/protein kinase B in epithelial cells by the Salmonella typhimurium effector sigD. *J. Biol. Chem.* 275, 37718–37724.

Steele-Mortimer, O., Meresse, S., Gorvel, J. P., Toh, B. H., and Finlay, B. B. (1999). Biogenesis of Salmonella typhimurium-containing vacuoles in epithelial cells involves interactions with the early endocytic pathway. *Cell. Microbiol.* 1, 33–49.

Stein, M. A., Leung, K. Y., Zwick, M., Garcia-Del Portillo, F., and Finlay, B. B. (1996). Identification of a Salmonella virulence gene required for formation of filamentous structures containing lysosomal membrane glycoproteins within epithelial cells. *Mol. Microbiol.* 20, 151–164.

Stender, S., Friebel, A., Linder, S., Rohde, M., Mirold, S., and Hardt, W. D. (2000). Identification of SopE2 from Salmonella typhimurium, a conserved guanine nucleotide exchange factor for Cdc42 of the host cell. *Mol. Microbiol.* 36, 1206–1221.

Stevens, M. P., Friebel, A., Taylor, L. A., Wood, M. W., Brown, P. J., Hardt, W. D., and Galyov, E. E. (2003). A Burkholderia pseudomallei type III secreted protein, BopE, facilitates bacterial invasion of epithelial cells and exhibits guanine nucleotide exchange factor activity. *J. Bacteriol.* 185, 4992–4996.

Terebiznik, M. R., Vieira, O. V., Marcus, S. L., Slade, A., Yip, C. M., Trimble, W. S., Meyer, T., Finlay, B. B., and Grinstein, S. (2002). Elimination of host cell PtdIns(4,5)P(2) by bacterial SigD promotes membrane fission during invasion by Salmonella. *Nat. Cell Biol.* 4, 766–773.

Trombert, A. N., Berrocal, L., Fuentes, J. A., and Mora, G. C. (2010). S. Typhimurium sseJ gene decreases the S. Typhi cytotoxicity toward cultured epithelial cells. *BMC Microbiol.* 10, 312. doi: 10.1186/1471-2180-10-312

Unsworth, K. E., Way, M., Mcniven, M., Machesky, L., and Holden, D. W. (2004). Analysis of the mechanisms of Salmonella-induced actin assembly during invasion of host cells and intracellular replication. *Cell. Microbiol.* 6, 1041–1055.

Van Weering, J. R., Verkade, P., and Cullen, P. J. (2010). SNX-BAR proteins in phosphoinositide-mediated, tubular-based endosomal sorting. *Semin. Cell Dev. Biol.* 21, 371–380.

Vinh, D. B., Ko, D. C., Rachubinski, R. A., Aitchison, J. D., and Miller, S. I. (2010). Expression of the Salmonella spp. *virulence factor SifA in yeast alters Rho*1 activity on peroxisomes. *Mol. Biol. Cell* 21, 3567–3577.

Wallis, T. S., Starkey, W. G., Stephen, J., Haddon, S. J., Osborne, M. P., and Candy, D. C. (1986). The nature and role of mucosal damage in relation to Salmonella typhimurium-induced fluid secretion in the rabbit ileum. *J. Med. Microbiol.* 22, 39–49.

Wasylnka, J. A., Bakowski, M. A., Szeto, J., Ohlson, M. B., Trimble, W. S., Miller, S. I., and Brumell, J. H. (2008). Role for myosin II in regulating positioning of Salmonella-containing vacuoles and intracellular replication. *Infect. Immun.* 76, 2722–2735.

Waterman, S. R., and Holden, D. W. (2003). Functions and effectors of the Salmonella pathogenicity island 2 type III secretion system. *Cell. Microbiol.* 5, 501–511.

Wileman, T. (2007). Aggresomes and pericentriolar sites of virus assembly: cellular defense or viral design? *Annu. Rev. Microbiol.* 61, 149–167.

Worby, C. A., and Dixon, J. E. (2002). Sorting out the cellular functions of sorting nexins. *Nat. Rev. Mol. Cell Biol.* 3, 919–931.

Xu, X., and Hensel, M. (2010). Systematic analysis of the SsrAB virulon of Salmonella enterica. *Infect. Immun.* 78, 49–58.

Zhou, D., and Galan, J. (2001). Salmonella entry into host cells: the work in concert of type III secreted effector proteins. *Microbes Infect.* 3, 1293–1298.

Zhou, D., Mooseker, M. S., and Galan, J. E. (1999). Role of the S. typhimurium actin-binding protein SipA in bacterial internalization. Science 283, 2092–2095.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 07 March 2011; accepted: 19 May 2011; published online: 03 June 2011.

Citation: Malik-Kale P, Jolly CE, Lathrop S, Winfree S, Luterbach C and Steele-Mortimer O (2011) Salmonella – at home in the host cell. Front. Microbio. ***2****:125. doi: 10.3389/fmicb.2011.00125*

This article was submitted to Frontiers in Cellular and Infection Microbiology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 20 April 2011
doi: 10.3389/fmicb.2011.00084

Nitric oxide and *Salmonella* pathogenesis

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Nitric oxide (NO) and its congeners contribute to the innate immune response to Salmonella. This enteric pathogen is exposed to reactive nitrogen species (RNS) in the environment and at different anatomical locations during its infectious cycle in vertebrate hosts. Chemical generation of RNS enhances the gastric barrier to enteropathogenic bacteria, while products of the Salmonella pathogenicity island 1 type III secretion system and Salmonella-associated molecular patterns stimulate transcription of inducible NO synthase (iNOS) by cells of the mononuclear phagocytic cell lineage. The resulting NO, or products that arise from its interactions with oxygen (O_2) or iron and low-molecular weight thiols, are preferentially bacteriostatic against Salmonella, while reaction of NO and superoxide (O_2^-) generates the bactericidal compound peroxynitrite ($ONOO^-$). The anti-Salmonella activity of RNS emanates from the modification of redox active thiols and metal prosthetic groups of key molecular targets of the electron transport chain, central metabolic enzymes, transcription factors, and DNA and DNA-associated proteins. In turn, Salmonella display a plethora of defenses that modulate the delivery of iNOS-containing vesicles to phagosomes, scavenge and detoxify RNS, and repair biomolecules damaged by these toxic species. Traditionally, RNS have been recognized as important mediators of host defense against Salmonella. However, exciting new findings indicate that Salmonella can exploit the RNS produced during the infection to foster virulence. More knowledge of the primary RNS produced in response to Salmonella infection, the bacterial processes affected by these toxic species, and the adaptive bacterial responses that protect Salmonella from nitrosative and oxidative stress associated with NO will increase our understanding of Salmonella pathogenesis. This information may assist in the development of novel therapeutics against this common enteropathogen.

Keywords: inducible nitric oxide synthase, macrophages, reactive nitrogen species, *Salmonella*, redox chemistry, virulence, intracellular, enteric bacteria

INTRODUCTION

Gram-negative bacilli of the species *Salmonella enterica* are frequent causes of disease in humans as well as domestic and wild animals. Invasive salmonellosis is commonly associated with a variety of syndromes that range from gastroenteritis to severe systemic infections. Although non-typhoidal *Salmonella* such as *S.* Typhimurium or *S.* Enteritidis are common causes of gastroenteritis in healthy individuals, these bacteria can cause life-threatening disseminated infections in immunocompromised hosts. *Salmonella* infections are primarily acquired through the ingestion of contaminated food or water. As food-borne pathogens capable of disseminating extraintestinally, *Salmonella* are exposed to a variety of innate defenses in the stomach, intestinal lumen, gastrointestinal mucosa, and the intracellular environment of phagocytes. The availability of immunocompromised strains of mice has revealed that reactive nitrogen species (RNS) produced by the enzymatic activity of the inducible NO synthase (iNOS) hemoprotein are integral components of the host armamentarium against *Salmonella*. As it has been shown for many phylogenetically diverse organisms, the contribution of RNS in resistance to *Salmonella* does not appear to be limited to direct cytotoxicity, but also involves host and pathogen signaling cascades that indirectly affect the outcome of the infection. Many of the mechanisms by which RNS contribute to *Salmonella* pathogenesis have been elucidated in murine models of infection using *S. enterica* serovar Typhimurium, although exciting evidence indicates that these reactive species are also used by diverse animal and human cells in their defense against multiple serovars of *S. enterica*. This review will discuss what we know about the contribution of RNS to *Salmonella* pathogenesis, paying particular attention to our current understanding of the mechanisms by which nitric oxide (NO) helps control *Salmonella* infections and the strategies used by this facultative intracellular pathogen to lessen the cytotoxicity of NO and its nitrosative and oxidative derivatives.

EXPOSURE OF ENTERIC PATHOGENS TO NO

As is the case for other enteropathogenic bacteria, *Salmonella* are exposed to NO at different stages during the infectious cycle. *Salmonella* encounter RNS in transit through the environment, the gastrointestinal lumen and mucosa, and phagosomes of mononuclear cells populating gut-associated lymphoid tissues or systemic sites.

EXPOSURE OF ENTERIC BACTERIA TO NO DURING TRANSIT IN THE ENVIRONMENT

Salmonella outbreaks are often associated with contaminated produce. This suggests that *Salmonella* must establish relationships with plants to ensure persistence in the environment while in transit between animal hosts. The plant surface is for the most part a

hostile environment in which bacteria can suffer the adverse effects of ultraviolet light and desiccation. Stomata (small openings in leaves involved in H_2O and gas exchange) provide a favorable niche for the survival of enteric bacteria in the plant host. Symmetrical guard cells are responsible for closing the pore of stomata, thereby limiting gas diffusion. Work with *E. coli* has shown that lipopolysaccharide (LPS) in the cell envelope triggers the closing of stomata, thus eliciting a protective response that limits bacterial colonization (Melotto et al., 2006). Elegant microscopic and pharmacological evidence has demonstrated that NO is the signal that triggers closing of stomata in response to *E. coli*. Given the similarities in the structure of LPS from *E. coli* and *Salmonella*, it is likely that the NO-dependent innate response that regulates the closing of stomata is not limited to *E. coli*, but represents a general strategy used by plants against *Salmonella* as well.

SOURCES OF NITROSATIVE STRESS IN THE GASTROINTESTINAL TRACT

Exposure of Salmonella to RNS in the gastric lumen

Whether associated with tainted vegetables, animal products, or water, *Salmonella* are encountered in the gastrointestinal tract by most vertebrate hosts. The acidic environment of the stomach is an insurmountable barrier for most microorganisms. *Salmonella* and many other enteropathogenic bacteria can resist the extreme acidity of the stomach for brief periods of time. In addition, *Salmonella*, either in response to moderate acidic environmental conditions or upon brief contact to mildly acidic pockets within the gastric content, express a genetic program known as the acid tolerance response (ATR) that enhances resistance to low pH and promotes oral virulence (Foster and Hall, 1990; Bourret et al., 2008). In addition to the accepted role of acidity as a nonspecific host defense of the gastric lumen, extensive work by Lundberg et al. (2004) indicates that nitrogen oxides represent another component of the antimicrobial arsenal of the stomach. Although the gastric mucosa can express iNOS in response to infection (Jones-Carson et al., 1995), the bulk of RNS in the gastric lumen originate via nitrate (NO_3^-). NO_3^- accumulates in blood from both endogenous inflammatory and physiological processes, and exogenous dietary sources. This anion is actively secreted in saliva from the enterosalivary circulation. Upon delivery in the mouth, salivary NO_3^- is reduced to nitrite (NO_2^-) by resident microbiota populating the posterior, anaerobic regions of the tongue (reaction 1; Duncan et al., 1995). In the low pH of the stomach, NO_2^- is protonated to its nitrous acid (HNO_2) conjugate, which serves as a precursor for various RNS such as NO, nitrogen dioxide (NO_2), and dinitrogen trioxide (N_2O_3; reactions 2–4).

$$NO_3^- \rightarrow NO_2^- \quad (1)$$

$$NO_2^- + H^+ \rightarrow HNO_2 \quad (2)$$

$$2HNO_2 \rightarrow NO_2 + NO + H_2O \quad (3)$$

$$NO_2 + NO \rightarrow N_2O_3 \quad (4)$$

The concentration of NO in the gastric space of human volunteers fluctuates from 100 μM under resting conditions to 500 μM after intake of the daily NO_3^- dietary consumption (McKnight et al., 1997). It is becoming increasingly clear that the battery of nitrogen oxides produced in the stomach adds to antimicrobial defense against diverse enteric pathogens. At pH 2.0, RNS are directly bactericidal against *Salmonella*, whereas at a more moderate pH 4.0, RNS exert indirect antimicrobial activity (Bourret et al., 2008). RNS encountered at pH 4.0 interfere with the ATR of rapidly growing *Salmonella* by selectively inhibiting the PhoPQ two-component regulatory signaling cascade. Similar to the phenotypes seen in bacteria lacking a functional PhoQ sensor kinase, the NO-dependent inhibition of the ATR prevents the adaptation of *Salmonella* to pH 3.0, and reduces both oral virulence and fecal shedding (Bourret et al., 2008). Despite the harsh host defenses present in the gastric lumen, some salmonellae must survive the rigors imposed by low pH and RNS, since these enteric bacteria are common causes of gastroenteritis in healthy individuals.

Exposure of Salmonella to RNS in the gut

Salmonella utilize the type III secretion system encoded within *Salmonella* pathogenicity island 1 (SPI1) to gain access to the mucosa of the small intestine. Secretion of SPI1 effector proteins into enterocytes and M cells of Peyer's patches of the ileum induces actin rearrangements that promote bacterial engulfment. In the lamina propria, *Salmonella* infect gut-associated macrophages, which undergo a proinflammatory cell death named pyroptosis (Bergsbaken et al., 2009). Molecular characterization of this event has shown that the SipB and SopE SPI1 effectors stimulate the formation of inflammasomes, multicellular complexes containing caspase-1, and the NOD-LRR family of proteins (Hersh et al., 1999; Muller et al., 2009). The proteolytic activity of caspase-1 activates the secretion of interleukin (IL)-1β and IL-18, stimulating the innate immune response against *Salmonella* (Raupach et al., 2006). Of importance to this review, the inflammatory response of the gut mucosa also induces the expression of iNOS and the consequent generation of NO. Perhaps unexpectedly, caspase-1, but not the proinflammatory cytokine IL-1β, induces the transcription of iNOS (Buzzo et al., 2010). The induction of iNOS appears to be a highly coveted event in the interaction of *Salmonella* with the gastrointestinal mucosa, because inflammasomes can be independently triggered through the injection of flagellin into the host cell cytosol (Gewirtz et al., 2001). Interestingly, flagellin is delivered into host cells via the SPI1 type III secretion system (Sun et al., 2007). The recognition of flagellin by the cytosolic NOD proteins Nirk4 and Naip5 induces the expression of iNOS (Sun et al., 2007; Buzzo et al., 2010). Therefore, it appears that several *Salmonella* effectors can independently trigger iNOS transcription.

The NO generated in response to *Salmonella* appears to play two seemingly contradictory roles in the gastrointestinal phase of the infection. On one hand, NO exerts important antimicrobial activity that helps limit the bacterial burden in an acute murine model of salmonellosis. Accordingly, the absence of iNOS increases both the numbers of *Salmonella* in Peyer's patches and the translocation of the bacteria into the underlying gut mucosa (Ackermann et al., 2008; Alam et al., 2008). On the other hand, NO and the ensuing proinflammatory cascade can be of benefit to *Salmonella*, because inflammation can paradoxically promote intestinal colonization and the systemic spread of *Salmonella* (Stecher et al., 2007). This observation appears to be in conflict with the more conventional view that intestinal inflammation protects against *Salmonella*. The effects of reactive species on the normal microbiota may explain this apparent paradox. Because the majority of the

intestinal flora consists of anaerobes, it is expected that reactive oxygen and nitrogen species generated in response to *Salmonella* are detrimental for the abundant microbiota that colonize the gastrointestinal tract. Following this line of reasoning, the inflammation caused by *Salmonella* eliminates a significant proportion of the gut microbiota, allowing *Salmonella* to more freely colonize the gastrointestinal mucosa (Stecher et al., 2007; Ackermann et al., 2008). Disrupting the microbial balance in the gastrointestinal tract is a SPI1-dependent process, because mutants in this type III secretion system are at a disadvantage for colonization and induction of inflammation (Barman et al., 2008).

The beneficial effects of NO may not be limited to the elimination of the competing microbiota. NO produced in the inflammatory process could potentiate *Salmonella* growth by supplying alternative electron acceptors. NO_3^- can arise in the gut as either an auto-oxidative product or through enzymatic detoxification of NO or nitrification reactions (Tannenbaum et al., 1978). NO_3^- could be used as alternative electron acceptor to maintain electron transport chain function in the O_2-limited environment of the gut (Jones et al., 2007). Failure of *Salmonella* to use O_2 as a terminal electron acceptor in the gut is likely compounded by the NO-dependent inhibition of the enzymatic activity of terminal quinol cytochrome oxidases in the electron transport chain (Husain et al., 2008). Under nitrosative stress in the O_2-depleted environment of the gastrointestinal lumen, *Salmonella* likely utilizes available alternative electron acceptors. In favor of this idea, the *Salmonella* NO_2^- transporter NirC contributes to *Salmonella* oral virulence (Das et al., 2009). In addition, the *Salmonella* genome encodes the membrane-bound *narGHJI* and *narZYWV* and periplasmic *nap* nitrate reductases that could use NO_3^- as a respiratory substrate (Prior et al., 2009). However, the role of these nitrate reductases to *Salmonella* pathogenesis awaits investigation. In contrast to NO_3^-, other metabolites generated through reaction with reactive oxygen species have been demonstrated to serve as alternative terminal electron acceptors. For instance, oxyradicals synthesized by the NADPH phagocyte oxidase react with sulfur compounds in the gut lumen, generating the alternative electron acceptor tetrathionate (Winter et al., 2010).

In conclusion, the growth advantage of *Salmonella* in the midst of an inflamed mucosa could be attributed to the ability of this enteric pathogen to utilize tetrathionate and NO_3^- as electron acceptors, while anaerobic residents are eradicated by the ensuing inflammation. In addition to disrupting the normal balance in the microbiota, RNS may contribute more directly to the gastroenteritis and diarrheal syndrome associated with *Salmonella* infection. Coexpression of iNOS and soluble guanylate cyclase (Closs et al., 1998), whose heme cofactor is allosterically stimulated by NO, could synergize with effectors of the SPI1 type III secretion system for the stimulation of secretion of fluid into the intestinal lumen (Tsolis et al., 1999).

Exposure of Salmonella to NO in systemic sites of infection

The ability of *Salmonella* to survive within professional phagocytes is a hallmark of the pathogenesis of this enteric bacterium (Fields et al., 1986). The interaction of *Salmonella* with professional phagocytes occurs shortly after infection, as *Salmonella* can be found within the confines of lamina propria $CD18^+$ phagocytic cells just minutes after challenge (Vazquez-Torres et al., 1999). In addition, *Salmonella* manipulate $CD18^+$ phagocytic cells as Trojan horses for their extraintestinal dissemination to systemic viscera (Vazquez-Torres et al., 1999; Worley et al., 2006). Phagocytes can provide a safe place for the intracellular replication of *Salmonella* (Fields et al., 1986; Jantsch et al., 2003; Das et al., 2009). However, cells of the mononuclear phagocytic cell lineage also serve as bottlenecks that eliminate a substantial number of intracellular *Salmonella* (Vazquez-Torres et al., 2000). Several effectors, of which the enzymatic production of reactive oxygen and nitrogen species are probably the best characterized, mediate the anti-*Salmonella* activity of macrophages. Importantly, both human and rodent macrophages have been shown to express iNOS in response to *Salmonella* (Witthoft et al., 1998; Eriksson et al., 2000; Khan et al., 2001; Stevanin et al., 2002; Giacomodonato et al., 2003; Bourret et al., 2008; Azenabor et al., 2009). The expression of iNOS takes place *in vivo* 3 days after intraperitoneal challenge (Umezawa et al., 1997). As discussed in more detail in the next section, the paucity in the expression of iNOS is indicative of the inducible nature of the response. The RNS generated by the enzymatic activity of iNOS are not simple markers of infection, but crucial host defenses that limit the replication of *Salmonella* in the spleen and liver (Mastroeni et al., 2000).

Investigations using a murine system have defined in detail the contribution of iNOS to the anti-*Salmonella* activity of professional phagocytes (Vazquez-Torres et al., 2000). The main anti-*Salmonella* activity of iNOS is expressed at later stages of the infection and manifests itself as an inhibition of *Salmonella* replication. Although more limited in scope, the NO congener $ONOO^-$, which is the product of the rate-limited reaction of O_2^- and NO, also contributes to the early killing of *Salmonella* by IFNγ-primed macrophages.

In addition to exerting direct anti-*Salmonella* activity, NO congeners regulate the ensuing innate immune response. For instance, NO produced in response to *Salmonella* prevents apoptosis (Cerquetti et al., 2002). Accordingly, *Salmonella*-infected, iNOS-deficient mice harbor abnormally high numbers of apoptotic cells in the liver and Peyer's patches (Alam et al., 2002, 2008). In addition, these immunodeficient mice suffer from enhanced septicemia, suggesting that the anti-apoptotic role associated with NO limits the extraintestinal dissemination of *Salmonella*.

INNATE AND IFNγ-DEPENDENT STIMULATION OF NO SYNTHESIS

The expression of iNOS is controlled at the transcriptional level. Both innate and IFNγ-dependent signaling cascades upregulate the expression of iNOS mRNA and protein in the course of *Salmonella* infection. The pattern recognition receptor Toll-like receptor 4 (TLR4) binds to lipid A acyl chains of LPS located in the outer leaflet of the outer membrane of Gram-negative bacteria. Binding of LPS to TLR4 activates MyD88, TRIF, and NFκB signaling that stimulates IFNβ production and STAT1 phosphorylation (Xie et al., 1994; Toshchakov et al., 2002; Talbot et al., 2009). On the other hand, erythropoietin antagonizes the activation of NFκB signaling in response to *Salmonella* (Nairz et al., 2011). The IFN/JAK/STAT-dependent activation of the IRF1 transcription factor, in turn, activates iNOS expression in response to *Salmonella* LPS (Kamijo et al., 1994). The importance that the TLR4-lipid A signaling cascade plays in *Salmonella* pathogenesis is demonstrated by the fact that

C3H/HeJ mice bearing a defective TLR4 allele are extraordinarily sensitive to *Salmonella* infection (Vazquez-Torres et al., 2004). The contribution of TLR4 signaling to host defense against *Salmonella* is partially mediated through the regulation of iNOS expression, because macrophages lacking TLR4 are not only low producers of NO, but are also less capable of controlling intracellular *Salmonella* (Vazquez-Torres et al., 2004). *Salmonella* have an invested interest in controlling TLR4 signaling as demonstrated by the fact that 3′-*O*-deacylation of *Salmonella* lipid A reduces both NO generation and antimicrobial activity of macrophages (Kawano et al., 2010).

The Nramp1 (Slc11a1) divalent metal transporter associated with phagosomal membranes also optimizes the innate expression of iNOS in response to *Salmonella* (Nairz et al., 2009). The mechanisms by which Nramp1 induces iNOS are, however, not completely understood, and may reflect the pleiotropic effects associated with disturbances in cytosolic metal concentrations associated with the Nramp1-dependent efflux of metals from the phagosome (reviewed in Cellier et al., 2007). The expression of this metal transporter can activate IRF1 (Fritsche et al., 2003), which, as seen above, is a positive signal of iNOS transcription. Nramp1 can also work synergistically with TNFα for the induction of iNOS expression (Ables et al., 2001). However, *Salmonella* can induce iNOS enzymatic activity in the absence of signaling through the TNFα p55 receptor (Vazquez-Torres et al., 2001). Finally, the increased NO synthesis seen in Nramp1$^+$ macrophages has been associated with decreases in IL-10 (Fritsche et al., 2008). Of biological importance, mice lacking Nramp1 are hyper-susceptible to *Salmonella* infection (Govoni and Gros, 1998). Possibly the most dramatic evidence linking Nramp1 to the NO-dependent host defense against *Salmonella* comes from the fact that attenuation of certain *Salmonella* mutants deficient in antinitrosative defenses is uniquely exposed in an iNOS-dependent manner in Nramp1$^+$ models of salmonellosis (Bang et al., 2006; Richardson et al., 2009; Husain et al., 2010).

Salmonella induce the production of both IL-12 by macrophages, and IFNγ by natural killer and T helper cells (Schwacha and Eisenstein, 1997). As is the case for other activation markers such as MHC class II, the expression of iNOS seen in response to IFNγ is optimal in the presence of a triggering signal. Findings in macrophages deficient in TLR4 indicate that the expression of iNOS seen in IFNγ-primed macrophages does not require LPS as the triggering signal (Vazquez-Torres et al., 2004). Given the direct activation of iNOS transcription by LPS, these findings might seem surprising. However, it is quite possible that in a hyper-activated state, *Salmonella* fimbriae, porins, and other surface structures may synergize with IFNγ in the activation of iNOS transcription (Vitiello et al., 2008). The elevated NO fluxes of IFNγ-primed macrophages are associated with enhanced anti-*Salmonella* activity (Vazquez-Torres et al., 2000).

PRODUCTION OF NO BY HUMAN MONONUCLEAR CELLS IN RESPONSE TO *SALMONELLA*

Human mononuclear cells, which similar to their murine counterparts activate iNOS expression in response to *Salmonella* LPS, use NO in their anti-*Salmonella* activity (Stevanin et al., 2002; Azenabor et al., 2009; Gomes et al., 2010). Despite these exciting findings generated with *in vitro* cell cultures, there still is a lack of clinical evidence correlating defects in iNOS with the predisposition of humans to *Salmonella* infection. Having said this, individuals bearing mutations in the IFNγ or IL-12 receptors, or IL-12 p40 are extremely susceptible to *Salmonella* (de Jong et al., 1998). It remains to be investigated whether and to what extent the IFNγ-signaling pathway contributes to the resistance of humans to salmonellosis through the upregulation of iNOS expression and high NO output.

MOLECULAR TARGETS AND BIOLOGICAL CHEMISTRY OF NO DURING THE COURSE OF *SALMONELLA* INFECTION

Despite being a radical, NO is remarkably unreactive. The biological chemistry of NO is derived from both direct and indirect effects of this radical with molecular targets (**Figure 1**). NO can react directly with a limited number of metalloproteins and organic radicals. Moreover, the auto-oxidation, nitrosative, and oxidative products of the reaction of NO with O_2, O_2^-, or iron and low-molecular weight thiols can diversify the biological chemistry of this diatomic radical. As presented above, most of the anti-*Salmonella* activity exerted

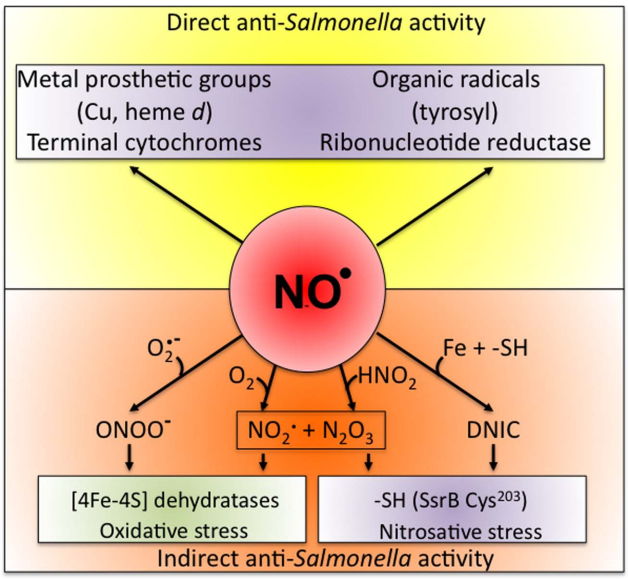

FIGURE 1 | Biological chemistry of NO and *Salmonella* targets. NO can react directly (yellow box) with metal prosthetic groups or other radicals. Binuclear centers containing copper (Cu) or iron heme d in terminal quinol cytochrome oxidases of the electron transport chain are directly nitrosylated by NO. The covalent attachment of NO to molecular targets causes nitrosative stress (purple boxes). Investigations in E. coli and mitochondria have shown that a tyrosyl radical in the active site of ribonucleotide reductase can be modified directly by NO. NO can also exert biological functions indirectly (orange box) by forming RNS through its interactions with superoxide anion (O_2^-), molecular oxygen (O_2), or iron and low-molecular weight thiols (-SH). Nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3) can independently arise from the condensation of acidified nitrite (HNO_2) or the auto-oxidation of NO in the presence of O_2. Peroxynitrite ($ONOO^-$), NO_2, and N_2O_3 are strong oxidants. [4Fe–4S] clusters of dehydratases are frequent targets of $ONOO^-$ (green box). N_2O_3 and dinitrosyl–iron complexes (DNIC), which frequently target thiol-containing proteins, are common sources of nitrosative stress. The redox active thiol of Cys^{203} in the dimerization domain of the SsrB response regulator that controls SPI2 gene transcription is a bona fide sensor of nitrosative stress in *Salmonella*.

by RNS is expressed in the form of bacteriostasis. The chemistries and the *Salmonella* molecular targets underlying the NO-mediated bacteriostasis are being elucidated.

DIRECT BIOLOGICAL CHEMISTRY OF NO AGAINST *SALMONELLA*

NO can react directly with metal prosthetic groups or with other radicals. Direct binding of NO to redox active iron cofactors is well characterized. For example, the high affinity of NO for terminal quinol cytochrome oxidases of the electron transport chain can inhibit the ability of *Salmonella* and *E. coli* to reduce O_2 to H_2O (Butler et al., 1997; Husain et al., 2008). *Salmonella* has four terminal cytochrome oxidases, of which *bd* and *bo* are the best characterized for their reactivities with NO and O_2. Work done in *E. coli* indicates that NO binds with higher affinity to cytochrome *bd* than cytochrome *bo* (Mason et al., 2009). So, it is expected that cytochrome *bd* will preferentially be inhibited at low NO rates, thereby protecting the proton-translocating and O_2-reducing capacities of cytochrome *bo*. Accordingly, cytochrome *bd* has been shown to be nitrosylated at heme *d* in *Salmonella* exposed to NO (Husain et al., 2008). At high levels of NO, such as those produced in the inflammatory response to *Salmonella*, both cytochromes would be expected to form metal nitrosyl compounds, and thus the overall respiratory activity ought to be repressed. Accordingly, *Salmonella* exposed to high levels of NO stop respiring (Husain et al., 2008). The NO-mediated repression of respiration could have dramatic consequences in the pathogenesis of *Salmonella*. First, carbon utilization in *Salmonella* experiencing intense nitrosative stress would be expected to flux toward fermentative pathways, which yield lower ATP per hexose molecule consumed. Second, reduced respiratory activity is likely to affect some signaling pathways that rely on the activity of the electron transport chain. For example, the ArcB sensor kinase in *E. coli* responds to the reduced quinone pool (Georgellis et al., 2001). Third, the NO-mediated inhibition of respiration may limit the transport of nutrients and other molecules across the membrane, which relies on the proton motive force. Fourth, the repression of respiration and a drop in proton motive force may, however, be of benefit to *Salmonella* in some settings, as shown by the fact that the NO-dependent repression of respiration increases resistance of *Salmonella* to aminoglycosides (McCollister et al., 2011). And, fifth, the NO-dependent repression of respiration can boost the antioxidant defenses of *Salmonella* (Husain et al., 2008).

In *E. coli*, NO forms iron–nitrosyl complexes with the ferric uptake regulatory protein Fur, thereby derepressing iron-regulated gene transcription (D'Autreaux et al., 2002). In addition to affecting iron metabolism, the nitrosylation of Fur could have broad implications in *Salmonella* gene expression. For example, Fur negatively regulates transcription of HN-S in *Salmonella* (Troxell et al., 2010). Because H-NS binds to AT-rich DNA and represses transcription through topological constraint (Lucchini et al., 2006; Navarre et al., 2006), the nitrosylation of Fur could influence the regulation of horizontally acquired *Salmonella* SPI1 and SPI2 genes. This interesting possibility awaits investigation.

Bearing an unpaired electron, NO reacts with high affinity and specificity with organic radicals. For example, NO directly reacts with the tyrosyl radical in the active site of ribonucleotide reductase (Lepoivre et al., 1991). The generation of the tyrosyl radical is a crucial step in the catalytic transfer of electrons to ribonucleotides for the reduction of the 2′ carbon of ribose-5-phosphate and formation of the deoxy derivative. Therefore, nitrosylation of ribonucleotide reductase disrupts the formation of deoxyribonucleotides needed for repair and synthesis of DNA. The concerted inhibition of respiratory activity and ribonucleotide reductase very likely contributes to the bacteriostatic effects of NO against *Salmonella*.

INDIRECT BIOLOGICAL CHEMISTRY OF NO AGAINST *SALMONELLA*

The indirect effects of NO on biological targets are mediated through the RNS generated from the reaction of NO with other molecules. NO can generate biologically relevant RNS through its interactions with molecular O_2 or O_2^-. Because of kinetic and temporal constraints, both of these chemistries appear to be limited to highly activated macrophages. IFNγ-activated macrophages synthesize about three- to fourfold higher amounts of NO than non-activated controls (Vazquez-Torres et al., 2000; McCollister et al., 2007). The increased NO fluxes of IFNγ-activated macrophages allow the following chemistries:

$$2NO + O_2 \rightarrow 2NO_2 \quad (5)$$

$$NO + NO_2 \rightarrow N_2O_3 \quad (6)$$

$$N_2O_3 + RSH \rightarrow RSNO + H^+ + NO_2^- \quad (7)$$

Since reaction (5) is second order for NO, it follows that N_2O_3 is only detected in the highly activated macrophages sustaining high NO fluxes (reaction 6; McCollister et al., 2007). In addition to being a potent oxidizing agent, N_2O_3 is a powerful S- and N-nitrosating species that promotes the formation of S-nitrosothiols (reaction 7) and N-nitrosamines. Similar to its formation in the gastric lumen, N_2O_3 can independently be generated in the phagosomal lumen through the condensation of HNO_2. In fact, about a third of the N_2O_3 generated in IFNγ-primed macrophages appears to form via HNO_2 (McCollister et al., 2007). Dinitrosyl–iron complexes (DNICs) generated from the reaction of NO with iron and low-molecular weight thiols are alternative nitrosating agents to N_2O_3. Lancaster and colleagues have argued that transnitrosation from DNICs is the primary means of S-nitrosothiol formation *in vivo* (Bosworth et al., 2009).

The reaction of NO with O_2^- is also limited to IFNγ-activated macrophages. The temporal dissociation between the activities of the NADPH oxidase and iNOS hemoproteins appears to be the main reason for the lack of $ONOO^-$ synthesis by non-activated macrophages. Treatment of macrophages with IFNγ stimulates iNOS expression during early phases of the infection, thereby allowing the simultaneous production of O_2^- and NO (Vazquez-Torres et al., 2000). A lack of $ONOO^-$ production in the absence of iNOS or NADPH oxidase demonstrates that the generation of $ONOO^-$ in IFNγ-primed macrophages results from the host response. Detection of nitrotyrosine formation in systemic sites suggests that the highly reactive oxidant $ONOO^-$ is indeed produced in the course of *Salmonella* infection (Alam et al., 2002). As seen in macrophages, the nitrotyrosine signature found in *Salmonella*-infected mice could reflect $ONOO^-$ generated from the interaction of NADPH oxidase and iNOS enzymatic activities. Nonetheless, it is also possible that the $ONOO^-$ detected *in vivo* could have been generated from NO produced by macrophages and O_2^- generated adventitiously from the reduction of O_2 by electrons in NADH

dehydrogenases of the electron transport chain. Conditions that reduce the flow of electrons diminish terminal cytochrome activity and lead to the stasis of electrons upstream in the electron transport chain, a situation that can stimulate O_2^- formation (Boveris and Chance, 1973). Moreover, nitrotyrosine could be a signature of peroxidase enzymatic activity using NO_2^- as a substrate (Eiserich et al., 1998). These interesting possibilities need to be investigated. It is also possible that reactive oxygen species such as H_2O_2 may synergize with NO for antimicrobial activity (Pacelli et al., 1995).

MOLECULAR TARGETS OF THE INDIRECT BIOLOGICAL CHEMISTRY OF NO

$ONOO^-$ generated by the reaction of NO with O_2^- preferentially targets [4Fe–4S] clusters of dehydratases (Castro et al., 1994; Hausladen and Fridovich, 1994; Keyer and Imlay, 1997). Several enzymes of intermediary metabolism containing [4Fe–4S] prosthetic groups in their catalytic cores are prime targets of $ONOO^-$. Aconitase and fumarase A of the citric acid cycle, dihydroxyacid dehydratase involved in branch chain amino acid synthesis, and phosphogluconate dehydratase of the Entner–Doudoroff pathway can all be inhibited with low concentrations of $ONOO^-$ (Keyer and Imlay, 1997; Hyduke et al., 2007). Alternatively, nitrosative species such as N_2O_3, S-nitrosoglutathione, and DNICs stimulate S-nitrosothiol formation. Some enzymes of intermediary metabolism utilize redox active cysteine residues for catalysis. For instance, redox active thiols in glyceraldehyde-3-phosphate dehydrogenase of glycolysis, the dihydrolipoamide acetyltransferase subunit of pyruvate dehydrogenase, ketol-acid reductoisomerase, and the small subunit of glutamate synthase responsible for amino acid synthesis are primary targets of nitrosative stress (Keyer and Imlay, 1997; Brandes et al., 2007). Collectively, the inhibition of redox active enzymes in central metabolism by oxidative and nitrosative stress likely induces global changes in bacterial physiology. *Salmonella* undergoing nitrosative stress downregulate translational machinery (Bourret et al., 2008), which not only is a key signature of the stringent response, but may also represent a physiological adaptation to RNS. Although the mechanism by which RNS activate the stringent response is not known, it is possible that the inhibition of amino acid synthesis by RNS could be the signal (Brandes et al., 2007; Hyduke et al., 2007).

Reactive nitrogen species can also disassemble zinc-fingers as suggested by the observation that NO-treated *Salmonella* accumulate chelatable zinc in their cytoplasms (Schapiro et al., 2003). It has been proposed that NO inactivates the zinc-finger-containing proteins PriA, DnaG, and DnaJ (Schapiro et al., 2003). However, biochemical evidence indicating that NO modifies the zinc-finger motifs in these DNA-binding proteins has not been experimentally demonstrated. Together with repression of the electron transport chain, inhibition of ribonucleotide reductase, and inhibition of enzymes in central metabolism, disruption of zinc-fingers in proteins associated with DNA replication could contribute to the bacteriostasis and cell filamentation of NO-treated *Salmonella* (De Groote et al., 1995; Vazquez-Torres et al., 2000; Schapiro et al., 2003).

Redox active thiols are preferred targets of the RNS generated during the auto-oxidation of NO. The thiol of Cys^{203} in the dimerization domain of the SPI2 master regulator SsrB is a *bona fide* example of a *Salmonella* target of S-nitrosylation (Husain et al., 2010). SsrB was recently recognized as a redox active protein that senses nitrosative stress. The relevance of the NO-sensing activity of SsrB in *Salmonella* pathogenesis is manifested by the fact that a strain of *Salmonella* expressing a redox resistant SsrB C203S variant is attenuated in an Nramp1 model of oral salmonellosis, and that Cys^{203} is conserved in SsrB of both typhoidal and non-typhoidal strains of *Salmonella.* The only exception is a strain of *S. enterica* sv. St. Paul, which instead has a tyrosine at position 203. This substitution is quite interesting since tyrosines and cysteines are preferred targets of RNS. It is still unknown how the sensing of NO congeners by SsrB Cys^{203} enhances *Salmonella* fitness. It is possible that, by decreasing either *Salmonella*-induced apoptosis or reducing recognition by T and B lymphocytes, the tight control of SPI2 expression exerted by a redox active SsrB may increase intracellular survival or limit the specific immune response to SPI2 effectors. The idea that the downregulation of SPI2 by the NO-sensing activity of SsrB is key to some aspect of *Salmonella* pathogenesis is in keeping with previous observations that showed that the repression of SPI2 is as important for *Salmonella* virulence as its positive regulation (Coombes et al., 2005).

ANTINITROSATIVE DEFENSES OF *SALMONELLA*

Many pathogenic bacteria have developed or adapted mechanisms to counteract RNS encountered in the host (**Figure 2**). As an intracellular pathogen, *Salmonella* possesses several strategies that avoid contact with iNOS-containing vesicles, detoxify NO, or repair lesions incurred by RNS.

AVOIDANCE OF RNS

Salmonella actively avoid iNOS-containing vesicles of professional phagocytes. SPI2 and iNOS are optimally expressed after 8 h of the innate response of macrophages to *Salmonella* (Eriksson et al., 2003). Effectors secreted through the SPI2 type III secretion system minimize trafficking of iNOS-containing vesicles to the proximity of phagosomes (Chakravortty et al., 2002). It might seem unclear why segregation of iNOS from the *Salmonella* phagosome might be advantageous, since NO diffuses freely through membranes. Moreover, others have noticed that iNOS, associated with cortical actin, vesicles, or cytosol, is not significantly mobilized in response to *Salmonella* infection (Webb et al., 2001; McCollister et al., 2007). Avoidance of iNOS-containing vesicles might be advantageous in the context of limiting exposure to $ONOO^-$ (Chakravortty et al., 2002), which being an anion does not cross freely through membranes. IFNγ-activated macrophages can, nonetheless, downregulate the intracellular expression of SPI2 (McCollister et al., 2005; Bourret et al., 2009). Biochemical and genetic lines of evidence indicate that NO congeners produced by the enzymatic activity of iNOS mediate repression of SPI2 in IFNγ-primed macrophages. This agrees with the fact that the chemical generation of NO represses SPI2 transcription (Bourret et al., 2009). Although it is not known how SPI2 transcription gets downregulated by RNS of activated macrophages, this process is independent of the repression of PhoPQ signaling (Bourret et al., 2009). It is also unclear if downregulation of SPI2 is a strategy sought by the bacteria or a target of host defense. The fact that NO congeners produced by IFNγ-activated macrophages promote the maturation of the *Salmonella* phagosome along the degradative pathway would argue to the latter. In this context, the NO-dependent repression of SPI2 transcription accounts for a great part of the enhanced killing of *Salmonella* by IFNγ-primed macrophages (McCollister et al., 2005).

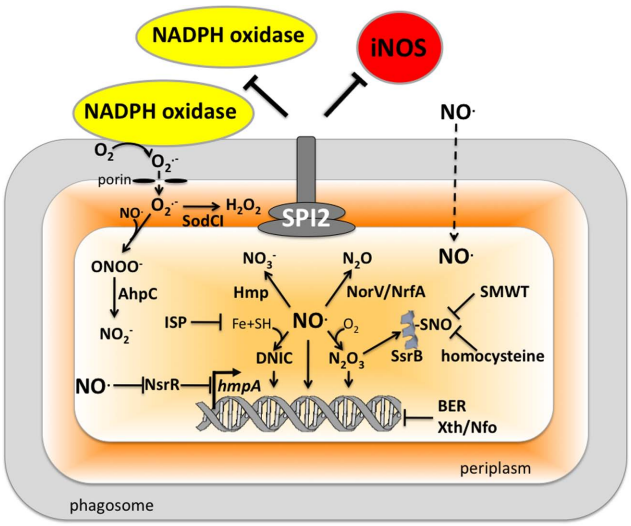

FIGURE 2 | Antinitrosative defenses of *Salmonella*. Effectors secreted by the type III secretion system encoded within SPI2 actively avoid NADPH oxidase- and iNOS-containing vesicles. Superoxide (O_2^-) produced by the enzymatic activity of NADPH oxidase enters the periplasmic space of *Salmonella* through porins, while NO can freely diffuse across membranes. Overlapping NADPH phagocyte oxidase and iNOS activities can generate the potent oxidant peroxynitrite ($ONOO^-$) through the reaction of NO with O_2^-. The negative effects of $ONOO^-$ are alleviated directly by the enzymatic activity of alkyl hydroperoxidase (AhpC) or indirectly by periplasmic SodCI, which detoxifies O_2^- and thereby limits $ONOO^-$ formation. NO can be directly reduced by the denitrosylase activity of the flavohemoprotein Hmp. Transcription of *hmpA* is de-repressed by the inactivation of the [2Fe–2S] redox active prosthetic group of the NsrR transcriptional repressor by NO. In hypoxic or anaerobic conditions, both the flavorubredoxin (NorV) and the cytochrome c nitrite reductase (NrfA) can reduce NO to nitrous oxide (N_2O). NO interacts with iron (Fe) and small-molecular weight thiols (SMWT) to generate dinitrosyl–iron complexes (DNICs), while its reaction with O_2 gives rise to dinitrogen trioxide (N_2O_3). Iron storage proteins (ISP) such as ferritins can limit the generation of DNICs by restricting available Fe. Homocysteine or SMWT antagonize the S-nitrosylation associated with N_2O_3 or DNIC. The SPI2 regulator SsrB is an example of a *Salmonella* protein that gets S-nitrosylated. A variety of RNS can oxidize purines and pyrimidines in the chromosome of *Salmonella*. The combined action of base excision repair (BER) glycosylases and Xth/Nfo endonucleases can repair these lesions.

CONSTITUTIVE DETOXIFICATION OF NO

Thiol-based scavenging systems serve as a means of directly removing RNS. Homocysteine, an intermediate in the methionine biosynthetic pathway, has been shown to enhance the resistance of *Salmonella* to S-nitrosothiols (De Groote et al., 1996). Furthermore, homocysteine adds to the antinitrosative defenses of *Salmonella* in a murine model of salmonellosis (De Groote et al., 1996). Other thiol-based scavengers, including cysteine and the tripeptide glutathione, could serve similar roles. Nonetheless, the contribution of these low-molecular weight thiols to the antinitrosative defenses of *Salmonella* awaits investigation.

INDUCIBLE DETOXIFICATION OF RNS

Enzymatic detoxification of NO

The flavohemoprotein Hmp is the primary means of NO detoxification in *Salmonella* (Bang et al., 2006). This flavohemoprotein, which is expressed by *Salmonella* within the intracellular environment of professional phagocytes (Eriksson et al., 2003), contributes to the inducible antinitrosative response of *Salmonella* and many other organisms by denitrosylating NO to NO_3^-, utilizing in the process O_2, NADH, and FAD (Crawford and Goldberg, 1998; Hausladen et al., 2001). Hmp limits the accumulation of low-molecular weight nitrosothiols in *Salmonella*-infected macrophages, and protects *Salmonella* against authentic NO while minimizing the anti-*Salmonella* activity of RNS generated by murine and human macrophages (Stevanin et al., 2002; Bang et al., 2006; Gilberthorpe et al., 2007; Laver et al., 2010). Moreover, Hmp contributes to the antinitrosative defenses of *Salmonella* in an Nramp1$^+$ murine model of salmonellosis (Bang et al., 2006). The constitutive expression of *hmpA*, nonetheless, leads to a loss of *Salmonella* fitness through its O_2^--producing capacity (McLean et al., 2010). Transcription of *hmpA* is greatly increased in response to NO (Crawford and Goldberg, 1998). Expression of *hmpA* is under the control of the redox active repressor NsrR (Bang et al., 2006), whose [Fe–S] cluster is reversibly inactivated by NO. In addition, *hmpA* expression is upregulated under iron-limiting conditions through NsrR, but independently of Fur (Bang et al., 2006).

Since significant antinitrosative activity of Hmp requires the consumption of O_2, the role of this flavohemoprotein might be limited in hypoxic or anoxic environments. The flavorubredoxin (NorV) and cytochrome *c* nitrite reductase (NrfA) not only reduce NO to nitrous oxide (N_2O), but are also important for the resistance of *Salmonella* to RNS under anaerobic conditions (Mills et al., 2005, 2008). However, these enzymes appear to contribute minimally to the antinitrosative defenses of *Salmonella in vivo*, because mutants lacking either *norV* or *nrfA* are virulent when inoculated intraperitoneally (Bang et al., 2006). It remains possible that NorV and NrfA may be important for resistance to nitrosative stress in the anoxic environment of the intestine. *Salmonella* also possess pathways for the denitrification of NO. The role of nitrogen metabolism in *Salmonella* has not been evaluated in the context of pathogenesis. However, similar to *E. coli* O157 (Jones et al., 2007), the terminal electron acceptor NO_3^- might also be important for colonization of the gastrointestinal tract by *Salmonella*. O_2 is thought to be limiting in the gut and in the intracellular environment of phagocytes and, therefore, alternative electron acceptors must be used instead. A limited respiratory activity can be aggravated under nitrosative stress that inhibits terminal cytochromes of the electron transport chain. Thus, the nitrate reductase complex NarGHIJ and the global regulator Fnr could help in maintaining NO homeostasis and resistance to RNS during some phases of the infection (Gilberthorpe and Poole, 2008).

Enzymatic detoxification of peroxynitrite

$ONOO^-$ is formed through the reaction of NO with O_2^- (Koppenol et al., 1992). $ONOO^-$ is a strong oxidant capable of modifying lipids, amino acids, DNA, and redox active metal centers of dehydratases (Radi, 2004). In analogy to the extensive functional overlap in the enzymatic detoxification of reactive oxygen species (Hebrard et al., 2009), *Salmonella* can antagonize $ONOO^-$ through both indirect and direct mechanisms. By consuming the O_2^- precursor, *Salmonella* periplasmic Cu/Zn superoxide dismutase SodCI prevents $ONOO^-$ formation (De Groote et al., 1997). SodCI has been shown to be crucial for *Salmonella* resistance to the synergistic cytotoxicity of

O_2^- and NO produced by NADPH oxidase and iNOS hemoproteins (De Groote et al., 1997; Sansone et al., 2002). Alternatively, $ONOO^-$ can be detoxified to NO_2^- by the peroxiredoxin-alkyl hydroperoxide reductase AhpC (Chen et al., 1998). In contrast to *sodCI*, *ahpC* is not essential for *Salmonella* pathogenesis (Taylor et al., 1998), raising the possibility that SodCI is the primary means of protection against $ONOO^-$ *in vivo*.

METABOLIC FLUX IN THE PROTECTIVE RESPONSE AGAINST NO

Given that NO can inhibit several enzymes of central metabolic pathways (Castro et al., 1994; Keyer and Imlay, 1997; Brandes et al., 2007; Hyduke et al., 2007), *Salmonella* likely coordinate a metabolic response to this diatomic radical. In accordance with this idea, glucose-6-phosphate dehydrogenase (Zwf) of the pentose phosphate pathway is important for resistance to RNS *in vitro* and *in vivo* (Lundberg et al., 1999). The gene encoding Zwf is part of the SoxR regulon, an [Fe–S] cluster-containing transcription factor activated by NO in *E. coli* (Ding and Demple, 2000). Zwf shuffles the flow of carbon through the pentose phosphate pathway, producing NADPH reducing equivalents in the process. NADPH could fuel glutathione oxidoreductase or thioredoxin reductase to repair damage caused by RNS. Moreover, the downstream non-oxidative branch of the pentose phosphate pathway generates precursors for the biosynthesis of nucleotides, which are needed for repair of RNS-mediated DNA damage.

REPAIR OF DNA LESIONS IN RESISTANCE TO RNS

Reactive nitrogen species produced in response to *Salmonella* can damage DNA by oxidizing purines and pyrimidines. The concerted actions of base excision repair glycosylases and apurinic/apyrimidinic Xth/Nfo endonucleases protect *Salmonella* against products of iNOS in macrophages and contribute to *Salmonella* virulence in an acute model of infection (Suvarnapunya et al., 2003; Richardson et al., 2009). Because the repair of damaged DNA requires nucleotides, *Salmonella* must use NO-resistant pathways for the biosynthesis of deoxyribonucleotides. As described above, NO can inhibit ribonucleotide reductase by reacting with the tyrosyl radical. Interestingly, *Salmonella* encodes other ribonucleotide reductases that may be resistant to the inhibitory effects of NO (Panosa et al., 2010).

CONCLUSIONS

Bacterial pathogens must adapt to changing environmental conditions to survive and cause disease. *Salmonella* experiences the stress imposed by RNS generated during the course of infection. NO produced by iNOS in response to *Salmonella* infection is involved in a broad range of pathophysiological processes, acting both as a signaling molecule and a potent antimicrobial mediator. RNS inhibit assorted bacterial targets involved in a variety of cellular processes. Given this strong selective pressure, *Salmonella* have developed mechanisms to counteract the cytotoxicity of RNS. Even more unexpectedly, recent investigations have shown that *Salmonella* can take advantage of the RNS to bolster growth in infected tissues.

By actively invading the gastrointestinal epithelia, *Salmonella* induce inflammation that promotes its colonization and spread. The NO produced as part of the inflammatory process may generate alternative electron acceptors that can be utilized by *Salmonella* in the O_2-depleted environment of the gut. The ability of *Salmonella* to utilize NO_3^- as an alternative electron acceptor may be especially important under nitrosative stress conditions that repress respiratory activity. Unexpectedly, independent lines of recent evidence indicate the RNS increase the fitness of *Salmonella* and allow the bacteria to outcompete intestinal microbiota (Stecher et al., 2007; Ackermann et al., 2008). Outcompeting the normal flora not only promotes colonization of the intestine and spread to systemic locations, but also promotes diarrhea that serves to disseminate this enteropathogen in the environment. NO sensing and regulation of virulence expression is a fascinating aspect of the emerging view that *Salmonella* do in fact co-opt RNS generated in the host response. The SPI2 regulator SsrB is the first example of a *Salmonella* regulatory protein required for fine-tuning virulence in the context of RNS generated in the host response. The redox active Cys^{203} in SsrB serves as a molecular switch to tightly control gene expression during the course of salmonellosis. It is highly likely that *Salmonella* possesses a variety of sensors, such as Fur, devoted to coordinating responses to NO.

Recent investigations have uncovered novel mechanisms by which *Salmonella* circumvent the detrimental effects of RNS produced during the host response to this facultative intracellular pathogen. Understanding the molecular mechanisms that coordinate *Salmonella* virulence in response to NO will advance our understanding of host–pathogen interactions taking place in the course of salmonellosis. This information can, in turn, illuminate novel therapeutic strategies to decrease the health burden that *Salmonella* infections inflict across the globe.

ACKNOWLEDGMENTS

This review was supported by grants from the Burroughs Welcome Fund, NIH project AI54959, and the Institutional Training grant T32 AI052066. We would like to thank Dr. Jessica Jones-Carson for discussions.

REFERENCES

Ables, G. P., Takamatsu, D., Noma, H., El-Shazly, S., Jin, H. K., Taniguchi, T., Sekikawa, K., and Watanabe, T. (2001). The roles of Nramp1 and Tnfα genes in nitric oxide production and their effect on the growth of *Salmonella* typhimurium in macrophages from Nramp1 congenic and tumor necrosis factor α–/– mice. *J. Interferon Cytokine Res.* 21, 53–62.

Ackermann, M., Stecher, B., Freed, N. E., Songhet, P., Hardt, W. D., and Doebeli, M. (2008). Self-destructive cooperation mediated by phenotypic noise. *Nature* 454, 987–990.

Alam, M. S., Akaike, T., Okamoto, S., Kubota, T., Yoshitake, J., Sawa, T., Miyamoto, Y., Tamura, F., and Maeda, H. (2002). Role of nitric oxide in host defense in murine salmonellosis as a function of its antibacterial and antiapoptotic activities. *Infect. Immun.* 70, 3130–3142.

Alam, M. S., Zaki, M. H., Sawa, T., Islam, S., Ahmed, K. A., Fujii, S., Okamoto, T., and Akaike, T. (2008). Nitric oxide produced in Peyer's patches exhibits antiapoptotic activity contributing to an antimicrobial effect in murine salmonellosis. *Microbiol. Immunol.* 52, 197–208.

Azenabor, A. A., Kennedy, P., and York, J. (2009). Free intracellular Ca^{2+} regulates bacterial lipopolysaccharide induction of iNOS in human macrophages. *Immunobiology* 214, 143–152.

Bang, I. S., Liu, L., Vazquez-Torres, A., Crouch, M. L., Stamler, J. S., and Fang, F. C. (2006). Maintenance of nitric oxide and redox homeostasis by the *Salmonella* flavohemoglobin hmp. *J. Biol. Chem.* 281, 28039–28047.

Barman, M., Unold, D., Shifley, K., Amir, E., Hung, K., Bos, N., and Salzman, N.

(2008). Enteric salmonellosis disrupts the microbial ecology of the murine gastrointestinal tract. *Infect. Immun.* 76, 907–915.

Bergsbaken, T., Fink, S. L., and Cookson, B. T. (2009). Pyroptosis: host cell death and inflammation. *Nat. Rev. Microbiol.* 7, 99–109.

Bosworth, C. A., Toledo, J. C. Jr., Zmijewski, J. W., Li, Q., and Lancaster, J. R. Jr. (2009). Dinitrosyliron complexes and the mechanism(s) of cellular protein nitrosothiol formation from nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4671–4676.

Bourret, T. J., Porwollik, S., M. McClelland, Zhao, R., Greco, T., Ischiropoulos, H., and Vazquez-Torres, A. (2008). Nitric oxide antagonizes the acid tolerance response that protects *Salmonella* against innate gastric defenses. *PLoS ONE* 3, e1833. doi: 10.1371/journal.pone.0001833

Bourret, T. J., Song, M., and Vazquez-Torres, A. (2009). Codependent and independent effects of nitric oxide-mediated suppression of PhoPQ and *Salmonella* pathogenicity island 2 on intracellular *Salmonella enterica* serovar typhimurium survival. *Infect. Immun.* 77, 5107–5115.

Boveris, A., and Chance, B. (1973). The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem. J.* 134, 707–716.

Brandes, N., Rinck, A., Leichert, L. I., and Jakob, U. (2007). Nitrosative stress treatment of *E. coli* targets distinct set of thiol-containing proteins. *Mol. Microbiol.* 66, 901–914.

Butler, C. S., Seward, H. E., Greenwood, C., and Thomson, A. J. (1997). Fast cytochrome bo from *Escherichia coli* binds two molecules of nitric oxide at CuB. *Biochemistry* 36, 16259–16266.

Buzzo, C. L., Campopiano, J. C., Massis, L. M., Lage, S. L., Cassado, A. A., Leme-Souza, R., Cunha, L. D., Russo, M., Zamboni, D. S., Amarante-Mendes, G. P., and Bortoluci, K. R. (2010). A novel pathway for inducible nitric oxide synthase activation through inflammasomes. *J. Biol. Chem.* 285, 32087–32095.

Castro, L., Rodriguez, M., and Radi, R. (1994). Aconitase is readily inactivated by peroxynitrite, but not by its precursor, nitric oxide. *J. Biol. Chem.* 269, 29409–29415.

Cellier, M. F., Courville, P., and Campion, C. (2007). Nramp1 phagocyte intracellular metal withdrawal defense. *Microbes Infect.* 9, 1662–1670.

Cerquetti, M. C., Goren, N. B., Ropolo, A. J., Grasso, D., Giacomodonato, M. N., and Vaccaro, M. I. (2002). Nitric oxide and apoptosis induced in Peyer's patches by attenuated strains of *Salmonella enterica* serovar Enteritidis. *Infect. Immun.* 70, 964–969.

Chakravortty, D., Hansen-Wester, I., and Hensel, M. (2002). *Salmonella* pathogenicity island 2 mediates protection of intracellular *Salmonella* from reactive nitrogen intermediates. *J. Exp. Med.* 195, 1155–1166.

Chen, L., Xie, Q. W., and Nathan, C. (1998). Alkyl hydroperoxide reductase subunit C (AhpC) protects bacterial and human cells against reactive nitrogen intermediates. *Mol. Cell* 1, 795–805.

Closs, E. I., Enseleit, F., Koesling, D., Pfeilschifter, J. M., Schwarz, P. M., and Forstermann, U. (1998). Coexpression of inducible NO synthase and soluble guanylyl cyclase in colonic enterocytes: a pathophysiologic signaling pathway for the initiation of diarrhea by gram-negative bacteria? *FASEB J.* 12, 1643–1649.

Coombes, B. K., Wickham, M. E., Lowden, M. J., Brown, N. F., and Finlay, B. B. (2005). Negative regulation of *Salmonella* pathogenicity island 2 is required for contextual control of virulence during typhoid. *Proc. Natl. Acad. Sci. U.S.A.* 102, 17460–17465.

Crawford, M. J., and Goldberg, D. E. (1998). Role for the *Salmonella* flavohemoglobin in protection from nitric oxide. *J. Biol. Chem.* 273, 12543–12547.

Das, P., Lahiri, A., and Chakravortty, D. (2009). Novel role of the nitrite transporter NirC in *Salmonella* pathogenesis: SPI2-dependent suppression of inducible nitric oxide synthase in activated macrophages. *Microbiology* 155, 2476–2489.

D'Autreaux, B., Touati, D., Bersch, B., Latour, J. M., and Michaud-Soret, I. (2002). Direct inhibition by nitric oxide of the transcriptional ferric uptake regulation protein via nitrosylation of the iron. *Proc. Natl. Acad. Sci. U.S.A.* 99, 16619–16624.

De Groote, M. A., Granger, D., Xu, Y., Campbell, G., Prince, R., and Fang, F. C. (1995). Genetic and redox determinants of nitric oxide cytotoxicity in a *Salmonella* typhimurium model. *Proc. Natl. Acad. Sci. U.S.A.* 92, 6399–6403.

De Groote, M. A., Ochsner, U. A., Shiloh, M. U., Nathan, C., McCord, J. M., Dinauer, M. C., Libby, S. J., Vazquez-Torres, A., Xu, Y., and Fang, F. C. (1997). Periplasmic superoxide dismutase protects *Salmonella* from products of phagocyte NADPH oxidase and nitric oxide synthase. *Proc. Natl. Acad. Sci. U.S.A.* 94, 13997–14001.

De Groote, M. A., Testerman, T., Xu, Y., Stauffer, G., and Fang, F. C. (1996). Homocysteine antagonism of nitric oxide-related cytostasis in *Salmonella* typhimurium. *Science* 272, 414–417.

de Jong, R., Altare, F., Haagen, I. A., Elferink, D. G., Boer, T., van Breda Vriesman, P. J., Kabel, P. J., Draaisma, J. M., van Dissel, J. T., Kroon, F. P., Casanova, J. L., and Ottenhoff, T. H. (1998). Severe mycobacterial and *Salmonella* infections in interleukin-12 receptor-deficient patients. *Science* 280, 1435–1438.

Ding, H., and Demple, B. (2000). Direct nitric oxide signal transduction via nitrosylation of iron–sulfur centers in the SoxR transcription activator. *Proc. Natl. Acad. Sci. U.S.A.* 97, 5146–5150.

Duncan, C., Dougall, H., Johnston, P., Green, S., Brogan, R., Leifert, C., Smith, L., Golden, M., and Benjamin, N. (1995). Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. *Nat. Med.* 1, 546–551.

Eiserich, J. P., Hristova, M., Cross, C. E., Jones, A. D., Freeman, B. A., Halliwell, B., and A. van der Vliet. (1998). Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* 391, 393–397.

Eriksson, S., Bjorkman, J., Borg, S., Syk, A., Pettersson, S., Andersson, D. I., and Rhen, M. (2000). *Salmonella* typhimurium mutants that downregulate phagocyte nitric oxide production. *Cell. Microbiol.* 2, 239–250.

Eriksson, S., Lucchini, S., Thompson, A., Rhen, M., and Hinton, J. C. (2003). Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. *Mol. Microbiol.* 47, 103–118.

Fields, P. I., Swanson, R. V., Haidaris, C. G., and Heffron, F. (1986). Mutants of *Salmonella* typhimurium that cannot survive within the macrophage are avirulent. *Proc. Natl. Acad. Sci. U.S.A.* 83, 5189–5193.

Foster, J. W., and Hall, H. K. (1990). Adaptive acidification tolerance response of *Salmonella* typhimurium. *J. Bacteriol.* 172, 771–778.

Fritsche, G., Dlaska, M., Barton, H., Theurl, I., Garimorth, K., and Weiss, G. (2003). Nramp1 functionality increases inducible nitric oxide synthase transcription via stimulation of IFN regulatory factor 1 expression. *J. Immunol.* 171, 1994–1998.

Fritsche, G., Nairz, M., Werner, E. R., Barton, H. C., and Weiss, G. (2008). Nramp1-functionality increases iNOS expression via repression of IL-10 formation. *Eur. J. Immunol.* 38, 3060–3067.

Georgellis, D., Kwon, O., and Lin, E. C. (2001). Quinones as the redox signal for the arc two-component system of bacteria. *Science* 292, 2314–2316.

Gewirtz, A. T., Simon, P. O. Jr., Schmitt, C. K., Taylor, L. J., Hagedorn, C. H., O'Brien, A. D., Neish, A. S., and Madara, J. L. (2001). *Salmonella* typhimurium translocates flagellin across intestinal epithelia, inducing a proinflammatory response. *J. Clin. Invest.* 107, 99–109.

Giacomodonato, M. N., Goren, N. B., Sordelli, D. O., Vaccaro, M. I., Grasso, D. H., Ropolo, A. J., and Cerquetti, M. C. (2003). Involvement of intestinal inducible nitric oxide synthase (iNOS) in the early stages of murine salmonellosis. *FEMS Microbiol. Lett.* 223, 231–238.

Gilberthorpe, N. J., Lee, M. E., Stevanin, T. M., Read, R. C., and Poole, R. K. (2007). NsrR: a key regulator circumventing *Salmonella enterica* serovar Typhimurium oxidative and nitrosative stress *in vitro* and in IFNγ-stimulated J774.2 macrophages. *Microbiology* 153, 1756–1771.

Gilberthorpe, N. J., and Poole, R. K. (2008). Nitric oxide homeostasis in *Salmonella* typhimurium: roles of respiratory nitrate reductase and flavohemoglobin. *J. Biol. Chem.* 283, 11146–11154.

Gomes, N. E., Brunialti, M. K., Mendes, M. E., Freudenberg, M., Galanos, C., and Salomao, R. (2010). Lipopolysaccharide-induced expression of cell surface receptors and cell activation of neutrophils and monocytes in whole human blood. *Braz. J. Med. Biol. Res.* 43, 853–858.

Govoni, G., and Gros, P. (1998). Macrophage NRAMP1 and its role in resistance to microbial infections. *Inflamm. Res.* 47, 277–284.

Hausladen, A., and Fridovich, I. (1994). Superoxide and peroxynitrite inactivate aconitases, but nitric oxide does not. *J. Biol. Chem.* 269, 29405–29408.

Hausladen, A., Gow, A., and Stamler, J. S. (2001). Flavohemoglobin denitrosylase catalyzes the reaction of a nitroxyl equivalent with molecular oxygen. *Proc. Natl. Acad. Sci. U.S.A.* 98, 10108–10112.

Hebrard, M., Viala, J. P., Meresse, S., Barras, F., and Aussel, L. (2009). Redundant hydrogen peroxide scavengers contribute to *Salmonella* virulence and oxidative stress resistance. *J. Bacteriol.* 191, 4605–4614.

Hersh, D., Monack, D. M., Smith, M. R., Ghori, N., Falkow, S., and Zychlinsky, A. (1999). The *Salmonella* invasin SipB induces macrophage apoptosis by binding to caspase-1. *Proc. Natl. Acad. Sci. U.S.A.* 96, 2396–2401.

Husain, M., Bourret, T. J., McCollister, B. D., Jones-Carson, J., Laughlin, J., and Vazquez-Torres, A. (2008). Nitric oxide evokes an adaptive response to oxidative stress by arresting respiration. *J. Biol. Chem.* 283, 7682–7689.

Husain, M., Jones-Carson, J., Song, M., McCollister, B. D., Bourret, T. J., and Vazquez-Torres, A. (2010). Redox sensor SsrB Cys_20_3 enhances *Salmonella* fitness against nitric oxide generated in the host immune response to oral infection. *Proc. Natl. Acad. Sci. U.S.A.* 107, 14396–14401.

Hyduke, D. R., Jarboe, L. R., Tran, L. M., Chou, K. J., and Liao, J. C. (2007). Integrated network analysis identifies nitric oxide response networks and dihydroxyacid dehydratase as a crucial target in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 8484–8489.

Jantsch, J., Cheminay, C., Chakravortty, D., Lindig, T., Hein, J., and Hensel, M. (2003). Intracellular activities of *Salmonella enterica* in murine dendritic cells. *Cell. Microbiol.* 5, 933–945.

Jones, S. A., Chowdhury, F. Z., Fabich, A. J., Anderson, A., Schreiner, D. M., House, A. L., Autieri, S. M., Leatham, M. P., Lins, J. J., Jorgensen, M., Cohen, P. S., and Conway, T. (2007). Respiration of *Escherichia coli* in the mouse intestine. *Infect. Immun.* 75, 4891–4899.

Jones-Carson, J., Vazquez-Torres, A., van der Heyde, H. C., Warner, T., Wagner, R. D., and Balish, E. (1995). γδ T cell-induced nitric oxide production enhances resistance to mucosal candidiasis. *Nat. Med.* 1, 552–557.

Kamijo, R., Harada, H., Matsuyama, T., Bosland, M., Gerecitano, J., Shapiro, D., Le, J., Koh, S. I., Kimura, T., Green, S. J., Mak, T. W., Taniguchi, T., and Vilcek, J. (1994). Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. *Science* 263, 1612–1615.

Kawano, M., Manabe, T., and Kawasaki, K. (2010). *Salmonella enterica* serovar Typhimurium lipopolysaccharide deacylation enhances its intracellular growth within macrophages. *FEBS Lett.* 584, 207–212.

Keyer, K., and Imlay, J. A. (1997). Inactivation of dehydratase [4Fe–4S] clusters and disruption of iron homeostasis upon cell exposure to peroxynitrite. *J. Biol. Chem.* 272, 27652–27659.

Khan, S. A., Strijbos, P. J., Everest, P., Moss, D., Stratford, R., Mastroeni, P., Allen, J., Servos, S., Charles, I. G., Dougan, G., and Maskell, D. J. (2001). Early responses to *Salmonella* typhimurium infection in mice occur at focal lesions in infected organs. *Microb. Pathog.* 30, 29–38.

Koppenol, W. H., Moreno, J. J., Pryor, W. A., Ischiropoulos, H., and Beckman, J. S. (1992). Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. *Chem. Res. Toxicol.* 5, 834–842.

Laver, J. R., Stevanin, T. M., Messenger, S. L., Lunn, A. D., Lee, M. E., Moir, J. W., Poole, R. K., and Read, R. C. (2010). Bacterial nitric oxide detoxification prevents host cell S-nitrosothiol formation: a novel mechanism of bacterial pathogenesis. *FASEB J.* 24, 286–295.

Lepoivre, M., Fieschi, F., Coves, J., Thelander, L., and Fontecave, M. (1991). Inactivation of ribonucleotide reductase by nitric oxide. *Biochem. Biophys. Res. Commun.* 179, 442–448.

Lucchini, S., Rowley, G., Goldberg, M. D., Hurd, D., Harrison, M., and Hinton, J. C. (2006). H-NS mediates the silencing of laterally acquired genes in bacteria. *PLoS Pathog.* 2, e81. doi: 10.1371/journal.ppat.0020081

Lundberg, B. E., Wolf, R. E. Jr., Dinauer, M. C., Xu, Y., and Fang, F. C. (1999). Glucose 6-phosphate dehydrogenase is required for *Salmonella* typhimurium virulence and resistance to reactive oxygen and nitrogen intermediates. *Infect. Immun.* 67, 436–438.

Lundberg, J. O., Weitzberg, E., Cole, J. A., and Benjamin, N. (2004). Nitrate, bacteria and human health. *Nat. Rev. Microbiol.* 2, 593–602.

Mason, M. G., Shepherd, M., Nicholls, P., Dobbin, P. S., Dodsworth, K. S., Poole, R. K., and Cooper, C. E. (2009). Cytochrome bd confers nitric oxide resistance to *Escherichia coli*. *Nat. Chem. Biol.* 5, 94–96.

Mastroeni, P., Vazquez-Torres, A., Fang, F. C., Xu, Y., Khan, S., Hormaeche, C. E., and Dougan, G. (2000). Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial proliferation and host survival *in vivo*. *J. Exp. Med.* 192, 237–248.

McCollister, B. D., Bourret, T. J., Gill, R., Jones-Carson, J., and Vazquez-Torres, A. (2005). Repression of SPI2 transcription by nitric oxide-producing, IFNγ-activated macrophages promotes maturation of *Salmonella* phagosomes. *J. Exp. Med.* 202, 625–635.

McCollister, B. D., Hoffman, M., Husain, M., and Vazquez-Torres, A. (2011). Nitric oxide protects bacteria from aminoglycosides by blocking the energy-dependent phases of drug uptake. *Antimicrob. Agents Chemother.* doi: 10.1128/AAC.01203-10. [Epub ahead of print].

McCollister, B. D., Myers, J. T., Jones-Carson, J., Husain, M., Bourret, T. J., and Vazquez-Torres, A. (2007). N_2O_3 enhances the nitrosative potential of IFNγ-primed macrophages in response to *Salmonella*. *Immunobiology* 212, 759–769.

McKnight, G. M., Smith, L. M., Drummond, R. S., Duncan, C. W., Golden, M., and Benjamin, N. (1997). Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans. *Gut* 40, 211–214.

McLean, S., Bowman, L. A., and Poole, R. K. (2010). Peroxynitrite stress is exacerbated by flavohaemoglobin-derived oxidative stress in *Salmonella* Typhimurium and relieved by NO. *Microbiology* 156, 3556–3565.

Melotto, M., Underwood, W., Koczan, J., Nomura, K. and He, S. Y. (2006). Plant stomata function in innate immunity against bacterial invasion. *Cell* 126, 969–980.

Mills, P. C., Richardson, D. J., Hinton, J. C., and Spiro, S. (2005). Detoxification of nitric oxide by the flavorubredoxin of *Salmonella enterica* serovar Typhimurium. *Biochem. Soc. Trans.* 33, 198–199.

Mills, P. C., Rowley, G., Spiro, S., Hinton, J. C., and Richardson, D. J. (2008). A combination of cytochrome c nitrite reductase (NrfA) and flavorubredoxin (NorV) protects *Salmonella enterica* serovar Typhimurium against killing by NO in anoxic environments. *Microbiology* 154, 1218–1228.

Muller, A. J., Hoffmann, C., Galle, M., Van Den Broeke, A., Heikenwalder, M., Falter, L., Misselwitz, B., Kremer, M., Beyaert, R., and Hardt, W. D. (2009). The Typhimurium, *S.* effector SopE induces caspase-1 activation in stromal cells to initiate gut inflammation. *Cell Host Microbe* 6, 125–136.

Nairz, M., Fritsche, G., Crouch, M. L., Barton, H. C., Fang, F. C., and Weiss, G. (2009). Slc11a1 limits intracellular growth of *Salmonella enterica* sv. Typhimurium by promoting macrophage immune effector functions and impairing bacterial iron acquisition. *Cell. Microbiol.* 11, 1365–1381.

Nairz, M., Schroll, A., Moschen, A. R., Sonnweber, T., Theurl, M., Theurl, I., Taub, N., Jamnig, C., Neurauter, D., Huber, L. A., Tilg, H., Moser, P. L., and Weiss, G. (2011). Erythropoietin contrastingly affects bacterial infection and experimental colitis by inhibiting nuclear factor-κB-inducible immune pathways. *Immunity* 31, 61–74.

Navarre, W. W., Porwollik, S., Wang, Y., McClelland, M., Rosen, H., Libby, S. J., and Fang, F. C. (2006). Selective silencing of foreign DNA with low GC content by the H-NS protein in *Salmonella*. *Science* 313, 236–238.

Pacelli, R., Wink, D. A., Cook, J. A., Krishna, M. C., DeGraff, W., Friedman, N., Tsokos, M., Samuni, A., and Mitchell, J. B. (1995). Nitric oxide potentiates hydrogen peroxide-induced killing of *Escherichia coli*. *J. Exp. Med.* 182, 1469–1479.

Panosa, A., Roca, I., and Gibert, I. (2010). Ribonucleotide reductases of *Salmonella* typhimurium: transcriptional regulation and differential role in pathogenesis. *PLoS ONE* 5, e11328. doi: 10.1371/journal.pone.0011328

Prior, K., Hautefort, I., Hinton, J. C., Richardson, D. J., and Rowley, G. (2009). All stressed out. *Salmonella* pathogenesis and reactive nitrogen species. *Adv. Microb. Physiol.* 56, 1–28.

Radi, R. (2004). Nitric oxide, oxidants, and protein tyrosine nitration. *Proc. Natl. Acad. Sci. U.S.A.* 101, 4003–4008.

Raupach, B., Peuschel, S. K., Monack, D. M., and Zychlinsky, A. (2006). Caspase-1-mediated activation of interleukin-1β (IL-1β) and IL-18 contributes to innate immune defenses against *Salmonella enterica* serovar Typhimurium infection. *Infect. Immun.* 74, 4922–4926.

Richardson, A. R., Soliven, K. C., Castor, M. E., Barnes, P. D., Libby, S. J., and Fang, F. C. (2009). The base excision repair system of *Salmonella enterica* serovar Typhimurium counteracts DNA damage by host nitric oxide. *PLoS Pathog.* 5, e1000451. doi: 10.1371/journal.ppat.1000451

Sansone, A., Watson, P. R., Wallis, T. S., Langford, P. R., and Kroll, J. S. (2002). The role of two periplasmic copper- and zinc-cofactored superoxide dismutases in the virulence of *Salmonella* choleraesuis. *Microbiology* 148, 719–726.

Schapiro, J. M., Libby, S. J., and Fang, F. C. (2003). Inhibition of bacterial DNA replication by zinc mobilization during nitrosative stress. *Proc. Natl. Acad. Sci. U.S.A.* 100, 8496–8501.

Schwacha, M. G., and Eisenstein, T. K. (1997). Interleukin-12 is critical for induction of nitric oxide-mediated immunosuppression following vaccination of mice with attenuated *Salmonella* typhimurium. *Infect. Immun.* 65, 4897–4903.

Stecher, B., Robbiani, R., Walker, A. W., Westendorf, A. M., Barthel, M., Kremer, M., Chaffron, S., Macpherson, A. J., Buer, J., Parkhill, J., Dougan, G., von Mering, C., and Hardt, W. D. (2007). *Salmonella enterica* serovar Typhimurium exploits inflammation to compete with the intestinal microbiota. *PLoS Biol.* 5, 2177–2189. doi: 10.1371/journal.pbio.0050244

Stevanin, T. M., Poole, R. K., Demoncheaux, E. A., and Read, R. C. (2002). Flavohemoglobin Hmp protects *Salmonella enterica* serovar Typhimurium from nitric oxide-related killing by human macrophages. *Infect. Immun.* 70, 4399–4405.

Sun, Y. H., Rolan, H. G., and Tsolis, R. M. (2007). Injection of flagellin into the host cell cytosol by *Salmonella enterica* serotype Typhimurium. *J. Biol. Chem.* 282, 33897–33901.

Suvarnapunya, A. E., Lagasse, H. A., and Stein, M. A. (2003). The role of DNA base excision repair in the

pathogenesis of *Salmonella enterica* serovar Typhimurium. *Mol. Microbiol.* 48, 549–559.

Talbot, S., Totemeyer, S., Yamamoto, M., Akira, S., Hughes, K., Gray, D., Barr, T., Mastroeni, P., Maskell, D. J., and Bryant, C. E. (2009). Toll-like receptor 4 signalling through MyD88 is essential to control *Salmonella enterica* serovar Typhimurium infection, but not for the initiation of bacterial clearance. *Immunology* 128, 472–483.

Tannenbaum, S. R., Fett, D., Young, V. R., Land, P. D., and Bruce, W. R. (1978). Nitrite and nitrate are formed by endogenous synthesis in the human intestine. *Science* 200, 1487–1489.

Taylor, P. D., Inchley, C. J., and Gallagher, M. P. (1998). The *Salmonella* typhimurium AhpC polypeptide is not essential for virulence in BALB/c mice but is recognized as an antigen during infection. *Infect. Immun.* 66, 3208–3217.

Toshchakov, V., Jones, B. W., Perera, P. Y., Thomas, K., Cody, M. J., Zhang, S., Williams, B. R., Major, J., Hamilton, T. A., Fenton, M. J., and Vogel, S. N. (2002). TLR4, but not TLR2, mediates IFNβ-induced STAT1α/β-dependent gene expression in macrophages. *Nat. Immunol.* 3, 392–398.

Troxell, B., Sikes, M. L., Fink, R. C., Vazquez-Torres, A., Jones-Carson, J., and Hassan, H. M. (2010). Fur negatively regulates hns and is required for the zxpression of HilA and virulence in *Salmonella enterica* serovar Typhimurium. *J. Bacteriol.* 193, 497–505.

Tsolis, R. M., Adams, L. G., Ficht, T. A., and Baumler, A. J. (1999). Contribution of *Salmonella* typhimurium virulence factors to diarrheal disease in calves. *Infect. Immun.* 67, 4879–4885.

Umezawa, K., Akaike, T., Fujii, S., Suga, M., Setoguchi, K., Ozawa, A., and Maeda, H. (1997). Induction of nitric oxide synthesis and xanthine oxidase and their roles in the antimicrobial mechanism against *Salmonella* typhimurium infection in mice. *Infect. Immun.* 65, 2932–2940.

Vazquez-Torres, A., Fantuzzi, G., Edwards, C. K. III, Dinarello, C. A., and Fang, F. C. (2001). Defective localization of the NADPH phagocyte oxidase to *Salmonella*-containing phagosomes in tumor necrosis factor p55 receptor-deficient macrophages. *Proc. Natl. Acad. Sci. U.S.A.* 98, 2561–2565.

Vazquez-Torres, A., Jones-Carson, J., Baumler, A. J., Falkow, S., Valdivia, R., Brown, W., Le, M., Berggren, R., Parks, W. T., and Fang, F. C. (1999). Extraintestinal dissemination of *Salmonella* by CD18-expressing phagocytes. *Nature* 401, 804–808.

Vazquez-Torres, A., Jones-Carson, J., Mastroeni, P., Ischiropoulos, H., and Fang, F. C. (2000). Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. Effects, I. on microbial killing by activated peritoneal macrophages *in vitro*. *J. Exp. Med.* 192, 227–236.

Vazquez-Torres, A., Vallance, B. A., Bergman, M. A., Finlay, B. B., Cookson, B. T., Jones-Carson, J., and Fang, F. C. (2004). Toll-like receptor 4 dependence of innate and adaptive immunity to *Salmonella*: importance of the Kupffer cell network. *J. Immunol.* 172, 6202–6208.

Vitiello, M., D'Isanto, M., Finamore, E., Ciarcia, R., Kampanaraki, A., and Galdiero, M. (2008). Role of mitogen-activated protein kinases in the iNOS production and cytokine secretion by *Salmonella enterica* serovar Typhimurium porins. *Cytokine* 41, 279–285.

Webb, J. L., Harvey, M. W., Holden, D. W., and Evans, T. J. (2001). Macrophage nitric oxide synthase associates with cortical actin but is not recruited to phagosomes. *Infect. Immun.* 69, 6391–6400.

Winter, S. E., Thiennimitr, P., Winter, M. G., Butler, B. P., Huseby, D. L., Crawford, R. W., Russell, J. M., Bevins, C. L., Adams, L. G., Tsolis, R. M., Roth, J. R., and Baumler, A. J. (2010). Gut inflammation provides a respiratory electron acceptor for *Salmonella*. *Nature* 467, 426–429.

Witthoft, T., Eckmann, L., Kim, J. M., and Kagnoff, M. F. (1998). Enteroinvasive bacteria directly activate expression of iNOS and NO production in human colon epithelial cells. *Am. J. Physiol.* 275, G564–G571.

Worley, M. J., Nieman, G. S., Geddes, K., and Heffron, F. (2006). *Salmonella* typhimurium disseminates within its host by manipulating the motility of infected cells. *Proc. Natl. Acad. Sci. U.S.A.* 103, 17915–17920.

Xie, Q. W., Kashiwabara, Y., and Nathan, C. (1994). Role of transcription factor NF-κ B/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* 269, 4705–4708.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 02 February 2011; accepted: 08 April 2011; published online: 20 April 2011.

Citation: Henard CA and Vázquez-Torres A (2011) Nitric oxide and Salmonella pathogenesis. Front. Microbio. ***2****:84. doi: 10.3389/fmicb.2011.00084*

This article was submitted to Frontiers in Cellular and Infection Microbiology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 02 June 2011
doi: 10.3389/fmicb.2011.00121

Technologies and approaches to elucidate and model the virulence program of *Salmonella*

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Salmonella is a primary cause of enteric diseases in a variety of animals. During its evolution into a pathogenic bacterium, *Salmonella* acquired an elaborate regulatory network that responds to multiple environmental stimuli within host animals and integrates them resulting in fine regulation of the virulence program. The coordinated action by this regulatory network involves numerous virulence regulators, necessitating genome-wide profiling analysis to assess and combine efforts from multiple regulons. In this review we discuss recent high-throughput analytic approaches used to understand the regulatory network of *Salmonella* that controls virulence processes. Application of high-throughput analyses have generated large amounts of data and necessitated the development of computational approaches for data integration. Therefore, we also cover computer-aided network analyses to infer regulatory networks, and demonstrate how genome-scale data can be used to construct regulatory and metabolic systems models of *Salmonella* pathogenesis. Genes that are coordinately controlled by multiple virulence regulators under infectious conditions are more likely to be important for pathogenesis. Thus, reconstructing the global regulatory network during infection or, at the very least, under conditions that mimic the host cellular environment not only provides a bird's eye view of *Salmonella* survival strategy in response to hostile host environments but also serves as an efficient means to identify novel virulence factors that are essential for *Salmonella* to accomplish systemic infection in the host.

Keywords: *Salmonella*, virulence, regulatory network, regulators, transcriptomics, proteomics, computational modeling

BACKGROUND

Salmonellae are Gram-negative facultative pathogens that live in diverse environments and infect a wide variety of animals. In addition they can use a great variety of compounds as carbon and energy sources. Gastrointestinal infections are the second most common cause of childhood mortality in the developing world and systemic disease typhoid alone (caused by serovar Typhi) is estimated to result in 500,000 deaths per year (Graham, 2002). In addition to fluid and electrolyte loss, non-typhoidal *Salmonella* often results in septicemia in children and in HIV infected adults in developing countries with a fatality rate of 25% or greater (Graham, 2010). *Salmonella* diverged from *E. coli* more than 100 million years ago (Vernikos et al., 2007), but the evolution within the *Salmonella* genus is more recent. Although there are now only two species of *Salmonella*; *S. enterica* and *S. bongori*, *S. enterica* has diverged into numerous subspecies and serovars that show a great variation in their infectivity for different cold blooded and warm blooded hosts but share greater than 95% DNA sequence homology. In fact, there are over 2500 serovars for *S. enterica* with close genetic homology and yet highly diverse regulatory and virulence expression patterns one can assume based on host preference.

Most of the human pathogenic *Salmonella* serovars, the general subject of virulence regulation in this article, belong to the *S. enterica enterica* subspecies. Often serovars are written with a capital letter and are not italicized, with only the genus name preceding. Thus *Salmonella enterica* subspecies enterica serovar Typhimurium is often written as *Salmonella* Typhimurium and we will use this convention throughout. *Salmonella* Pullorum and Gallinarum are primarily avian pathogens whereas many serotypes such as Rubislaw, Agona, Infantis, and Panama are found in reptiles and amphibians (Pedersen et al., 2009). *S. enterica* Typhi and Paratyphi are host restricted to humans, but Typhimurium can infect a variety of animals including cattle. Possibly because of the diversity of potential hosts and environmental conditions it encounters, the regulatory network for virulence in *Salmonella* is complicated and involves many regulators and a large number of virulence proteins.

One of the earliest genetic-screening approaches to identify the *Salmonella* genes required for virulence was carried out in the Heffron laboratory by infecting primary peritoneal macrophages with *Salmonella* transposon mutants (Fields et al., 1986). After incubation the host cells were lysed and plated on selective bacteriological plates to determine if a specific mutant strain was lost. Over 9500 independently derived transposon insertions were screened one by one in elicited peritoneal macrophages. The most important mutation derived in this first screen was located in *phoP/phoQ*, a two-component regulator, that controls resistance to defensins (Groisman et al., 1989). Many of the genes regulated by *phoP/phoQ* are required for virulence. Other mutations showed increased

sensitivity to oxidizing agents that are found within macrophages. When virulence genes are often present within a contiguous stretch of DNA and are missing in closely related and often non-pathogenic bacteria, it suggests that they were acquired horizontally. These so called pathogenicity islands are about the size that would be carried by a transducing bacteriophage and are often flanked by sequences such as transposons or t-RNA that could permit homologous recombination events. In *Salmonella* two of these regions important in virulence are called *Salmonella* pathogenicity island (SPI)-1 and 2.

Both SPI-1 and SPI-2 encode the structural components of their respective type III secretion system (T3SS) apparatus and translocate effectors for distinct purposes. SPI-1-secreted effectors play essential roles for invasion into epithelial cells and promote intestinal inflammation and gastroenteritis. SPI-1, is required for persistent infections in mice (Monack et al., 2004) and for cell invasion (Steele-Mortimer, 2008; McGhie et al., 2009), but is not required following intraperitoneal infection (i.e., systemic infection models) in BALB/c or C57/BL6 mice (rev. in Zhou and Galan, 2001). SPI-2 is induced during intracellular *Salmonella* infection of a variety of cell types (Geddes et al., 2007) and secretes dozens of distinct effector proteins (Niemann et al., 2011). Mutation of this T3SS does not have a huge effect on intramacrophage survival, however it does result in completely abrogating infection in mice (Buchmeier and Heffron, 1991; Poh et al., 2008). Following internalization into host cells, the SPI-2 T3SS secretes effectors into the cytoplasmic space of epithelial cells or professional phagocytes such as macrophages. SPI-2-secreted effectors prevent maturation of the phagocytic vesicle thereby blocking phago-lysosome fusions and exposure of *Salmonella* to oxidative and non-oxidative microbicidal mechanisms (Buchmeier and Heffron, 1991; Ramsden et al., 2007; Poh et al., 2008). While most known *Salmonella* effectors have been found to be secreted specifically by the SPI-2 or SPI-1 T3SS (Steele-Mortimer, 2008; McGhie et al., 2009; Niemann et al., 2011), some have been shown to utilize both the SPI-1 and SPI-2 T3SSs for efficient secretion (Haraga et al., 2008). This complex mixture of secretion processes for *Salmonella* effectors suggests that there are multiple levels of regulation from transcription to translation to the secretion apparatus each level is critical for the virulence program of these pathogens.

Besides the complex T3SS, *Salmonella* possesses alternative mechanisms to translocate virulence factors into the extracellular milieu. These include outer membrane vesicles (OMV), the two-partner secretion system ZirT/ZirS, and a type VI secretion system. OMV are observed in a variety of Gram-negative bacteria including pathogenic bacteria and deliver specific periplasmic proteins as well as some outer membrane components to the host cells, promoting proinflammatory responses (Ellis et al., 2010). The ZirT/ZirS system, conserved throughout the *Salmonella* genus, secretes ZirS via a hydrophilic β-barrel pore formed by ZirT (Gal-Mor et al., 2008). *Salmonella* lacking this system was hypervirulent, implying a unique function of ZirT/ZirS as an antivirulence modulator during systemic infection. *Salmonella* also horizontally acquired a type VI secretion system (Bingle et al., 2008). SciS, a component homologous to IcmF in *Legionella*, appeared to attenuate virulence to prevent acute infections for a long-term dissemination (Parsons and Heffron, 2005). Due to the variety of secretion mechanisms in *Salmonella*, the virulence program is in many ways surprisingly robust and often challenging to dissect.

Salmonella dwells a variety of environments, thus being able to quickly recognize and respond to the environmental stimuli is essential for its survival. During the course of systemic infection in mice, bacteria are found within neutrophils, monocytes, dendritic cells, and B and T cells but are not found extracellularly until the last 1 or 2 days immediately before death of the host (Dunlap et al., 1994; Yrlid and Wick, 2002; Geddes et al., 2007). The mechanisms of how *Salmonella* survives and replicates within the host and how it regulates virulence genes at the appropriate time during systemic infection is imperfectly understood and the subject of this work. In fact, *Salmonella* encodes more than 300 annotated transcriptional and translational regulators that govern expression of *Salmonella* genes under a myriad of parasitic and free-living conditions (McClelland et al., 2001). By comparison the eukaryote *Saccharomyces cerevisiae* has nearer 100 (Goffeau et al., 1996).

The fact that *Salmonella* can successfully survive in these disparate environments even within a single host is likely one reason for the large number of regulators it encodes. Recently, we have deleted all genes annotated as transcriptional or translational regulators in *Salmonella* and have tested all of them in an acute mouse infection model. There are many regulators that are involved in chemotaxis and cell motility. These have not been examined in detail except for FliA, the flagellar sigma factor (Lockman and Curtiss, 1990). Previous work suggests that with the exception of one or two genes none of the genes required for chemotaxis and motility are necessary for virulence in a BALB/c mouse. In all we have identified 26 regulators that are required for potent virulence in our mouse infection model. This is a surprisingly small number given that 330 were tested and many might have been assumed to result in less fit *Salmonella* generally. In fact, only in-frame deletions in 20 regulator genes conferred a strong virulence deficient phenotype following intraperitoneal (i.p.) infection (**Table 1**), while the remaining deletions affected virulence only in intragastric (i.g.) infection or long-term persistence (l.p.) test, likely suggesting the requirement of those regulators for adaptation to the intestinal environment (i.g. attenuated strains) or for evading the adaptive immune system (l.p. attenuated strains). About 90 of the 330 regulators have been tested by i.g. infection and thus there are likely to be several more than the three listed that are unable to effect by this route.

In this review we discuss recent efforts to understand the regulatory network of *Salmonella* that controls a robust set of virulence processes and mechanisms. We focus here on the application of high-throughput and/or global methodologies to measure virulence and regulatory aspects of *Salmonella*, the computational approaches used to determine regulatory networks, and how this information can be used to construct systems models/simulations of *Salmonella* pathogenesis (**Figure 1**).

EXPERIMENTAL METHODS TO DETERMINE REGULATORY NETWORKS

OVERVIEW

In the first part of this review we cover some of the experimental methods that have been employed to determine virulence patterns and regulatory networks in *Salmonella*, or are currently being used

Table 1 | Regulators involved in virulence regulation.

Gene	Description	Virulence attenuation*	Reference
TRANSCRIPTIONAL REGULATORS			
STM2575	LysR family regulator	i.g.	Unpubl.
STM2912	LysR family regulator	i.g.	Unpubl.
STM0604 (*ybdM*)	Transcriptional regulator related to SpoOJ	i.g.	Unpubl.
hilD	AraC family regulator that controls expression of SPI-1	I.p.	Lucas and Lee (2001), Schechter and Lee (2001), Teplitski et al. (2003)
fis	DNA bending protein required for site specific recombination of the flagellar phase variation protein hin; regulates SPI-1	I.p.	Wilson et al. (2001)
STM3096 (*yqgE*)	Transcriptional regulator containing a highly conserved domain of unknown function	I.p.	Unpubl.
phoP/phoQ	Two-component regulator that responds to low Mg and defensins	i.g.; i.p.	Groisman et al. (1989)
ssrA/ssrB	SPI-2 encoded two-component regulator required for systemic infection	i.g.; i.p.	Hensel et al. (1998), Ochman et al. (1996)
slyA	Tunes regulation of SPI-2 more precisely than SsrA/SsrB alone; controls regulation of many virulence factors	i.g.; i.p.	Buchmeier et al. (1997)
crp	Responds to cAMP levels which are determined in part by external glucose concentration	i.g.; i.p.	Curtiss and Kelly (1987), Teplitski et al. (2006)
ompR/envZ	Two-component regulator that responds to osmolarity	i.g.; i.p.	Dorman et al. (1989)
fruR	Controls carbon metabolism	i.p.	Chin et al. (1987)
ihf	Required for bacteriophage lambda integration; bends DNA and significantly changes transcriptional regulation of many genes	i.g.; i.p.	Mangan et al. (2006)
relA/spoT	Required for the bacterial stringent response that results in reduced transcription in the presence of uncharged t-RNA	i.p.; ND in i.g./l.p.	Munro et al. (1995), Song et al. (2004)
STM1547	MarR family transcription regulator	i.p.; ND in i.g./l.p.	Unpubl.
STM3121	LysR regulator of the adjacent operon (STM3117-3120); regulates additional genes but only observed during intracellular growth	i.p.; ND in i.g./l.p.	Shi et al. (2006)
rpoE	Sigma factor for envelope-stress	i.g.; i.p.	Crouch et al. (2005), Osborne and Coombes (2009)
rpoS	Sigma factor for stationary-phase	i.g.; i.p.	Fang et al. (1996), Kowarz et al. (1994)
spvR	Controls expression of effectors encoded on the virulence plasmid; Virulence effect is dependent on strain of mouse	i.g.; i.p.	Krause et al. (1992), Yoon and Gros Heffron (2011)
*rcsA***	Positive transcriptional regulator of capsular/exo-polysaccharide synthesis	i.p.; ND in i.g./l.p.	Virlogeux et al. (1996)
hnr (mviA)*	Response regulator with CheY-like receiver domains	i.g.; i.p.	Bearson et al. (1996)
*fliA***	Sigma factor for flagella synthesis	i.p.; ND in i.g./l.p.	Ohnishi et al. (1990)
TRANSLATIONAL REGULATORS			
smpB*	Together with tmRNA binds to stalled bacterial ribosome permitting trans-translation and addition of a short coding sequence encoded by tmRNA; affects translation of approximately 14% of total *Salmonella* messages	i.p.	Ansong et al. (2009), Chin et al. (1987)
csrA	Global carbon metabolism regulator that controls glycolysis and gluconeogenesis by binding a specific RNA motif to block translation	i.p.	Lawhon et al. (2003), Teplitski et al. (2006)
hfq	Host factor for Qβ replication; a factor that controls translation of many mRNA in bacteria	i.g.; i.p.	Ansong et al. (2009), Figueroa-Bossi et al. (2006), Sittka et al. (2009)
rseA	Anti-sigma E factor post-translational control of *rpoE*	i.p.; ND in i.g./l.p.	Alba and Gross (2004)

**Virulence attenuation was examined in a mouse model by intragastric (i.g.) infection, intraperitoneal (i.p.) infection, and long-term persistence (l.p.) test. ND means "not determined yet."*

***Strains with 10–100 × LD_{50}, indicating modest virulence attenuation compared to others.*

Strains in bold were analyzed by global transcriptomic profiling (Yoon et al., 2009).

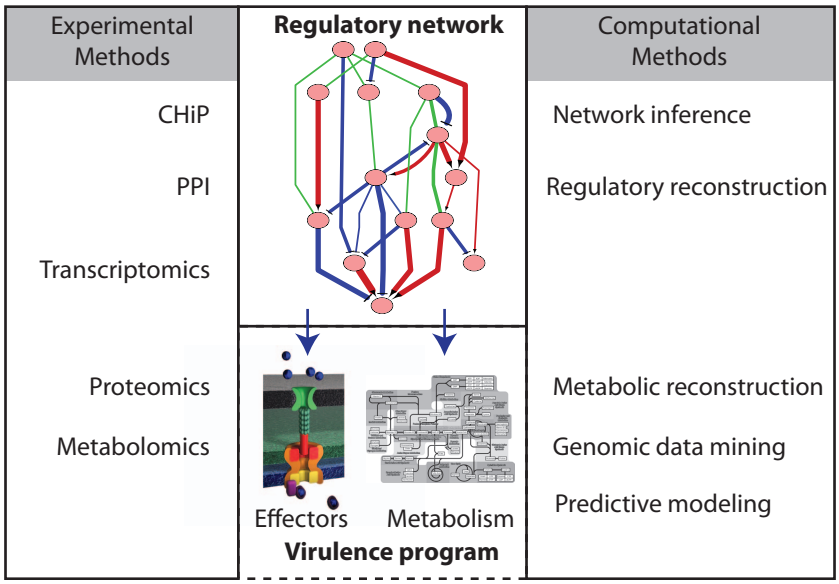

FIGURE 1 | Overview of high-throughput and computational methods to elucidation of the regulatory networks governing *Salmonella* virulence.

to develop these networks, focusing on high-throughput techniques. We begin with a short description of the types of approaches used to characterize virulence in *Salmonella*. We then discuss transcriptomic and chromatin immunoprecipitation (ChIP) approaches, proteomic, and protein–protein interactions (PPIs).

CONSIDERATIONS OF EXPERIMENTAL APPROACHES TO STUDYING VIRULENCE

Regulatory interactions in *Salmonella* are studied in three principal ways. The first is based on using *in vitro* media conditions that simulate infectious and non-infectious conditions to elicit changes that can be studied and perturbed (Beuzon et al., 1999; Coombes et al., 2005; Adkins et al., 2006; Shi et al., 2009a). The second is in infected cultured macrophages (depending on perspective this is referred to as *in vivo* or *ex vivo*; Eriksson et al., 2003; Shi et al., 2009b). And the third is using model organisms such as mice or cattle, though this is generally aimed at answering relatively narrow questions of virulence (Chan et al., 2005; Lawley et al., 2006). The choice of experimental system used to investigate regulatory networks is driven by the need for coverage and depth of measurements versus the correctness of the biological environment. That is, *in vitro* culture conditions, while biologically imperfect, are capable of generating large quantities of samples required for many of the assays described in this section. Advances in methodology are allowing more work to be done *ex vivo* and *in vivo*, such as in infected macrophages or by direct measurement within the mouse. These advances include those related to next-generation sequencing for expression profiling, improvements in the sensitivity of proteomics, and ChIP-based methods. As more *ex vivo* data becomes available it will be possible to compare responses in *in vitro* conditions with those in macrophages to refine regulatory networks and to refine the culture conditions to better reflect the intracellular milieu.

TRANSCRIPTOME PROFILING

Changes in gene expression are attributed to the DNA-binding activity of regulators responding to environmental stimuli. Transcriptional regulation is often a complex process composed of multiple regulatory factors and thus systems biology approaches are necessary to integrate the activities of multiple regulators. With the development of the microarray where thousands of sequences are spotted on a chip and the expression of numerous genes is simultaneously compared, the inference of regulatory networks could be accomplished in a high-throughput manner (Faith et al., 2007; Bonneau, 2008). Furthermore, related approaches including ChIP–microarray (ChIP–chip) and ChIP-sequencing (ChIP-seq) methods, discussed below, have accelerated defining the transcriptional regulatory network (Macquarrie et al., 2011). We have used a transcriptomic approach to decipher the regulatory network governed by virulence regulators during *Salmonella* systemic infection. Regulators sensing the multiple environmental cues execute defense programs and coordinately tune the expression of genes involved in virulence. By deleting *Salmonella* regulators across the chromosome and its virulence-related plasmid, we defined 20 regulators that were required for *Salmonella* systemic infection in mice (avirulent strains in i.p. infection in **Table 1**). These virulence regulators were varied, including two-component regulators (PhoP/PhoQ, SsrA/SsrB, and OmpR/EnvZ), alternative sigma factors (RpoE, RpoS, and FliA), post-transcriptional/post-translational regulators (SmpB, CsrA, Hfq, and RseA), a response regulator for which the signal sensor is unknown (Hnr), a bending protein essential for some types of recombination (IHF), and an assortment of other transcriptional/putative transcriptional regulators (SlyA, Crp, FruR, RelA/SpoT, STM1547, STM3121, SpvR, and RcsA). We chose 14 *Salmonella* regulators whose absence caused severe survival defect in mice (see strains in bold in **Table 1**) and determined the global transcriptional changes by each virulence

regulator in intracellular-mimicking conditions. Collective transcriptomic data revealed an interaction network among virulence regulators and furthermore suggested a group of genes that were coordinately controlled by virulence regulators and are likely to be important for *Salmonella* virulence (Yoon et al., 2009). We show a simplified version of the inferred regulatory network that was obtained in this study in **Figure 2**. This network shows that the primary regulator responsible for SPI-2 virulence expression, SsrB, integrates signals from many regulators and two-component regulatory systems. Interestingly, this network showed that SlyA is directly upstream of SsrB, but that it also seemed to be directly regulating SPI-2 expression. Both these predictions were validated in the study, elaborating the role that SlyA plays in virulence in *Salmonella*.

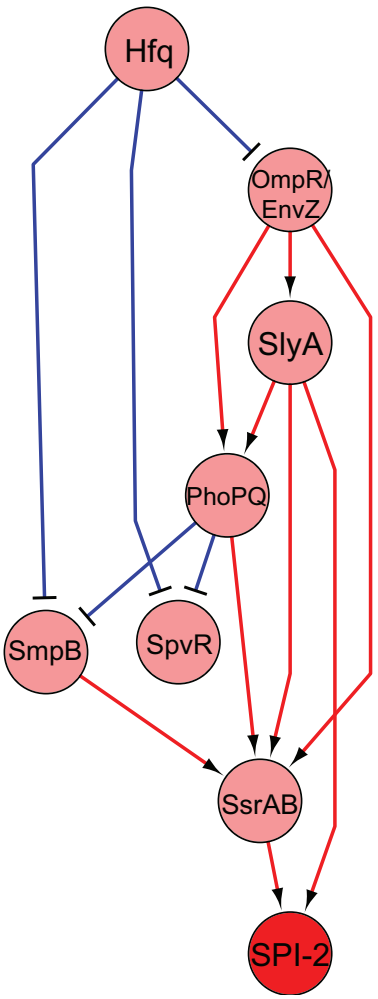

FIGURE 2 | Regulatory network of selected transcription factors essential for virulence. Regulators essential in systemic infection were deleted and microarray expression data under SPI-2 inducing conditions were used to construct a regulatory network. The figure shown represents a selected subset of all the regulators examined (see Yoon et al., 2009 for the complete network). The nodes indicate regulators, with the red node indicating the SPI-2 genes. Edges indicate activation (red) or repression (blue). Predictions made by this model were validated experimentally (Yoon et al., 2009).

There have been many studies that characterize the global expression of *Salmonella* under different conditions that are relevant to virulence (Clements et al., 2002; Eriksson et al., 2003; Lawhon et al., 2003; Kelly et al., 2004; Mangan et al., 2006; Sheng et al., 2009). While these studies do not directly address the global regulatory network driving virulence, they have been used to elucidate novel virulence factors, functional pathways involved in pathogenesis and regulons activated by virulence conditions.

Recent advances in high-throughput DNA sequencing technology have also been applied to the studies on bacterial transcriptome (Passalacqua et al., 2009; Perkins et al., 2009) as well as the eukaryotic transcriptome (Nagalakshmi et al., 2008; Wilhelm et al., 2008). Using an RNA-sequencing (RNA-seq) method, it became possible to define the composition of a whole mRNA population and evaluate gene regulation in detail at the transcriptional level. By converting RNA to DNA, Perkins et al. (2009) analyzed the transcription profile of *S.* Typhi in a strand-specific manner and identified small RNA and RNA transcripts originating from overlapping DNA sequences as well, which is not possible using low-density microarrays. This analysis revealed a number of novel non-coding RNAs and was used to refine the regulon of the OmpR regulator, which plays important roles in virulence.

PROTEOME PROFILING

Although transcriptomics is more commonly applied to determine gene regulatory networks, global profiling of protein abundance by proteomic analysis can also be very useful in this regard. A large-scale view of protein abundances from regulatory mutants combined with different stimuli can help identify co-regulated groups, and be used to link these groups to specific regulators. The reasons that transcriptomics are more often used to study gene regulatory networks are (i) microarray analysis has more complete coverage; and (ii) protein abundances are complex being regulated by gene expression, post-transcriptional modifications, and degradation. Recent advances in nanoflow liquid chromatography and mass spectrometry (LC–MS) have tremendously improved proteome coverage (Nilsson et al., 2010). For instance, with current technology and applying extensive protein or peptide fraction previous to LC–MS analysis (two-dimensional LC–MS or 2D-LC–MS) it is possible to routinely obtain proteome coverage of better than ~50% of the predicted ORFs for prokaryotic organisms (Adkins et al., 2006; Malmstrom et al., 2009). Recent analysis of the *S.* Typhi and *S.* Typhimurium proteomes led to the identification of 47 and 51% of all predicted ORFs, respectively (Adkins et al., 2006; Ansong et al., 2008). It is also worth noting that a number of genes are expressed only under specific conditions and some of the predicted ORFs may not even be functional, thus the coverage of the actual expressible proteome is likely higher.

Relative to traditional transcriptomic studies, increasing depth of proteome coverage is frequently associated with labor-intensive, low-throughput analysis, thus limiting the throughput and number of samples to be studied. More commonly applied multi-dimensional LC–MS/MS experiments can detect a great number of peptides; however, the approach relies on decisions made automatically by the MS instrument on which peptides will be selected and fragmented for identification. To circumvent this issue, our team utilizes a methodology that separates the peptide

identifications from the quantitative analysis, in a process called accurate mass and time (AMT) tag approach (Zimmer et al., 2006). The AMT tag approach reduces labor-intensive experimental steps and enables relatively comprehensive analysis of large number of samples with deep proteome coverage.

The AMT tag approach has enabled a number of proteomic analyses of *Salmonella* that would have been difficult and highly labor-intensive otherwise. This includes analyses of the combined *Salmonella* and host proteomes during time course analyses (Shi et al., 2006, 2009b) and analysis of post-transcriptional regulatory mutants lacking Hfq and SmpB under multiple conditions (Ansong et al., 2009). In a more recent study, we have utilized the AMT tag approach to revisit the 14 virulence regulators studied by transcriptomic analysis (Yoon et al., 2011). All 14 mutant strains lacking virulence regulators were grown in conditions that mimic intracellular environments and global proteomics measurements were made. We then inferred networks from proteomics data and used the structure and topology of these networks to find virulence effector candidates that were co-regulated with known virulence factors, such as the SPI-2 T3SS. Five of these candidates were found to be virulence proteins secreted through a non-T3SS mechanism, validating our approach and demonstrating the utility of regulatory networks. These examples show that high dimensional proteomic datasets can be useful to help elucidate gene regulatory networks or determine more complex mechanisms of protein abundance controls.

CHROMATIN IMMUNOPRECIPITATION-BASED METHODS

Chromatin immunoprecipitation is a popular methodology to study gene regulation. ChIP–chip and ChIP-seq enable the determination of the genes regulated by certain known regulator. In these procedures DNA is cross-linked with interacting proteins and the regulator of interest is pulled down by immunoprecipitation (Gilchrist et al., 2009). The interacting regions of the genome are determined by either microarray (ChIP–chip) or by next-generation sequencing (ChIP-seq; Gilchrist et al., 2009). An elegant example of a ChIP–chip experiment has been demonstrated by Tomljenovic-Berube et al. (2010) in which they combine microarray analysis of *ssrB*-mutant bacteria with ChIP–chip experiments to determine the set of genes under regulatory control of SsrA–SsrB, which are major regulators of SPI-2 genes. In another example, the regulatory network of the transcription silencer H-NS was determined by ChIP–chip analysis (Dillon et al., 2010).

Although transcription factors (TFs) are key elements of expression control, they are often associated with large protein complexes. In this context, a valuable approach consists of analyzing the immunoprecipitated protein complex-DNA by mass spectrometry (ChIP–MS) leading to the identification of gene regulator partners (Lambert et al., 2009). An approach was recently introduced that enables the determination of loci-specific regulatory machinery. This method is based on the cross-linking of DNA–protein complexes; then the DNA is hybridized with region-specific probes and pull-down by affinity purifications (AP). This method became known as reverse-ChIP since rather than immunoprecipitating the regulators to discover their interacting loci in the genome, probes are designed to pull-down proteins bound to specific DNA regions (Dejardin and Kingston, 2009).

PROTEIN–PROTEIN INTERACTION-BASED METHODS

Protein–protein interactions play an essential role in cells because many proteins are part of larger complexes (Nooren and Thornton, 2003). There are many approaches to study PPIs including *in silico* predictions, yeast two-hybrid (Y2H) systems and AP (Perkins et al., 2010). Although *in silico* predictions are very useful, experimental validation is always required to ensure data accuracy (Perkins et al., 2010). Y2H screening is a very popular methodology to study PPIs, but may present high rates of false-positive and false-negative interactions (Perkins et al., 2010). Chowdhury et al. (2009) studied PPIs for three *S.* Typhimurium proteins (HimD, PduB, and PhoP), by expressing them fused with a histidine–biotin–histidine tag, chemical cross-link, and two-steps AP followed by proteomic analysis. With this approach several proteins were found, including previously characterized partners for the targeted proteins (Chowdhury et al., 2009). The advantage of cross-linking proteins is that depending on the type of AP, the purification can be performed in very stringent denaturing conditions, thus decreasing undesired unspecific interactions of proteins to the affinity beads (Guerrero et al., 2006, 2008). Another AP approach to study PPIs is by performing the purifications of non-cross-linked samples in much milder conditions. However, this method can lead to more "noisy" data by having proteins binding non-specifically to beads. Recently, progress has been made to distinguish the specific interacting proteins from the background. Based on quantitative proteomic analysis and statistical analysis, Choi et al. designed a computational tool to determine the proteins that are significantly enriched compared to the background (Breitkreutz et al., 2010; Choi et al., 2011).

Although, PPI and ChIP approaches are similar in many aspects, we believe that they are complementary. Thus, the combination of both PPI and ChIP may in the future lead to a more comprehensive view of the regulatory processes in *Salmonella*, which will lead to the ability to predict environmental responses by *Salmonella*.

COMPUTATIONAL ANALYSIS OF DATA TO DETERMINE REGULATORY NETWORKS

OVERVIEW

High-throughput data acquisition methods have been effectively used to define regulons and regulatory networks in *Salmonella* as discussed above. However, these approaches require sophisticated computational analysis methods to provide reliable regulatory network predictions. Additionally, analysis of other kinds of data, for example genomic sequences, can provide corroborating or supporting information about virulence regulation in *Salmonella*. In this section we describe some of the computational approaches that have been applied to supply supporting information about virulence regulation, to derive regulatory networks from high-throughput data, and to develop predictive models of regulatory processes in *Salmonella* virulence. This kind of analysis is essential to complete a systems biology approach and to make sense of the complicated high-throughput data being generated (Aderem et al., 2011).

GENOMIC DATA INTEGRATION AND INFERENCE

Mining genomic sequence information has proven to be very informative about virulence in *Salmonella*, and this information directly supports virulence-related regulatory network analysis.

Spatial organization of genes on the chromosome has been used to identify the *Salmonella* pathogenicity islands, which are essential for virulence and largely co-regulated (Salama and Falkow, 1999; Vernikos and Parkhill, 2006; Hensel, 2007; Chatterjee et al., 2008). An additional approach to identifying virulence-related genes from sequence information is by using the nucleotide composition of the gene, relying on the observation that higher G + C content is associated with horizontally transferred virulence genes (Collmer et al., 2002; Srividhya et al., 2007). Analysis of the promoter sequences for several TFs involved in virulence has further expanded their regulons (Wozniak and Hughes, 2008; Harari et al., 2009). Finally, we (Samudrala et al., 2009; McDermott et al., 2011) and others (Arnold et al., 2009; Lower and Schneider, 2009) have recently described methods to integrate this kind of genomic data to provide accurate prediction of type III secreted effectors, which do not share easily recognizable signal sequences that direct their secretion. These approaches have led to the definition of a number of novel effectors (Niemann et al., 2011) and have provided insights about the conservation and regulation of T3SSs across organisms (Arnold et al., 2009; Buchko et al., 2010; McDermott et al., 2011). Since type III effectors are a primary means by which *Salmonella* promotes virulence in the host, better definition of the secreted effector repertoire is expected to shed light on virulence regulation and evolutionary patterns associated with virulence.

NETWORK INFERENCE FROM HIGH-THROUGHPUT DATA

Prediction of regulatory interactions between genes based on high-throughput data is called network inference. Network inference is a data-driven process that relies on the association of genes based on similar expression patterns over a collection of different experimental conditions (De Smet and Marchal, 2010). Regulatory networks can be inferred from high-throughput data with good accuracy, if sufficient data is available. As discussed above we have used network inference approaches to identify interactions between regulators essential to virulence (Yoon et al., 2009). In that study we applied an inference method that relies on the similarity between gene profiles as well as a network inference method that assesses the impact of regulator deletions on the expression levels of other regulators to derive a network of regulatory interactions (Yoon et al., 2009; Prill et al., 2010) that was validated using RT-PCR. This regulatory network highlighted the redundancy and interdependency present in the virulence regulatory network in *Salmonella* (**Figure 2**), defined points of control for the regulatory network (McDermott et al., 2009), and identified groups of important virulence genes with conserved regulatory patterns (Yoon et al., 2011). Network inference is useful as a relatively unbiased approach to determining regulatory networks, but does not make use of the considerable amount of information available about regulation in bacterial systems.

GENOME-SCALE RECONSTRUCTION OF REGULATORY AND METABOLIC NETWORKS

A variety of approaches to modeling transcriptional regulation have been developed over the years, many of which are reviewed by Karlebach and Shamir (2008). When it comes to analyzing transcriptional output or developing predictive integrated models of regulation and metabolism on the genome-scale, constraint-based approaches such as network component analysis (NCA; Liao et al., 2003) and Boolean networks (Kauffman, 1969; Covert et al., 2001, 2008; Gianchandani et al., 2006; Klamt et al., 2006; Shlomi et al., 2007; Graudenzi et al., 2011) and more recently probabilistic models (Chandrasekaran and Price, 2010) have been used. **Figure 3** provides an overview of these methods. These methods all require a regulatory network structure as an input that may be assembled from manual curation of the literature (Gama-Castro et al., 2008), ChIP studies (Cho et al., 2009), or inference methods (Sabatti et al., 2002, 2005; Gardner et al., 2003; Margolin et al., 2006; Faith et al., 2007) discussed above and in a variety of other reviews (Chou and Voit, 2009; De Smet and Marchal, 2010). The regulatory network structure is often referred to as the connectivity matrix as it is modeled as a matrix where all of the TFs are on one axis, all of the genes

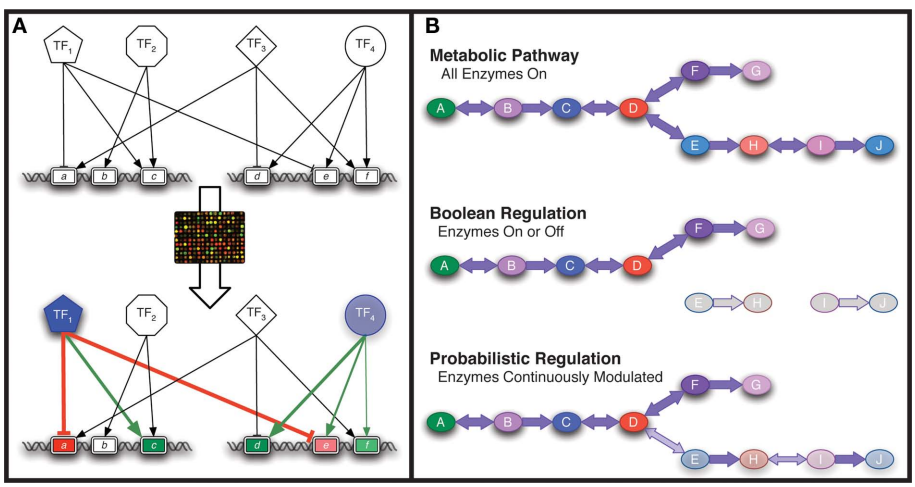

FIGURE 3 | Systems modeling approaches. (A) Network component analysis uses transcriptome data to deduce transcription factor (TF) activities given a TF/gene (g) connectivity network. **(B)** Genome-scale metabolic models are constructed with all enzymes. With Boolean regulatory constraints, enzymes are either expressed or not expressed. With probabilistic regulatory constraints, the enzyme expression levels are modulated based on expression level of the regulators.

on the other axis, and a non-zero element in the matrix indicates that a TF is known to influence the expression of the corresponding gene. A second common feature for these methods is that they were designed to only approximate the interactions between a TF and its target genes: NCA employs a Hill-type equation for each TF-gene interaction, Boolean models represent each gene as on or off based on the sum of Boolean interactions for all TFs that interact with the gene, and probabilistic regulation of metabolism (PROM) uses linear weights derived from prior transcriptome data to constrain flux through a gene's associated enzymatic activities based on current TF expression profiles.

It is possible to integrate a variety of data types, such as transcriptome measurements, motifs, promoter regions, ChIP data, and growth phenotypes, with these modeling tools to update the connectivity matrix for a specific condition or during analysis to uncover additional hypothetical interactions (Foat et al., 2005; Sabatti and James, 2006; Hyduke et al., 2007; Wang et al., 2008; Barua et al., 2010; Tran et al., 2010). These methods have been predominantly used with *E. coli* due to the availability of a global regulatory network, but as *Salmonella*'s regulatory network is delineated through the methods described above it will be possible to apply these methods to the massive amounts of *Salmonella* high-throughput data. Since classical virulence genes such as adhesins, toxins, and secreted effectors are not included in standard metabolic models the integration of metabolic models with more descriptive data-driven models will be of paramount importance to fully model virulence in *Salmonella*.

There have been several studies in *Salmonella* that have modeled regulation of the SPI-1 T3SS, which is important for virulence as described above. Two studies (Temme et al., 2008; Saini et al., 2010) described experimental approaches to define parameters for computational models of the TFs (HilA, HilC, HilD, and RtsA) and their downstream targets (components of the T3SS and effector proteins). These studies allowed determination of how individual components in the SPI-1 regulatory network coordinate its virulence program. Though these studies are not aimed at providing a comprehensive, global view of the virulence regulatory network in *Salmonella* they provide valuable insight into the details of the regulatory network driving SPI-1 expression.

PREDICTIVE MODELING

Computational models can be used to predict behaviors of the system under conditions not previously considered. Integrated models of regulation and metabolism may be used to simulate the growth phenotypes of microbes in a variety of conditions, including those relevant to infection (Bumann, 2009). Metabolic network reconstructions aim to be comprehensive biochemically, genetically, and genomically (BiGG) consistent knowledge bases for an organism (Reed et al., 2006; Schellenberger et al., 2010). The recent publication of a BiGG metabolic knowledgebase for *Salmonella* Typhimurium LT2 represents a resource that may be used as a platform for integrated modeling of metabolic activities during infection (Bumann, 2009; Thiele et al., 2011).

Mathematical models derived from BiGG knowledge bases will often predict capabilities that are not biologically realizable; this is due to the lack of regulatory constraints. Integration of Boolean regulatory constraints (Kauffman, 1969; Covert et al., 2001; Covert and Palsson, 2002) with the metabolic models increases their accuracy (Covert et al., 2004). In Covert et al. (2004), Boolean regulatory constraints were integrated with a metabolic network model for *E. coli* and used to simulate growth phenotypes for over 13,000 conditions described in the ASAP database (Glasner et al., 2006). The integrated model accurately simulated growth for ~79% of the conditions; when the regulatory constraints were removed accuracy dropped to ~65%. Amongst the set of failures, Covert et al. (2004) noticed that low oxygen (O_2) concentration was a common theme. Pathogens will likely encounter low O_2 levels when interacting with the innate immune response. This highlights the need to improve our knowledge of regulatory interactions in microaerobic and anaerobic conditions.

Recently, Barua et al. (2010) developed an automated approach for using global phenotypic (phenomic) data in conjunction with BiGG metabolic models to update Boolean regulatory rules. Additionally, they used their approach along with sequence homology data to construct and refine a putative regulatory network for *S.* Typhimurium LT2. Integrating this putative regulatory network with experimental and inference methods described above may result in an initial global regulatory network for *Salmonella* that can be used with NCA to interpret transcriptome data or with BiGG metabolic models to develop predictive models.

Chandrasekaran and Price (Covert and Palsson, 2002) devised another promising method to incorporate regulation with metabolism at the genome-scale. This method "PROM" integrates omics data with *a priori* regulatory network information to model regulation in a continuous fashion. The two attractive features of PROM are that it does not model gene expression as Boolean and that it can be used with an inferred regulatory network to make qualitative phenotypic predictions. While direct measurements of TF/target interactions are preferred it is often not possible to acquire the information for organisms of interest.

Finally, it is possible to use networks inferred from global gene expression profiling to generate models capable of predicting gene expression under novel conditions (Bonneau et al., 2007). In this approach the relationships between regulators and their target regulons are represented as mathematical equations. This set of equations can then be used to generate predictions of system behavior when growth conditions have changed or when individual regulators have been inactivated.

FUTURE PROSPECTS

METABOLOME PROFILING

In addition to the explicit effectors of the virulence program (T3SS effectors, etc.), *Salmonella* must tightly regulate its metabolism to allow survival in the host environment. Changes in *Salmonella* metabolism can be addressed using metabolomics approaches, which can provide untargeted, quantitative measurements of perturbations in the metabolite complement of an integrated biological system in response to stimuli (Nicholson et al., 1999). In this respect, metabolomics analyses have not yet been widely implemented in the study of pathogenic bacteria (Bundy et al., 2005; Boroujerdi et al., 2009; Henderson et al., 2009; Himmelreich et al., 2009; de Carvalho et al., 2010; Eylert et al., 2010; Raghunathan et al., 2010; Simon et al., 2010). Indeed, only two recent papers report the application of metabolomics in the study of *Salmonella*: Xu et al.

(2011) used a volatile organic compound (VOC)-based metabolic profiling approach to differentiate between natural spoiled pork and pork contaminated with *Salmonella*, and White et al. (2010) employed gas chromatography–mass spectrometry and nuclear magnetic resonance spectroscopy to identify metabolite profiles specific for *Salmonella* cells in radar (red, dry, and rough morphotype) colony biofilms and for cells that do not produce an extracellular matrix due to a *csgD* deletion mutant. Neither study addressed the *Salmonella* virulence regulatory network.

The role of metabolites in *Salmonella* virulence is two-fold. First and foremost, they represent nutrients necessary to sustain cellular growth and division and to provide the basic building blocks of virulence effectors. Studies using metabolic mutants have revealed insights into the nutritional requirements of *Salmonella* both *in vitro* and *in vivo*. For example, glucose is required for efficient intracellular growth in the *Salmonella*-containing vacuole (Tchawa Yimga et al., 2006; Gotz et al., 2010); however, a mutant unable to uptake glucose was still able to replicate within cells at a reduced rate, indicating that other carbon sources can be utilized when necessary (Bowden et al., 2009). Similar studies revealed various degrees of attenuation in mice infected by mutants lacking a complete TCA cycle (Tchawa Yimga et al., 2006), defective in converting malate to pyruvate, or lacking isocitrate lyase (Mercado-Lubo et al., 2009). The second role of metabolites in *Salmonella* virulence is as signaling molecules. Quorum sensing, or the recognition of bacterial cell populations based on concentrations of key molecules, is the small-molecule signaling mechanism in bacteria (Vendeville et al., 2005; Boyen et al., 2009). While the majority of Gram-negative bacteria utilize *N*-acylhomoserine lactones (AHLs), or autoinducer-1, as the signaling molecules (Boyen et al., 2009), *Salmonella* does not produce AHLs (Soares and Ahmer, 2011). Instead, *Salmonella* relies on autoinducer-2 molecules, such as 4,5-dihydroxy-2,3-pentanedione (DPD; Choi et al., 2007). Alternatively, the nucleotide alarmones guanosine 5′-diphosphate-3′-diphosphate and guanosine 5′-triphosphate-3′-diphosphate (collectively referred to as ppGpp) play a comprehensive role in *Salmonella* virulence (reviewed in Dalebroux et al., 2010). An as yet relatively unexplored area of research is the role played by host cell signaling molecules in virulence. In this respect, epinephrine was recently reported to play an important role in modulating *Salmonella* virulence (Spencer et al., 2010). Thus, comprehensive metabolomics analyses should be able to provide additional insights into the mechanisms by which *Salmonella* receives nutrition during infection and the role of small signaling molecules in virulence.

ANALYZING TRANSCRIPTIONAL OUTPUT

Given their ability to make predictions about the outputs of a network (e.g., virulence processes, metabolic function) given inputs (e.g., growth conditions, carbon source, environmental cures) genome-scale reconstructions will be able to provide a more complete model of *Salmonella* virulence. One goal of constraint-based regulatory network analysis is to be able to deduce the activity of all of an organism's TFs in a given condition. The TF activities (TFAs) will provide insight into how the TF network responds to a given perturbation. Once the TFA changes are known in response to a specific perturbation, it may be possible to determine the underlying factors that drive these changes. One perturbation that is essential to pathogenesis is exposure to reactive nitrogen species, such as nitric oxide (NO) and nitrosothiols (RSNOs), which are produced by the innate immune in response to the presence of a pathogen and induce cytolysis of the invading pathogen (MacMicking et al., 1997; Fang, 2004). In an effort to unravel the full effects of RNS on *E. coli*, a variety of transcriptomic surveys were made (Mukhopadhyay et al., 2004; Flatley et al., 2005; Justino et al., 2005) and with *ad hoc* analysis important interactions were implicated. However, the precise targets and regulatory response have not yet been elucidated.

Based on the hypothesis that it would be possible to trace a shift in regulatory network activity in response to a perturbation back to its originating source, we designed a series of experiments where we measured the transcriptomic response of *E. coli* to NO and *S*-nitrosoglutathione and used NCA to identify the TFA perturbations (Hyduke et al., 2007). A number of the significantly perturbed TFs (IscR, NorR, NsrR) have been associated with the NO-response, however, they had not be examined as an integrated system (D'Autreaux et al., 2005; Rodionov et al., 2005; Pullan et al., 2007). Next, for the significantly perturbed TFs we constructed deletion mutants to validate the NCA calculations and observe the changes in phenotypes. From the NCA-deduced perturbations in TFAs and a series of phenotyping studies we were able to identify the iron–sulfur cluster of dihydroxyacid dehydratase as a crucial enzyme that innate immune system targets to inhibit bacterial growth. *Salmonella* may exhibit similar responses due to its relatively recent divergence from *E. coli*, however, the larger number of regulators and greater variety of pathogenic strains indicate that there will be key differences. After *Salmonella*'s global regulatory network is further delineated, it will be possible to use NCA to identify the crucial differences.

CONCLUSION

Salmonella systemic infection is accomplished by a sophisticated regulatory network orchestrating activities of multiple regulators. Virulence regulators process and integrate a variety of hostile environmental cues including acidic pH, antimicrobial peptides, and reactive oxygen species within macrophages, and trigger induction of specific subsets of genes required for successful replication and evading lysosomal fusion. The coordinated action by this regulatory network involves numerous virulence factors including SPI-1 and -2 T3SSs. Genes that are coordinately controlled by multiple virulence regulators under infectious conditions and show a similar expression profile to that of well-known virulence genes are more likely to be important for pathogenesis. Thus, reconstructing the global regulatory network during infection or, at the very least, under conditions that mimic the host cellular environment can serve as an efficient strategy for the identification of genes that are required for *Salmonella* virulence (Yoon et al., 2011).

Impairing microbial virulence could be an interesting strategy to fight infections, so we propose that these secreted effectors among others could be excellent targets for the development of new antimicrobial therapies. Drugs could be designed to interfere in their binding to host proteins or even to disrupt their enzymatic activities. In this context, small compound libraries can be screened to select the drug candidates. Rasko et al. (2008) used a library of small organic compounds to screen for molecules that bind and inhibit QseC. QseC is a sensor histidine kinase that recognizes host adrenergic

signals by binding to epinephrine and norepinephrine (Rasko et al., 2008). After binding to any of these signaling molecules, QseC autophosphorylates and subsequently phosphorylates the TF QseB leading to a cascade that results in the expression of virulence factors. After screening the library, one compound named LED209 was found to bind with high affinity and to strongly inhibit QseC autophosphorylation disrupting the expression of known virulence factors. Furthermore, LED209 was also shown to decrease bacterial virulence in murine infections. Understanding regulatory networks and virulence processes at the system level will allow development of more sophisticated and nuanced approaches to target specific proteins and entire pathways. Information about the regulatory and metabolic networks controlling virulence can be used to identify better targets for drug development. Small-molecule inhibitors that can target interactions between proteins, enzymes, effector, and host proteins, or transcriptional regulatory interactions, will allow finer control of virulence processes and thus better antibiotics.

Another approach to inhibiting virulence in *Salmonella* is to target the T3SS apparatus itself with inhibitors. Several small-molecule inhibitors have been developed that target the T3SS and impair secretion (Nordfelth et al., 2005; Muschiol et al., 2006; Bailey et al., 2007; Negrea et al., 2007). Additionally, we have undertaken preliminary structural studies of the disordered N-terminal secretion signal of T3SS effectors in *Salmonella* (Buchko et al., 2010) and this information could potentially be used for future drug discovery efforts. The ability of *Salmonella* to introduce proteins directly into host cells has raised the possibility of targeted therapeutic approaches for other diseases using *Salmonella* as the delivery vehicle (Chamekh, 2010). Finally, there have been efforts to use *Salmonella* for biotechnology applications, such as exporting spider silk monomers (Widmaier et al., 2009). Clearly, the complicated regulatory control of virulence proteins will need to be better understood in order for progress to be made in these efforts.

In this review we have discussed various systems biology approaches to determination of regulatory networks involved in virulence in *Salmonella*. With the increasing body of knowledge and data arising from the high-throughput approaches discussed, and those that are currently being developed, it is very important that more sophisticated computational approaches to use this information be developed. Comprehensive systems models of *Salmonella* pathogenesis will have applications for antibiotic development, new strategies for therapeutic treatments, and further understanding of the complex interplay between pathogen and host during infection. Improved understanding of virulence regulation will be essential to fully realize the potential of each of these applications.

ACKNOWLEDGMENTS

This work was supported by the National Institute of Allergy and Infectious Diseases, NIH/DHHS, through interagency agreement Y1-AI-8401-01 and NIH RO1 AI022933.

REFERENCES

Aderem, A., Adkins, J. N., Ansong, C., Galagan, J., Kaiser, S., Korth, M. J., Law, G. L., McDermott, J. G., Proll, S. C., Rosenberger, C., Schoolnik, G., and Katze, M. G. (2011). A systems biology approach to infectious disease research: innovating the pathogen-host research paradigm. *MBio* 2, e00325–e00410.

Adkins, J. N., Mottaz, H. M., Norbeck, A. D., Gustin, J. K., Rue, J., Clauss, T. R., Purvine, S. O., Rodland, K. D., Heffron, F., and Smith, R. D. (2006). Analysis of the *Salmonella typhimurium* proteome through environmental response toward infectious conditions. *Mol. Cell Proteomics* 5, 1450–1461.

Alba, B. M., and Gross, C. A. (2004). Regulation of the *Escherichia coli* sigma-dependent envelope stress response. *Mol. Microbiol.* 52, 613–619.

Ansong, C., Yoon, H., Norbeck, A. D., Gustin, J. K., McDermott, J. E., Mottaz, H. M., Rue, J., Adkins, J. N., Heffron, F., and Smith, R. D. (2008). Proteomics analysis of the causative agent of typhoid fever. *J. Proteome Res.* 7, 546–557.

Ansong, C., Yoon, H., Porwollik, S., Mottaz-Brewer, H., Petritis, B. O., Jaitly, N., Adkins, J. N., McClelland, M., Heffron, F., and Smith, R. D. (2009). Global systems-level analysis of Hfq and SmpB deletion mutants in *Salmonella*: implications for virulence and global protein translation. *PLoS ONE* 4, e4809. doi:10.1371/journal.pone.0004809

Arnold, R., Brandmaier, S., Kleine, F., Tischler, P., Heinz, E., Behrens, S., Niinikoski, A., Mewes, H. W., Horn, M., and Rattei, T. (2009). Sequence-based prediction of type III secreted proteins. *PLoS Pathog.* 5, e1000376. doi: 10.1371/journal.ppat.1000376

Bailey, L., Gylfe, A., Sundin, C., Muschiol, S., Elofsson, M., Nordström, P., Henriques-Normark, B., Lugert, R., Waldenström, A., Wolf-Watz, H., and Bergström, S. (2007). Small molecule inhibitors of type III secretion in *Yersinia* block the *Chlamydia pneumoniae* infection cycle. *FEBS Lett.* 581, 587–595.

Barua, D., Kim, J., and Reed, J. (2010). An automated phenotype-driven approach (GeneForce) for refining metabolic and regulatory models. *PLoS Comput. Biol.* 6, e1000970. doi: 10.1371/journal.pcbi.1000970

Bearson, S. M. D., Benjamin, W. H. Jr., Swords, W. E., and Foster, J. W. (1996). Acid shock induction of RpoS is mediated by the mouse virulence gene mviA of *Salmonella typhimurium*. *J. Bacteriol.* 178, 2572–2579.

Beuzon, C. R., Banks, G., Deiwick, J., Hensel, M., and Holden, D. W. (1999). pH-dependent secretion of SseB, a product of the SPI-2 type III secretion system of *Salmonella typhimurium*. *Mol. Microbiol.* 33, 806–816.

Bingle, L. E., Bailey, C. M., and Pallen, M. J. (2008). Type VI secretion: a beginner's guide. *Curr. Opin. Microbiol.* 11, 3–8.

Bonneau, R. (2008). Learning biological networks: from modules to dynamics. *Nat. Chem. Biol.* 4, 658–664.

Bonneau, R., Facciotti, M. T., Reiss, D. J., Schmid, A. K., Pan, M., Kaur, A., Thorsson, V., Shannon, P., Johnson, M. H., Bare, J. C., Longabaugh, W., Vuthoori, M., Whitehead, K., Madar, A., Suzuki, L., Mori, T., Chang, D. E., Diruggiero, J., Johnson, C. H., Hood, L., and Baliga, N. S. (2007). A predictive model for transcriptional control of physiology in a free living cell. *Cell* 131, 1354–1365.

Boroujerdi, A. F., Vizcaino, M. I., Meyers, A., Pollock, E. C., Huynh, S. L., Schock, T. B., Morris, P. J., and Bearden, D. W. (2009). NMR-based microbial metabolomics and the temperature-dependent coral pathogen *Vibrio coralliilyticus*. *Environ. Sci. Technol.* 43, 7658–7664.

Bowden, S. D., Rowley, G., Hinton, J. C., and Thompson, A. (2009). Glucose and glycolysis are required for the successful infection of macrophages and mice by *Salmonella enterica* serovar typhimurium. *Infect. Immun.* 77, 3117–3126.

Boyen, F., Eeckhaut, V., Van Immerseel, F., Pasmans, F., Ducatelle, R., and Haesebrouck, F. (2009). Quorum sensing in veterinary pathogens: mechanisms, clinical importance and future perspectives. *Vet. Microbiol.* 135, 187–195.

Breitkreutz, A., Choi, H., Sharom, J. R., Boucher, L., Neduva, V., Larsen, B., Lin, Z. Y., Breitkreutz, B. J., Stark, C., Liu, G., Ahn, J., Dewar-Darch, D., Reguly, T., Tang, X., Almeida, R., Qin, Z. S., Pawson, T., Gingras, A. C., Nesvizhskii, A. I., and Tyers, M. (2010). A global protein kinase and phosphatase interaction network in yeast. *Science* 328, 1043–1046.

Buchko, G. W., Niemann, G., Baker, E. S., Belov, M. E., Smith, R. D., Heffron, F., Adkins, J. N., and McDermott, J. E. (2010). A multi-pronged search for a common structural motif in the secretion signal of *Salmonella enterica* serovar Typhimurium type III effector proteins. *Mol Biosyst.* 6, 2448–2458.

Buchmeier, N., Bossie, S., Chen, C. Y., Fang, F. C., Guiney, D. G., and Libby, S. J. (1997). SlyA, a transcriptional regulator of *Salmonella typhimurium*, is required for resistance to oxidative stress and is expressed in the intracellular environment of macrophages. *Infect. Immun.* 65, 3725–3730.

Buchmeier, N. A., and Heffron, F. (1991). Inhibition of macrophage phagosome-lysosome fusion by *Salmonella typhimurium*. *Infect. Immun.* 59, 2232–2238.

Bumann, D. (2009). System-level analysis of *Salmonella* metabolism during infection. *Curr. Opin. Microbiol.* 12, 559–567.

Bundy, J. G., Willey, T. L., Castell, R. S., Ellar, D. J., and Brindle, K. M. (2005). Discrimination of pathogenic clinical isolates and laboratory strains of *Bacillus cereus* by NMR-based metabolomic profiling. *FEMS Microbiol. Lett.* 242, 127–136.

Chamekh, M. (2010). Immunomodulation using genetically engineered bacteria for type III-mediated delivery of heterologous antigens and cytokines: potential application in vaccine and therapeutical developments. *Immunopharmacol. Immunotoxicol.* 32, 1–4.

Chan, K., Kim, C. C., and Falkow, S. (2005). Microarray-based detection of *Salmonella enterica* serovar Typhimurium transposon mutants that cannot survive in macrophages and mice. *Infect. Immun.* 73, 5438–5449.

Chandrasekaran, S., and Price, N. (2010). Probabilistic integrative modeling of genome-scale metabolic and regulatory networks in *Escherichia coli* and *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.* 107, 17845–17850.

Chatterjee, R., Chaudhuri, K., and Chaudhuri, P. (2008). On detection and assessment of statistical significance of genomic islands. *BMC Genomics* 9, 150. doi: 10.1186/1471-2164-9-150

Chin, A., Feucht, B., and Saier, M. J. (1987). Evidence for regulation of gluconeogenesis by the fructose phosphotransferase system in *Salmonella typhimurium*. *J. Bacteriol.* 169, 897–899.

Cho, B., Zengler, K., Qiu, Y., Park, Y. S., Knight, E. M., Barrett, C. L., Gao, Y., and Palsson, B. Ø. (2009). The transcription unit architecture of the *Escherichia coli* genome. *Nat. Biotechnol.* 27, 1043–1049.

Choi, H., Larsen, B., Lin, Z. Y., Breitkreutz, A., Mellacheruvu, D., Fermin, D., Qin, Z. S., Tyers, M., Gingras, A. C., and Nesvizhskii, A. I. (2011). SAINT: probabilistic scoring of affinity purification-mass spectrometry data. *Nat. Methods* 8, 70–73.

Choi, J., Shin, D., and Ryu, S. (2007). Implication of quorum sensing in *Salmonella enterica* serovar typhimurium virulence: the luxS gene is necessary for expression of genes in pathogenicity island 1. *Infect. Immun.* 75, 4885–4890.

Chou, I., and Voit, E. (2009). Recent developments in parameter estimation and structure identification of biochemical and genomic systems. *Math. Biosci.* 219, 57–83.

Chowdhury, S. M., Shi, L., Yoon, H., Ansong, C., Rommereim, L. M., Norbeck, A. D., Auberry, K. J., Moore, R. J., Adkins, J. N., Heffron, F., and Smith, R. D. (2009). A method for investigating protein-protein interactions related to *Salmonella typhimurium* pathogenesis. *J. Proteome Res.* 8, 1504–1514.

Clements, M. O., Eriksson, S., Thompson, A., Lucchini, S., Hinton, J. C., Normark, S., and Rhen, M. (2002). Polynucleotide phosphorylase is a global regulator of virulence and persistency in *Salmonella enterica*. *Proc. Natl. Acad. Sci. U.S.A.* 99, 8784–8789.

Collmer, A., Lindeberg, M., Petnicki-Ocwieja, T., Schneider, D. J., and Alfano, J. R. (2002). Genomic mining type III secretion system effectors in *Pseudomonas syringae* yields new picks for all TTSS prospectors. *Trends Microbiol.* 10, 462–469.

Coombes, B. K., Wickham, M. E., Lowden, M. J., Brown, N. F., and Finlay, B. B. (2005). Negative regulation of *Salmonella* pathogenicity island 2 is required for contextual control of virulence during typhoid. *Proc. Natl. Acad. Sci. U.S.A.* 102, 17460–17465.

Covert, M., Knight, E. M., Reed, J. L., Herrgard, M. J., and Palsson, B. O. (2004). Integrating high-throughput and computational data elucidates bacterial networks. *Nature* 429, 92–96.

Covert, M., and Palsson, B. (2002). Transcriptional regulation in constraints-based metabolic models of *Escherichia coli*. *J. Biol. Chem.* 277, 28058–28064.

Covert, M., Schilling, C., and Palsson, B. (2001). Regulation of gene expression in flux balance models of metabolism. *J. Theor. Biol.* 213, 73–88.

Covert, M., Xiao, N., Chen, T. J., and Karr, J. R. (2008). Integrating metabolic, transcriptional regulatory and signal transduction models in *Escherichia coli*. *Bioinformatics* 24, 2044–2050.

Crouch, M., Becker, L. A., Bang, I. S., Tanabe, H., Ouellette, A. J., and Fang, F. C. (2005). The alternative sigma factor sigma is required for resistance of *Salmonella enterica* serovar Typhimurium to anti-microbial peptides. *Mol. Microbiol.* 56, 789–799.

Curtiss, R. R., and Kelly, S. (1987). *Salmonella typhimurium* deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. *Infect. Immun.* 55, 3035–3043.

Dalebroux, Z. D., Svensson, S. L., Gaynor, E. C., and Swanson, M. S. (2010). ppGpp conjures bacterial virulence. *Microbiol. Mol. Biol. Rev.* 74, 171–199.

D'Autreaux, B., Tucker, N. P., Dixon, R., and Spiro, S. (2005). A non-haem iron centre in the transcription factor NorR senses nitric oxide. *Nature* 437, 769–772.

de Carvalho, L. P., Fischer, S. M., Marrero, J., Nathan, C., Ehrt, S., and Rhee, K. Y. (2010). Metabolomics of *Mycobacterium tuberculosis* reveals compartmentalized co-catabolism of carbon substrates. *Chem. Biol.* 17, 1122–1131.

De Smet, R., and Marchal, K. (2010). Advantages and limitations of current network inference methods. *Nat. Rev. Microbiol.* 8, 717–729.

Dejardin, J., and Kingston, R. E. (2009). Purification of proteins associated with specific genomic loci. *Cell* 136, 175–186.

Dillon, S. C., Cameron, A. D., Hokamp, K., Lucchini, S., Hinton, J. C., and Dorman, C. J. (2010). Genome-wide analysis of the H-NS and Sfh regulatory networks in *Salmonella typhimurium* identifies a plasmid-encoded transcription silencing mechanism. *Mol. Microbiol.* 76, 1250–1265.

Dorman, C. J., Chatfield, S., Higgins, C. F., Hayward, C., and Dougan, G. (1989). Characterization of porin and ompR mutants of a virulent strain of *Salmonella typhimurium*: ompR mutants are attenuated in vivo. *Infect. Immun.* 57, 2136–2140.

Dunlap, N., Benjamin, W. J., and Briles, D. (1994). The intracellular nature of *Salmonella* infection during the early stages of mouse typhoid. *Immunol. Ser.* 60, 303–312.

Ellis, T. N., Leiman, S. A., and Kuehn, M. J. (2010). Naturally produced outer membrane vesicles from *Pseudomonas aeruginosa* elicit a potent innate immune response via combined sensing of both lipopolysaccharide and protein components. *Infect. Immun.* 78, 3822–3831.

Eriksson, S., Lucchini, S., Thompson, A., Rhen, M., and Hinton, J. C. (2003). Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. *Mol. Microbiol.* 47, 103–118.

Eylert, E., Herrmann, V., Jules, M., Gillmaier, N., Lautner, M., Buchrieser, C., Eisenreich, W., and Heuner, K. (2010). Isotopologue profiling of *Legionella pneumophila*: role of serine and glucose as carbon substrates. *J. Biol. Chem.* 285, 22232–22243.

Faith, J. J., Hayete, B., Thaden, J. T., Mogno, I., Wierzbowski, J., Cottarel, G., Kasif, S., Collins, J. J., and Gardner, T. S. (2007). Large-scale mapping and validation of *Escherichia coli* transcriptional regulation from a compendium of expression profiles. *PLoS Biol.* 5, e8. doi: 10.1371/journal.pbio.0050008

Fang, F. (2004). Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat. Rev. Microbiol.* 2, 820–832.

Fang, F., Chen, C. Y., Guiney, D. G., and Xu, Y. (1996). Identification of sigma S-regulated genes in *Salmonella typhimurium*: complementary regulatory interactions between sigma S and cyclic AMP receptor protein. *J. Bacteriol.* 178, 5112–5120.

Fields, P., Swanson, R. V., Haidaris, C. G., and Heffron, F. (1986). Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc. Natl. Acad. Sci. U.S.A.* 83, 5189–5193.

Figueroa-Bossi, N., Lemire, S., Maloriol, D., Balbontín, R., Casadesús, J., and Bossi, L. (2006). Loss of Hfq activates the sigmaE-dependent envelope stress response in *Salmonella enterica*. *Mol. Microbiol.* 62, 838–852.

Flatley, J., Barrett, J., Pullan, S. T., Hughes, M. N., Green, J., and Poole, R. K. (2005). Transcriptional responses of *Escherichia coli* to S-nitrosoglutathione under defined chemostat conditions reveal major changes in methionine biosynthesis. *J. Biol. Chem.* 280, 10065–10072.

Foat, B., Houshmandi, S. S., Olivas, W. M., and Bussemaker, H. J. (2005). Profiling condition-specific, genome-wide regulation of mRNA stability in yeast. *Proc. Natl. Acad. Sci. U.S.A.* 102, 17675–17680.

Gal-Mor, O., Gibson, D. L., Baluta, D., Vallance, B. A., and Finlay, B. B. (2008). A novel secretion pathway of *Salmonella enterica* acts as an antivirulence modulator during salmonellosis. *PLoS Pathog.* 4, e1000036. doi: 10.1371/journal.ppat.1000036

Gama-Castro, S., Jiménez-Jacinto, V., Peralta-Gil, M., Santos-Zavaleta, A., Peñaloza-Spinola, M. I., Contreras-Moreira, B., Segura-Salazar, J., Muñiz-Rascado, L., Martínez-Flores, I., Salgado, H., Bonavides-Martínez, C., Abreu-Goodger, C., Rodríguez-Penagos, C., Miranda-Ríos, J., Morett, E., Merino, E., Huerta, A. M., Treviño-Quintanilla, L., and Collado-Vides, J. (2008). RegulonDB (version 6.0): gene regulation model of *Escherichia* coli K-12 beyond transcription, active (experimental) annotated promoters and Textpresso navigation. *Nucleic Acids Res.* 36, D120–D124.

Gardner, T., di Bernardo, D., Lorenz, D., and Collins, J. J. (2003). Inferring genetic networks and identifying compound mode of action via expression profiling. *Science* 301, 102–105.

Geddes, K., Cruz, F., and Heffron, F. (2007). Analysis of cells targeted by *Salmonella* type III secretion in vivo. *PLoS Pathog.* 3, e196. doi: 10.1371/journal.ppat.0030196

Gianchandani, E., Papin, J. A., Price, N. D., Joyce, A. R., and Palsson, B. O. (2006). Matrix formalism to describe functional states of transcriptional regulatory systems. *PLoS Comput.*

Biol. 2, e101. doi: 10.1371/journal.pcbi.0020101

Gilchrist, D. A., Fargo, D. C., and Adelman, K. (2009). Using ChIP-chip and ChIP-seq to study the regulation of gene expression: genome-wide localization studies reveal widespread regulation of transcription elongation. *Methods* 48, 398–408.

Glasner, J., Rusch, M., Liss, P., Plunkett, G. III, Cabot, E. L., Darling, A., Anderson, B. D., Infield-Harm, P., Gilson, M. C., and Perna, N. T. (2006). ASAP: a resource for annotating, curating, comparing, and disseminating genomic data. *Nucleic Acids Res.* 34, D41–D45.

Goffeau, A., Barrell, B. G., Bussey, H., Davis, R. W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J. D., Jacq, C., Johnston, M., Louis, E. J., Mewes, H. W., Murakami, Y., Philippsen, P., Tettelin, H., and Oliver, S. G. (1996). Life with 6000 genes. *Science* 274, 563–567.

Gotz, A., Eylert, E., Eisenreich, W., and Goebel, W. (2010). Carbon metabolism of enterobacterial human pathogens growing in epithelial colorectal adenocarcinoma (Caco-2) cells. *PLoS ONE* 5, e10586. doi: 10.1371/journal.pone.0010586

Graham, S. (2002). Salmonellosis in children in developing and developed countries and populations. *Curr. Opin. Infect. Dis.* 15, 507–512.

Graham, S. (2010). Nontyphoidal salmonellosis in Africa. *Curr. Opin. Infect. Dis.* 23, 409–414.

Graudenzi, A., Serra, R., Villani, M., Damiani, C., Colacci, A., and Kauffman, S. A. (2011). Dynamical properties of a Boolean model of gene regulatory network with memory. *J. Comput. Biol.* doi: 10.1089/cmb.2010.0069. [Epub ahead of print].

Groisman, E., Chiao, E., Lipps, C. J., and Heffron, F. (1989). *Salmonella typhimurium* phoP virulence gene is a transcriptional regulator. *Proc. Natl. Acad. Sci. U.S.A.* 86, 7077–7081.

Guerrero, C., Milenkovic, T., Przulj, N., Kaiser, P., and Huang, L. (2008). Characterization of the proteasome interaction network using a QTAX-based tag-team strategy and protein interaction network analysis. *Proc. Natl. Acad. Sci. U.S.A.* 105, 13333–13338.

Guerrero, C., Tagwerker, C., Kaiser, P., and Huang, L. (2006). An integrated mass spectrometry-based proteomic approach: quantitative analysis of tandem affinity-purified in vivo cross-linked protein complexes (QTAX) to decipher the 26 S proteasome-interacting network. *Mol. Cell Proteomics* 5, 366–378.

Haraga, A., Ohlson, M. B., and Miller, S. I. (2008). Salmonellae interplay with host cells. *Nat. Rev. Microbiol.* 6, 53–66.

Harari, O., del Val, C., Romero-Zaliz, R., Shin, D., Huang, H., Groisman, E. A., and Zwir, I. (2009). Identifying promoter features of co-regulated genes with similar network motifs. *BMC Bioinformatics* 10(Suppl. 4), S1. doi: 10.1186/1471-2105-10-S4-S1

Henderson, J. P., Crowley, J. R., Pinkner, J. S., Walker, J. N., Tsukayama, P., Stamm, W. E., Hooton, T. M., and Hultgren, S. J. (2009). Quantitative metabolomics reveals an epigenetic blueprint for iron acquisition in uropathogenic *Escherichia coli*. *PLoS Pathog.* 5, e1000305. doi: 10.1371/journal.ppat.1000305

Hensel, M. (2007). Genome-based identification and molecular analyses of pathogenicity islands and genomic islands in *Salmonella enterica*. *Methods Mol. Biol.* 394, 77–88.

Hensel, M., Shea, J. E., Waterman, S. R., Mundy, R., Nikolaus, T., Banks, G., Vazquez-Torres, A., Gleeson, C., Fang, F. C., and Holden, D. W. (1998). Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. *Mol. Microbiol.* 30, 163–174.

Himmelreich, U., Malik, R., Kühn, T., Daniel, H. M., Somorjai, R. L., Dolenko, B., and Sorrell, T. C. (2009). Rapid etiological classification of meningitis by NMR spectroscopy based on metabolite profiles and host response. *PLoS ONE* 4, e5328. doi: 10.1371/journal.pone.0005328

Hyduke, D., Jarboe, L. R., Tran, L. M., Chou, K. J., and Liao, J. C. (2007). Integrated network analysis identifies nitric oxide response networks and dihydroxyacid dehydratase as a crucial target in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 8484–8489.

Justino, M., Vicente, J. B., Teixeira, M., and Saraiva, L. M. (2005). New genes implicated in the protection of anaerobically grown *Escherichia coli* against nitric oxide. *J. Biol. Chem.* 280, 2636–2643.

Karlebach, G., and Shamir, R. (2008). Modelling and analysis of gene regulatory networks. *Nat. Rev. Mol. Cell Biol.* 9, 770–780.

Kauffman, S. (1969). Metabolic stability and epigenesis in randomly constructed genetic nets. *J. Theor. Biol.* 22, 437–467.

Kelly, A., Goldberg, M. D., Carroll, R. K., Danino, V., Hinton, J. C., and Dorman, C. J. (2004). A global role for Fis in the transcriptional control of metabolism and type III secretion in *Salmonella enterica* serovar Typhimurium. *Microbiology* 150(Pt 7), 2037–2053.

Klamt, S., Saez-Rodriguez, J., Lindquist, J. A., Simeoni, L., and Gilles, E. D. (2006). A methodology for the structural and functional analysis of signaling and regulatory networks. *BMC Bioinformatics* 7, 56. doi: 10.1186/1471-2105-7-56

Kowarz, L., Coynault, C., Robbe-Saule, V., and Norel, F. (1994). The *Salmonella typhimurium* katF (rpoS) gene: cloning, nucleotide sequence, and regulation of spvR and spvABCD virulence plasmid genes. *J. Bacteriol.* 176, 6852–6860.

Krause, M., Fang, F., and Guiney, D. (1992). Regulation of plasmid virulence gene expression in *Salmonella* Dublin involves an unusual operon structure. *J. Bacteriol.* 174, 4482–4489.

Lambert, J. P., Mitchell, L., Rudner, A., Baetz, K., and Figeys, D. (2009). A novel proteomics approach for the discovery of chromatin-associated protein networks. *Mol. Cell Proteomics* 8, 870–882.

Lawhon, S., Frye, J. G., Suyemoto, M., Porwollik, S., McClelland, M., and Altier, C. (2003). Global regulation by CsrA in *Salmonella typhimurium*. *Mol. Microbiol.* 48, 1633–1645.

Lawley, T. D., Chan, K., Thompson, L. J., Kim, C. C., Govoni, G. R., and Monack, D. M. (2006). Genome-wide screen for *Salmonella* genes required for long-term systemic infection of the mouse. *PLoS Pathog.* 2, e11. doi: 10.1371/journal.ppat.0020011

Liao, J., Boscolo, R., Yang, Y. L., Tran, L. M., Sabatti, C., and Roychowdhury, V. P. (2003). Network component analysis: reconstruction of regulatory signals in biological systems. *Proc. Natl. Acad. Sci. U.S.A.* 100, 15522–15527.

Lockman, H., and Curtiss, R. R. (1990). *Salmonella typhimurium* mutants lacking flagella or motility remain virulent in BALB/c mice. *Infect. Immun.* 58, 137–143.

Lower, M., and Schneider, G. (2009). Prediction of type III secretion signals in genomes of gram-negative bacteria. *PLoS ONE* 4, e5917. doi: 10.1371/journal.pone.0005917

Lucas, R., and Lee, C. (2001). Roles of hilC and hilD in regulation of hilA expression in *Salmonella enterica* serovar Typhimurium. *J. Bacteriol.* 183, 2733–2745.

MacMicking, J., Xie, Q., and Nathan, C. (1997). Nitric oxide and macrophage function. *Annu. Rev. Immunol.* 15, 323–350.

Macquarrie, K. L., Fong, A. P., Morse, R. H., and Tapscott, S. J. (2011). Genome-wide transcription factor binding: beyond direct target regulation. *Trends Genet.* 27, 141–148.

Malmstrom, J., Beck, M., Schmidt, A., Lange, V., Deutsch, E. W., and Aebersold, R. (2009). Proteome-wide cellular protein concentrations of the human pathogen *Leptospira interrogans*. *Nature* 460, 762–765.

Mangan, M., Lucchini, S., Danino, V., Cróinín, T. O., Hinton, J. C., and Dorman, C. J. (2006). The integration host factor (IHF) integrates stationary-phase and virulence gene expression in *Salmonella enterica* serovar Typhimurium. *Mol. Microbiol.* 59, 1831–1847.

Margolin, A. A., Nemenman, I., Basso, K., Wiggins, C., Stolovitzky, G., Dalla Favera, R., and Califano, A. (2006). ARACNE: an algorithm for the reconstruction of gene regulatory networks in a mammalian cellular context. *BMC Bioinformatics* 7(Suppl. 1), S7. doi: 10.1186/1471-2105-7-S1-S7

McClelland, M., Sanderson, K. E., Spieth, J., Clifton, S. W., Latreille, P., Courtney, L., Porwollik, S., Ali, J., Dante, M., Du, F., Hou, S., Layman, D., Leonard, S., Nguyen, C., Scott, K., Holmes, A., Grewal, N., Mulvaney, E., Ryan, E., Sun, H., Florea, L., Miller, W., Stoneking, T., Nhan, M., Waterston, R., and Wilson, R. K. (2001). Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature* 413, 852–856.

McDermott, J. E., Corrigan, A., Peterson, E., Oehmen, C., Niemann, G., Cambronne, E. D., Sharp, D., Adkins, J. N., Samudrala, R., and Heffron, F. (2011). Computational prediction of type III and IV secreted effectors in gram-negative bacteria. *Infect. Immun.* 79, 23–32.

McDermott, J. E., Taylor, R. C., Yoon, H., and Heffron, F. (2009). Bottlenecks and hubs in inferred networks are important for virulence in *Salmonella typhimurium*. *J. Comput. Biol.* 16, 169–180.

McGhie, E. J., Brawn, L. C., Hume, P. J., Humphreys, D., and Koronakis, V. (2009). *Salmonella* takes control: effector-driven manipulation of the host. *Curr. Opin. Microbiol.* 12, 117–124.

Mercado-Lubo, R., Leatham, M. P., Conway, T., and Cohen, P. S. (2009). *Salmonella enterica* serovar Typhimurium mutants unable to convert malate to pyruvate and oxaloacetate are avirulent and immunogenic in BALB/c mice. *Infect. Immun.* 77, 1397–1405.

Monack, D., Bouley, D., and Falkow, S. (2004). *Salmonella typhimurium* persists within macrophages in the mesenteric lymph nodes of chronically infected Nramp1+/+ mice and can be reactivated by IFNgamma neutralization. *J. Exp. Med.* 199, 231–241.

Mukhopadhyay, P., Zheng, M., Bedzyk, L. A., LaRossa, R. A., and Storz, G. (2004). Prominent roles of the NorR and Fur regulators in the *Escherichia*

coli transcriptional response to reactive nitrogen species. *Proc. Natl. Acad. Sci. U.S.A.* 101, 745–750.

Munro, P., Flatau, G. N., Clément, R. L., and Gauthier, M. J. (1995). Influence of the RpoS (KatF) sigma factor on maintenance of viability and culturability of *Escherichia coli* and *Salmonella typhimurium* in seawater. *Appl. Environ. Microbiol.* 61, 1853–1858.

Muschiol, S., Bailey, L., Gylfe, A., Sundin, C., Hultenby, K., Bergström, S., Elofsson, M., Wolf-Watz, H., Normark, S., and Henriques-Normark, B. (2006). A small-molecule inhibitor of type III secretion inhibits different stages of the infectious cycle of *Chlamydia trachomatis. Proc. Natl. Acad. Sci. U.S.A.* 103, 14566–14571.

Nagalakshmi, U., Wang, Z., Waern, K., Shou, C., Raha, D., Gerstein, M., and Snyder, M. (2008). The transcriptional landscape of the yeast genome defined by RNA sequencing. *Science* 320, 1344–1349.

Negrea, A., Bjur, E., Ygberg, S. E., Elofsson, M., Wolf-Watz, H., and Rhen, M. (2007). Salicylidene acylhydrazides that affect type III protein secretion in *Salmonella enterica* serovar typhimurium. *Antimicrob. Agents Chemother.* 51, 2867–2876.

Nicholson, J. K., Lindon, J. C., and Holmes, E. (1999). "Metabonomics": understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* 29, 1181–1189.

Niemann, G., Brown, R. N., Gustin, J. K., Stufkens, A., Shaikh-Kidwai, A. S., Li, J., McDermott, J. E., Brewer, H. M., Schepmoes, A., Smith, R. D., Adkins, J. N., and Heffron, F. (2011). Discovery of novel secreted virulence factors from *Salmonella enterica* serovar Typhimurium by proteomic analysis of culture supernatants. *Infect. Immun.* 79, 33–43.

Nilsson, T., Mann, M., Aebersold, R., Yates, J. R. III, Bairoch, A., and Bergeron, J. J. (2010). Mass spectrometry in high-throughput proteomics: ready for the big time. *Nat. Methods* 7, 681–685.

Nooren, I. M., and Thornton, J. M. (2003). Diversity of protein-protein interactions. *EMBO J.* 22, 3486–3492.

Nordfelth, R., Kauppi, A. M., Norberg, H. A., Wolf-Watz, H., and Elofsson, M. (2005). Small-molecule inhibitors specifically targeting type III secretion. *Infect. Immun.* 73, 3104–3114.

Ochman, H., Soncini, F. C., Solomon, F., and Groisman, E. A. (1996). Identification of a pathogenicity island required for *Salmonella* survival in host cells. *Proc. Natl. Acad. Sci. U.S.A.* 93, 7800–7804.

Ohnishi, K., Kutsukake, K., Suzuki, H., and Iino, T. (1990). Gene fliA encodes an alternative sigma factor specific for flagellar operons in *Salmonella typhimurium. Mol. Gen. Genet.* 221, 139–147.

Osborne, S., and Coombes, B. (2009). RpoE fine tunes expression of a subset of SsrB-regulated virulence factors in *Salmonella enterica* serovar Typhimurium. *BMC Microbiol.* 9, 45. doi: 10.1186/1471-2180-9-45

Parsons, D. A., and Heffron, F. (2005). sciS, an icmF homolog in *Salmonella enterica* serovar Typhimurium, limits intracellular replication and decreases virulence. *Infect. Immun.* 73, 4338–4345.

Passalacqua, K. D., Varadarajan, A., Ondov, B. D., Okou, D. T., Zwick, M. E., and Bergman, N. H. (2009). Structure and complexity of a bacterial transcriptome. *J. Bacteriol.* 191, 3203–3211.

Pedersen, K., Lassen-Nielsen, A. M., Nordentoft, S., and Hammer, A. S. (2009). Serovars of *Salmonella* from captive reptiles. *Zoonoses Public Health* 56, 238–242.

Perkins, J. R., Diboun, I., Dessailly, B. H., Lees, J. G., and Orengo, C. (2010). Transient protein-protein interactions: structural, functional, and network properties. *Structure* 18, 1233–1243.

Perkins, T. T., Kingsley, R. A., Fookes, M. C., Gardner, P. P., James, K. D., Yu, L., Assefa, S. A., He, M., Croucher, N. J., Pickard, D. J., Maskell, D. J., Parkhill, J., Choudhary, J., Thomson, N. R., and Dougan, G. (2009). A strand-specific RNA-Seq analysis of the transcriptome of the typhoid bacillus *Salmonella typhi. PLoS Genet.* 5, e1000569. doi: 10.1371/journal.pgen.1000569

Poh, J., Odendall, C., Spanos, A., Boyle, C., Liu, M., Freemont, P., and Holden, D. W. (2008). SteC is a *Salmonella* kinase required for SPI-2-dependent F-actin remodelling. *Cell. Microbiol.* 10, 20–30.

Prill, R. J., Marbach, D., Saez-Rodriguez, J., Sorger, P. K., Alexopoulos, L. G., Xue, X., Clarke, N. D., Altan-Bonnet, G., and Stolovitzky, G. (2010). Towards a rigorous assessment of systems biology models: the DREAM3 challenges. *PLoS ONE* 5, e9202. doi: 10.1371/journal.pone.0009202

Pullan, S., Gidley, M. D., Jones, R. A., Barrett, J., Stevanin, T. M., Read, R. C., Green, J., and Poole, R. K. (2007). Nitric oxide in chemostat-cultured *Escherichia coli* is sensed by Fnr and other global regulators: unaltered methionine biosynthesis indicates lack of S nitrosation. *J. Bacteriol.* 189, 1845–1855.

Raghunathan, A., Shin, S., and Daefler, S. (2010). Systems approach to investigating host-pathogen interactions in infections with the biothreat agent *Francisella*. Constraints-based model of *Francisella tularensis. BMC Syst. Biol.* 4, 118. doi: 10.1186/1752-0509-4-118

Ramsden, A. E., Mota, L. J., Münter, S., Shorte, S. L., and Holden, D. W. (2007). The SPI-2 type III secretion system restricts motility of *Salmonella*-containing vacuoles. *Cell. Microbiol.* 9, 2517–2529.

Rasko, D. A., Moreira, C. G., Li de, R., Reading, N. C., Ritchie, J. M., Waldor, M. K., Williams, N., Taussig, R., Wei, S., Roth, M., Hughes, D. T., Huntley, J. F, Fina, M. W., Falck, J. R., and Sperandio, V. (2008). Targeting QseC signaling and virulence for antibiotic development. *Science* 321, 1078–1080.

Reed, J., Patel, T. R., Chen, K. H., Joyce, A. R., Applebee, M. K., Herring, C. D., Bui, O. T., Knight, E. M., Fong, S. S., and Palsson, B. O. (2006). Systems approach to refining genome annotation. *Proc. Natl. Acad. Sci. U.S.A.* 103, 17480–17484.

Rodionov, D., Dubchak, I. L., Arkin, A. P., Alm, E. J., and Gelfand, M. S. (2005). Dissimilatory metabolism of nitrogen oxides in bacteria: comparative reconstruction of transcriptional networks. *PLoS Comput. Biol.* 1, e55. doi: 10.1371/journal.pcbi.0010055

Sabatti, C., and James, G. (2006). Bayesian sparse hidden components analysis for transcription regulation networks. *Bioinformatics* 22, 739–746.

Sabatti, C., Rohlin, L., Lange, K., and Liao, J. C. (2005). Vocabulon: a dictionary model approach for reconstruction and localization of transcription factor binding sites. *Bioinformatics* 21, 922–931.

Sabatti, C., Rohlin, L., Oh, M. K., and Liao, J. C. (2002). Co-expression pattern from DNA microarray experiments as a tool for operon prediction. *Nucleic Acids Res.* 30, 2886–2893.

Saini, S., Ellermeier, J. R., Slauch, J. M., and Rao, C. V. (2010). The role of coupled positive feedback in the expression of the SPI1 type three secretion system in *Salmonella. PLoS Pathog.* 6, e1001025. doi: 10.1371/journal.ppat.1001025

Salama, N. R., and Falkow, S. (1999). Genomic clues for defining bacterial pathogenicity. *Microbes Infect.* 1, 615–619.

Samudrala, R., Heffron, F., and McDermott, J. E. (2009). Accurate prediction of secreted substrates and identification of a conserved putative secretion signal for type III secretion systems. *PLoS Pathog.* 5, e1000375. doi: 10.1371/journal.ppat.1000375

Schechter, L., and Lee, C. (2001). AraC/XylS family members, HilC and HilD, directly bind and derepress the *Salmonella typhimurium* hilA promoter. *Mol. Microbiol.* 40, 1289–1299.

Schellenberger, J., Park, J. O., Conrad, T. M., and Palsson, B. Ø. (2010). BiGG: a biochemical genetic and genomic knowledgebase of large scale metabolic reconstructions. *BMC Bioinformatics* 11, 213. doi: 10.1186/1471-2105-11-213

Sheng, X., Huang, X., Li, J., Xie, X., Xu, S., Zhang, H., and Xu, H. (2009). Regulation of sulfur assimilation pathways in *Salmonella enterica* serovar Typhi upon up-shift high osmotic treatment: the role of UhpA revealed through transcriptome profiling. *Curr. Microbiol.* 59, 628–635.

Shi, L., Adkins, J. N., Coleman, J. R., Schepmoes, A. A., Dohnkova, A., Mottaz, H. M., Norbeck, A. D., Purvine, S. O., Manes, N. P., Smallwood, H. S., Wang, H., Forbes, J., Gros, P., Uzzau, S., Rodland, K. D., Heffron, F., Smith, R. D., and Squier, T. C. (2006). Proteomic analysis of *Salmonella enterica* serovar typhimurium isolated from RAW 264.7 macrophages: identification of a novel protein that contributes to the replication of serovar typhimurium inside macrophages. *J. Biol. Chem.* 281, 29131–29140.

Shi, L., Ansong, C., Smallwood, H., Rommereim, L., McDermott, J. E., Brewer, H. M., Norbeck, A. D., Taylor, R. C., Gustin, J. K., Heffron, F., Smith, R. D., and Adkins, J. N. (2009a). Proteome of *Salmonella enterica* serotype Typhimurium grown in a low Mg2+/pH medium. *J. Proteomics Bioinform.* 2, 388–397.

Shi, L., Chowdhury, S. M., Smallwood, H. S., Yoon, H., Mottaz-Brewer, H. M., Norbeck, A. D., McDermott, J. E., Clauss, T. R., Heffron, F., Smith, R. D., and Adkins, J. N. (2009b). Proteomic investigation of the time course responses of RAW 264.7 macrophages to infection with *Salmonella enterica. Infect. Immun.* 77, 3227–3233.

Shlomi, T., Eisenberg, Y., Sharan, R., and Ruppin, E. (2007). A genome-scale computational study of the interplay between transcriptional regulation and metabolism. *Mol. Syst. Biol.* 3, 101.

Simon, C., Langlois-Meurinne, M., Bellvert, F., Garmier, M., Didierlaurent, L., Massoud, K., Chaouch, S., Marie, A., Bodo, B., Kauffmann, S., Noctor, G., and Saindrenan, P. (2010). The differential spatial distribution of secondary metabolites in *Arabidopsis* leaves reacting hypersensitively to *Pseudomonas syringae* pv. tomato is dependent on the oxidative burst. *J. Exp. Bot.* 61, 3355–3370.

Sittka, A., Sharma, C. M., Rolle, K., and Vogel, J. (2009). Deep sequencing of *Salmonella* RNA associated with

heterologous Hfq proteins in vivo reveals small RNAs as a major target class and identifies RNA processing phenotypes. *RNA Biol.* 6, 266–275.

Soares, J. A., and Ahmer, B. M. (2011). Detection of acyl-homoserine lactones by *Escherichia* and *Salmonella*. *Curr. Opin. Microbiol.* 14, 188–193.

Song, M., Kim, H. J., Kim, E. Y., Shin, M., Lee, H. C., Hong, Y., Rhee, J. H., Yoon, H., Ryu, S., Lim, S., and Choy, H. E. (2004). ppGpp-dependent stationary phase induction of genes on *Salmonella* pathogenicity island 1. *J. Biol. Chem.* 279, 34183–34190.

Spencer, H., Karavolos, M. H., Bulmer, D. M., Aldridge, P., Chhabra, S. R., Winzer, K., Williams, P., and Khan, C. M. (2010). Genome-wide transposon mutagenesis identifies a role for host neuroendocrine stress hormones in regulating the expression of virulence genes in *Salmonella*. *J. Bacteriol.* 192, 714–724.

Srividhya, K. V., Alaguraj, V., Poornima, G., Kumar, D., Singh, G. P., Raghavenderan, L., Katta, A. V., Mehta, P., and Krishnaswamy, S. (2007). Identification of prophages in bacterial genomes by dinucleotide relative abundance difference. *PLoS ONE* 2, e1193. doi: 10.1371/journal.pone.0001193

Steele-Mortimer, O. (2008). The *Salmonella*-containing vacuole: moving with the times. *Curr. Opin. Microbiol.* 11, 38–45.

Tchawa Yimga, M., Leatham, M. P., Allen, J. H., Laux, D. C., Conway, T., and Cohen, P. S. (2006). Role of gluconeogenesis and the tricarboxylic acid cycle in the virulence of *Salmonella enterica* serovar Typhimurium in BALB/c mice. *Infect. Immun.* 74, 1130–1140.

Temme, K., Salis, H., Tullman-Ercek, D., Levskaya, A., Hong, S. H., and Voigt, C. A. (2008). Induction and relaxation dynamics of the regulatory network controlling the type III secretion system encoded within *Salmonella* pathogenicity island 1. *J. Mol. Biol.* 377, 47–61.

Teplitski, M., Goodier, R., and Ahmer, B. (2003). Pathways leading from BarA/SirA to motility and virulence gene expression in *Salmonella*. *J. Bacteriol.* 185, 7257–7265.

Teplitski, M., Goodier, R., and Ahmer, B. (2006). Catabolite repression of the SirA regulatory cascade in *Salmonella enterica*. *Int. J. Med. Microbiol.* 296, 449–466.

Thiele, I., Hyduke, D. R., Steeb, B., Fankam, G., Allen, D. K., Bazzani, S., Charusanti, P., Chen, F. C., Fleming, R. M., Hsiung, C. A., De Keersmaecker, S. C., Liao, Y. C., Marchal, K., Mo, M. L., Özdemir, E., Raghunathan, A., Reed, J. L., Shin, S. I., Sigurbjörnsdóttir, S., Steinmann, J., Sudarsan, S., Swainston, N., Thijs, I. M., Zengler, K., Palsson, B. O., Adkins, J. N., and Bumann, D. (2011). A community effort towards a knowledge-base and mathematical model of the human pathogen *Salmonella typhimurium* LT2. *BMC Syst. Biol.* 5, 8. doi: 10.1186/1752-0509-5-8

Tomljenovic-Berube, A. M., Mulder, D. T., Whiteside, M. D., Brinkman, F. S., and Coombes, B. K. (2010). Identification of the regulatory logic controlling *Salmonella* pathoadaptation by the SsrA-SsrB two-component system. *PLoS Genet.* 6, e1000875. doi: 10.1371/journal.pgen.1000875

Tran, L., Hyduke, D., and Liao, J. (2010). Trimming of mammalian transcriptional networks using network component analysis. *BMC Bioinformatics* 11, 511. doi: 10.1186/1471-2105-11-511

Vendeville, A., Winzer, K., Heurlier, K., Tang, C. M., and Hardie, K. R. (2005). Making "sense" of metabolism: autoinducer-2, LuxS and pathogenic bacteria. *Nat. Rev. Microbiol.* 3, 383–396.

Vernikos, G., Thomson, N., and Parkhill, J. (2007). Genetic flux over time in the *Salmonella* lineage. *Genome Biol.* 8, R100.

Vernikos, G. S., and Parkhill, J. (2006). Interpolated variable order motifs for identification of horizontally acquired DNA: revisiting the *Salmonella* pathogenicity islands. *Bioinformatics* 22, 2196–2203.

Virlogeux, I., Waxin, H., Ecobichon, C., Lee, J. O., and Popoff, M. Y. (1996). Characterization of the rcsA and rcsB genes from *Salmonella typhi*: rcsB through tviA is involved in regulation of Vi antigen synthesis. *J. Bacteriol.* 178, 1691–1698.

Wang, C., Xuan, J., Chen, L., Zhao, P., Wang, Y., Clarke, R., and Hoffman, E. (2008). Motif-directed network component analysis for regulatory network inference. *BMC Bioinformatics* 9(Suppl. 1), S21. doi: 10.1186/1471-2105-9-S1-S21

White, A. P., Weljie, A. M., Apel, D., Zhang, P., Shaykhutdinov, R., Vogel, H. J., and Surette, M. G. (2010). A global metabolic shift is linked to *Salmonella* multicellular development. *PLoS ONE* 5, e11814. doi: 10.1371/journal.pone.0011814

Widmaier, D. M., Tullman-Ercek, D., Mirsky, E. A., Hill, R., Govindarajan, S., Minshull, J., and Voigt, C. A. (2009). Engineering the *Salmonella* type III secretion system to export spider silk monomers. *Mol. Syst. Biol.* 5, 309.

Wilhelm, B. T., Marguerat, S., Watt, S., Schubert, F., Wood, V., Goodhead, I., Penkett, C. J., Rogers, J., and Bähler, J. (2008). Dynamic repertoire of a eukaryotic transcriptome surveyed at single-nucleotide resolution. *Nature* 453, 1239–1243.

Wilson, R., Libby, S. J., Freet, A. M., Boddicker, J. D., Fahlen, T. F., and Jones, B. D. (2001). Fis, a DNA nucleoid-associated protein, is involved in *Salmonella typhimurium* SPI-1 invasion gene expression. *Mol. Microbiol.* 39, 79–88.

Wozniak, C. E., and Hughes, K. T. (2008). Genetic dissection of the consensus sequence for the class 2 and class 3 flagellar promoters. *J. Mol. Biol.* 379, 936–952.

Xu, Y., Cheung, W., Winder, C. L., Dunn, W. B., and Goodacre, R. (2011). Metabolic profiling of meat: assessment of pork hygiene and contamination with *Salmonella typhimurium*. *Analyst* 136, 508–514.

Yoon, H., Ansong, C., Adkins, J. N., and Heffron, F. (2011). Secretion of *Salmonella* virulence factors into host cytoplasm via outer membrane vesicles. *BMC Syst. Biol.* (in press).

Yoon, H., Gros,, and Heffron, F. (2011). Quantitative PCR-based competitive index for high-throughput screening of *Salmonella* virulence factors. *Infect. Immun.* 79, 360–368.

Yoon, H., McDermott, J. E., Porwollik, S., McClelland, M., and Heffron, F. (2009). Coordinated regulation of virulence during systemic infection of *Salmonella enterica* serovar Typhimurium. *PLoS Pathog.* 5, e1000306. doi: 10.1371/journal.ppat.1000306

Yrlid, U., and Wick, M. (2002). Antigen presentation capacity and cytokine production by murine splenic dendritic cell subsets upon *Salmonella* encounter. *J. Immunol.* 169, 108–116.

Zhou, D., and Galan, J. (2001). *Salmonella* entry into host cells: the work in concert of type III secreted effector proteins. *Microbes Infect.* 3, 1293–1298.

Zimmer, J. S., Monroe, M. E., Qian, W. J., and Smith, R. D. (2006). Advances in proteomics data analysis and display using an accurate mass and time tag approach. *Mass Spectrom. Rev.* 25, 450–482.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 March 2011; accepted: 15 May 2011; published online: 02 June 2011.
Citation: McDermott JE, Yoon H, Nakayasu ES, Metz TO, Hyduke DR, Kidwai AS, Palsson BO, Adkins JN and Heffron F (2011) Technologies and approaches to elucidate and model the virulence program of Salmonella. Front. Microbio. ***2****:121. doi: 10.3389/fmicb.2011.00121*
This article was submitted to Frontiers in Cellular and Infection Microbiology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 14 June 2011
doi: 10.3389/fmicb.2011.00129

The role of the *spv* genes in *Salmonella* pathogenesis

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Salmonella strains cause three main types of diseases in people: gastroenteritis, enteric (typhoid) fever, and non-typhoid extra-intestinal disease with bacteremia. Genetic analysis indicates that each clinical syndrome requires distinct sets of virulence genes, and *Salmonella* isolates differ in their constellation of virulence traits. The *spv* locus is strongly associated with strains that cause non-typhoid bacteremia, but are not present in typhoid strains. The *spv* region contains three genes required for the virulence phenotype in mice: the positive transcriptional regulator *spvR* and two structural genes *spvB* and *spvC*. SpvB and SpvC are translocated into the host cell by the *Salmonella* pathogenicity island-2 type-three secretion system. SpvB prevents actin polymerization by ADP-ribosylation of actin monomers, while SpvC has phosphothreonine lyase activity and has been shown to inhibit MAP kinase signaling. The exact mechanisms by which SpvB and SpvC act in concert to enhance virulence are still unclear. SpvB exhibits a cytotoxic effect on host cells and is required for delayed cell death by apoptosis following intracellular infection. Strains isolated from systemic infections of immune compromised patients, particularly HIV patients, usually carry the *spv* locus, strongly suggesting that CD4 T cells are required to control disease due to *Salmonella* that are *spv* positive. This association is not seen with typhoid fever, indicating that the pathogenesis and immunology of typhoid have fundamental differences from the syndrome of non-typhoid bacteremia.

Keywords: *Salmonella, spv,* virulence, non-typhoid bacteremia, apoptosis, ADP-ribosylation, phosphothreonine lyase, CD4

Salmonella enterica strains are facultative intracellular pathogens that can produce both localized and disseminated, systemic disease in humans and a variety of other vertebrates (Guiney et al., 1994, 1995; Fierer and Guiney, 2001; Coburn et al., 2007). Non-typhoid *Salmonella* strains are endemic in domestic agricultural animal populations throughout the world, and provide a huge reservoir for human infection. An extensive body of work has shown that all *S. enterica* strains share common, core features of pathogenesis. This analysis has also shown that the ability to produce the distinctive clinical syndromes of typhoid fever, enteritis, or non-typhoid disseminated infection is due to the presence of specific sets of genes that are frequently located in pathogenicity islands and on phage or virulence plasmids (Fierer and Guiney, 2001).

SALMONELLA TYPE III PROTEIN SECRETION SYSTEMS IN PATHOGENESIS

Common features of *Salmonella* pathogenesis include the ability to induce intestinal epithelial cells to take up the organism into a *Salmonella*-containing vacuole (SCV), and the ability to manipulate the intracellular trafficking of the vacuole to promote survival and replication of the pathogen. These core virulence processes depend on the function of two distinct type-three secretion systems (abbreviated TTSS or T3SS) that transfer multiple effector proteins into the host cell cytoplasm. Following ingestion, *Salmonella* attach to intestinal epithelial cells and induce uptake of the bacteria into specialized membrane-bound vesicles termed SCV (reviewed in Galan, 2001; Ly and Casanova, 2007). This invasion process requires the TTSS encoded in the *Salmonella* pathogenicity island-1 (SPI-1) locus. The SPI-1 TTSS transfers a number of effector proteins into host epithelial cells, acting on Rho family GTPases and actin to produce physiological and structural changes in the cytoskeleton leading to bacterial uptake. The Rho family GTPases lead to activation of pro-inflammatory signaling pathways involving MAP kinases and NF-κB. Inside the SCV, *Salmonella* strains express a second TTSS encoded by the SPI-2 locus (Knodler and Steele-Mortimer, 2003; Waterman and Holden, 2003; Abrahams and Hensel, 2006). A variety of SPI-2 effectors regulate the trafficking of the SCV and interactions with the endosomal vesicular sorting process. These effectors position the SCV in a perinuclear position closely associated with the Golgi apparatus (Abrahams et al., 2006; Deiwick et al., 2006), stimulate the accumulation of actin filaments around the SCV (Poh et al., 2007), and also induce the formation of long, filamentous vesicular structures called Sifs (Brumell et al., 2002). *Salmonella* are able to replicate inside intestinal epithelial cells and induce apoptosis after 18–24 h (Kim et al., 1998; Paesold et al., 2002). In polarized intestinal monolayers, *Salmonella* can also pass through the epithelial cells and exit on the basolateral side before monolayer disruption occurs. Mucosal macrophages ingest *Salmonella*, but a massive influx of neutrophils quickly ensues in animals that develop clinical enteritis. *Salmonella* can also pass through the intestinal mucosa and disseminate by an SPI-1 independent process requiring CD18 positive cells (Vazquez-Torres et al., 1999).

The SPI-2 TTSS and a subset of effectors have been shown to be required to produce systemic disease (Knodler and Steele-Mortimer, 2003; Waterman and Holden, 2003; Abrahams and Hensel, 2006). A large body of experimental evidence indicates that extra-intestinal *Salmonella* infection occurs inside tissue macrophages (Fields et al., 1986; Vassiloyanakopoulos et al., 1998). Both clinical experience in humans and experimental studies in mice indicate that extracellular antibiotics such as the aminoglycosides are not effective against systemic *Salmonella* infections, demonstrating that *Salmonella* can grow within cells and spread from cell-to-cell without significant exposure to the extracellular space (Fierer et al., 1990; Vassiloyanakopoulos et al., 1998). SPI-2 TTSS effectors appear to act to promote intracellular infection by increasing the ability to *Salmonella* to resist the antimicrobial activity of macrophages and to grow within the cells. In macrophages, *Salmonella* appear to inhibit the recruitment of the NADPH oxidase to the phagosome, and this inhibition requires the SPI-2 TTSS function (Vazquez-Torres et al., 2000; Gallois et al., 2001). In support of the interaction between the SPI-2 virulence system and oxidative killing, gp91 phox−/− knockout mice lacking oxidase activity are fully susceptible to infection with SPI-2 mutants, while SPI-2 mutants are severely attenuated in mice with an intact NADPH oxidase (Vazquez-Torres et al., 2000). Unfortunately, the specific SPI-2 effectors involved in downregulating the NADPH oxidase have not been identified.

THE *spv* VIRULENCE LOCUS IN *SALMONELLA*

Non-typhoid *Salmonella* strains associated with extra-intestinal infections in humans and animals carry an additional locus termed *spv* (Guiney et al., 1994, 1995; Fierer and Guiney, 2001). The *spv* genes are located within a highly homologous region contained on virulence plasmids found in the subspecies 1 lineage of *S. enterica*, and were named for the designation *Salmonella* plasmid virulence (**Figure 1**). The *spv* locus enhances mouse virulence by several orders of magnitude in LD_{50}, depending on the *Salmonella* serovar and the mouse strain. The virulence phenotype is seen in both ItyS and ItyR mice, also referred to as Nramp1 (Slc11a1) mutant and wild-type mice respectively. Furthermore the *spv* effect has also been documented experimentally in calves and pigs and by molecular epidemiology in humans (Fierer et al., 1992; Libby et al., 1997). Subsequent work showed that the *spv* genes are located in the chromosome in certain other *S. enterica* lineages (Boyd and Hartl, 1998; Libby et al., 2002). *spv*-carrying serovars associated with human disease include Typhimurium, Enteritidis, Choleraesuis, Dublin, and Arizona. Certain host-adapted animal pathogens, such as Gallinarum/Pullorum and Abortusovis, also contain the *spv* locus. The plasmid *spvABCD* genes are arranged in an operon positively regulated by the upstream *spvR* gene, as shown in **Figure 1** (Fang et al., 1991; Krause et al., 1991, 1992). The *spvD* gene is missing in the chromosomal locus as found in serovar Arizona (Libby et al., 2002). The SpvR protein is a positive transcriptional regulator of the LysR family and binds to inverted repeat recognition sequences upstream of its own promoter and the *spvA* promoter (Krause et al., 1991, 1995; Grob and Guiney, 1996; Grob et al., 1997). Transcription initiation at both promoters requires the alternative sigma factor RpoS ("stationary phase sigma factor"; Fang et al., 1992; Chen et al., 1995). Expression of the *spv* operon is induced by the intracellular environment of host cells and is dependent on both SpvR and RpoS (Fierer et al., 1993; Chen et al., 1996). Genetic analysis demonstrates that the *spvR* and *spvBC* genes are required for the virulence phenotype of the *spv* locus, while mutations in *spvA* and *spvD* do not have a reproducible virulence phenotype in mice (Roudier et al., 1992).

Biochemical activities for SpvB and SpvC have been identified (Lesnick et al., 2001; Li et al., 2007). As shown in **Figure 1**, the SpvB protein has two distinct domains separated by a run of seven proline residues (nine in Dublin; Guiney and Lesnick, 2005). The N-terminal domain belongs to a large family of bacterial proteins identified by genome sequencing. Early work found members of

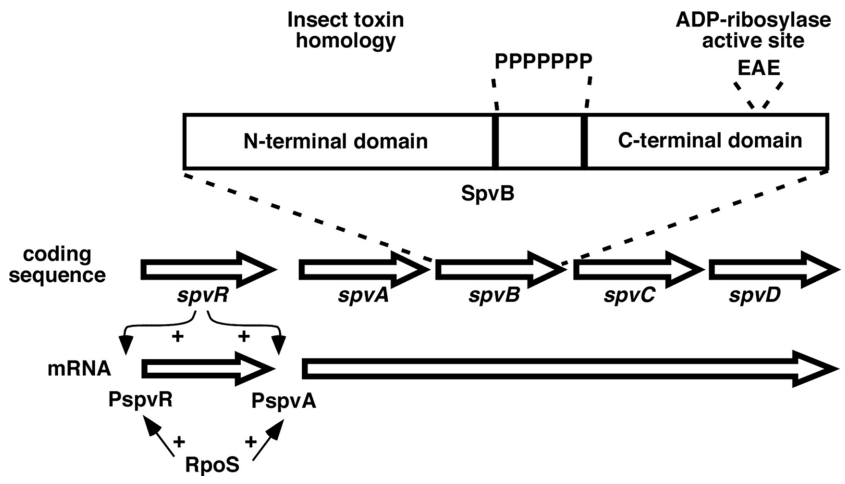

FIGURE 1 | Map of the *spv* region found on virulence plasmids in subspecies 1 strains, not drawn to scale. The *spvR* gene is transcribed separately from the *spvABCD* genes. SpvR activates transcription at both promoters in concert with the RpoS sigma factor. The expansion shows the general structure of the SpvB protein, with N- and C-terminal domains separated by a run of proline residues (P). EAE denotes residues at the active site for the ADP-ribosylation activity.

this family in certain insect toxins from Enterobacteriaceae that infect insects, but the regions of homology do not involve the toxin activity (Lesnick and Guiney, 2001). At present, the functions of this widely distributed protein family are unknown. In contrast, the C-terminal domain of SpvB contains ADP-ribosyltransferase activity that covalently modifies G-actin monomers and prevents their polymerization into F-actin filaments (Lesnick et al., 2001; Hochmann et al., 2006). Since F-actin is continuously formed and depolymerized in the cell, the activity of SpvB in the host cell cytoplasm leads to loss of the F-actin cytoskeleton (Lesnick et al., 2001; Browne et al., 2002). The C-terminal domain is both necessary and sufficient to depolymerize cellular F-actin, and the N-terminal domain is not required (Lesnick et al., 2001). A site-specific mutation at the active site (see **Figure 1**) was used to show that the actin ADP-ribosylating activity of SpvB is required for the virulence phenotype in mice and for actin depolymerization in infected macrophages (Lesnick et al., 2001). SpvB is required for *Salmonella* proliferation in a subset of monocyte-derived human macrophages, and is required for the late apoptosis seen in host cells during *Salmonella* infection (Libby et al., 2000; Paesold et al., 2002). Purified, enzymatically active SpvB protein does not enter intact eukaryotic cells (Lesnick et al., 2001). Instead, SpvB is translocated from *Salmonella* in the SCV into the host cell cytoplasm by a process dependent on SPI-2 (Browne et al., 2002, 2008). Recently, SpvC and the related *Shigella* effector OspF have been shown to have phosphothreoninelyase activity that irreversibly inactivates host cell MAP kinases by removal of phosphate and modification of the target threonine (Li et al., 2007). The role of this activity in the virulence phenotype of SpvC has yet to be determined. SpvC is also translocated by the SPI-2 TTSS (Mazurkiewicz et al., 2008). Although SpvB and SpvC have distinct and seemingly unrelated biochemical actions, both are required to produce the *spv* virulence phenotype (Roudier et al., 1992). Cloned *spvB* can complement a non-polar *spvB* mutant, but *spvB* without *spvC* does not have a detectable virulence phenotype (Guiney and Fierer, unpublished). These results strongly suggest that SpvB and SpvC act at different stages to affect a common pathway or process in the host cell required for resistance to infection.

HOST CELL DEATH INDUCED BY *SALMONELLA*

Salmonella strains produce host cell death during infection of cell cultures by several different mechanisms. *Salmonella* grown under conditions to express the SPI-1 TTSS activate the NLRC4 inflammasome in macrophages by a mechanism that depends on the SPI-1 TTSS rod component PrgJ and flagellin subunits (Franchi et al., 2006; Miao et al., 2006, 2010). The inflammasome activates caspase-1 leading to processing and release of IL-1β and IL-18, and cell death by a process termed pyroptosis (Fink and Cookson, 2007). The macrophages lyse, and bacteria are released to the extracellular space. The significance of this process for *Salmonella* pathogenesis is unknown. Recent studies show that caspase-1 apparently has a protective role for the host during systemic *Salmonella* infection, suggesting that caspase-1 activation by *Salmonella* would be detrimental to the organism in disseminated disease (Lara-Tejero et al., 2006; Raupach et al., 2006). However, these studies do not distinguish the roles of caspase-1 in the release of cytokines versus the induction of pyroptosis. Furthermore, SPI-1 TTSS mutants (including SipB mutants) have normal virulence for systemic disease in mice, indicating that SipB-mediated macrophage pyroptosis may not have a significant role in systemic infection (Guiney, 2005). The SPI-2 TTSS, which is essential for systemic virulence, does not appear to translocate flagellin nor activate the inflammasome (Miao et al., 2010). In contrast, human intestinal epithelial cells do not undergo pyroptosis even though *Salmonella* invasion involves the SPI-1 TTSS and SipB (Kim et al., 1998; Paesold et al., 2002). Instead, *Salmonella* strains proliferate inside epithelial cells and induce delayed cell death (16–24 h after infection) by a process that depends on both the SPI-2 TTSS and the spv locus (Paesold et al., 2002). *Salmonella*-induced epithelial cell death has features of classical apoptosis including cell surface exposure of phosphatidylserine, mitochondrial membrane depolarization, caspase-3 activation, cytokeritin-3 cleavage, and DNA fragmentation between nucleosomes (Kim et al., 1998; Paesold et al., 2002). A similar process occurs in macrophages infected with *Salmonella* that are grown under conditions that do not induce SPI-1 expression, or when SPI-1 mutants are used (Libby et al., 2000; Browne et al., 2002; Hsu et al., 2004). An important role for this delayed process of macrophage apoptosis during systemic *Salmonella* infection *in vivo* is supported by (1) histologic evidence of apoptosis in liver macrophages (Richter-Dahlfors et al., 1997), and (2) correspondence between the bacterial factors (such as SpvB) required for apoptosis in cell culture and for virulence *in vivo* (Libby et al., 2000; Browne et al., 2002; Hsu et al., 2004).

Regulation of apoptosis in macrophages is complex, with competing pathways leading to pro- and anti-apoptotic factors that govern the ultimate outcome. In general, primary macrophages exposed to bacterial products and/or pro-inflammatory, death-inducing cytokines such as TNF become activated rather than undergo apoptosis, due to the induction of factors that favor cell survival (Park et al., 2002; Hsu et al., 2004). Clearly, macrophages have to survive at sites of infection in order to function as immune effector cells. However, certain bacterial pathogens have evolved specific virulence mechanisms that block the pro-survival pathways of macrophages, tipping the balance of signals in favor of apoptosis and resulting in the death of the macrophage. **Figure 2** shows a schematic representation of the key pathways leading to the induction of apoptosis during infection of macrophages with *Salmonella*. For clarity, many factors and co-factors in the individual pathways are not shown. The data in support for this scheme were obtained primarily through the use of macrophages from specific knockout and transgenic mice in combination with specific bacterial mutants. As seen in **Figure 2**, apoptosis of wild-type macrophages in this system requires the SPI-2 TTSS but not the SPI-1 TTSS (SipB). Therefore, the process of apoptotic cell death clearly differs from pyroptosis dependent on SipB as described above (Fink and Cookson, 2007). *Salmonella* infection triggers activation of TLR4 by LPS. Although *Salmonella* produces ligands for other TLRs such as TLR2, TLR5, and TLR9, TLR4 has been shown to be required for apoptosis induced by *Salmonella*, so we have not shown the other TLRs in this scheme. TLR4 ligation triggers activation of the adapter proteins MyD88 and TRIF (also known as lps2). As seen in **Figure 2**, the TRIF

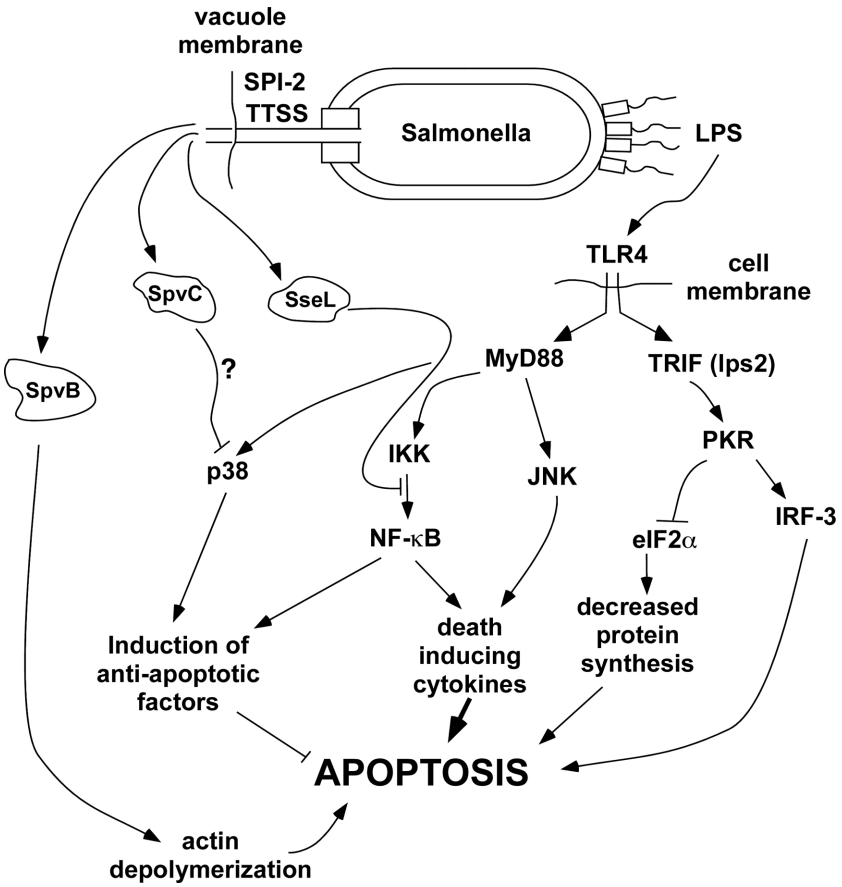

FIGURE 2 | General scheme of pathways affecting delayed apoptosis in *Salmonella*-infected host cells. Roles for each of the host factors were demonstrated using knockout mice or inhibitors. SpvB is known to be required and presumably acts through actin depolymerization, but the exact mechanism connecting SpvB to the induction of apoptosis remains to be clarified. Although SpvC and SseL have been shown to inhibit p38 and NF-κB respectively, the roles of these effectors in apoptosis is hypothetical at present.

pathway is a major activator of apoptosis, through a key intermediate kinase, PKR (also known as dsRNA responsive protein kinase because of its involvement in TLR3 signaling). Macrophages deficient in TRIF or PKR show marked reduction in *Salmonella*-induced apoptosis. PKR activates interferon-response factor-3 (IRF-3) and also phosphorylates eukaryotic translation initiation factor 2α (eIF2α), inhibiting protein synthesis and therefore blocking the expression of anti-apoptotic factors. Macrophages lacking IRF-3, and transgenic knock-in macrophages expressing a mutant eIF2α that is not phosphorylated by PKR both show reduced apoptosis after *Salmonella* infection (data in reference Hsu et al., 2004). These results clearly establish the TRIF pathway as a major transducer of pro-apoptotic signaling during *Salmonella* infection.

Ligation of TLR4 also activates pathways dependent on the MyD88 adapter protein, leading to activation of MAP kinases and NF-κB. The MAP kinase isoform JNK acts with NF-κB to induce pro-inflammatory, death-inducing cytokines such as TNF. However, NF-κB and the MAP kinase p38 have critical roles in the induction of anti-apoptotic factors leading to macrophage survival after exposure to LPS. Macrophages treated with a p38 inhibitor, or that have knockouts in MyD88 or IKKα undergo apoptosis after exposure to LPS alone (Park et al., 2002; Hsu et al., 2004). However, it is important to note that normal macrophages do not undergo apoptosis after treatment with LPS because the anti-apoptotic signaling dependent on p38 and NF-κB predominates.

ROLES OF *SALMONELLA* VIRULENCE FACTORS IN HOST CELL APOPTOSIS

The delayed apoptosis of macrophages after *Salmonella* infection requires PhoP (Valle and Guiney, 2005) and the SPI-2 TTSS, but not SPI-1 (Hsu et al., 2004). PhoP is a master regulatory factor that controls the expression of a large number of genes during intracellular infection. We found that PhoP is required independent of SPI-2 for *Salmonella* to induce death by a novel mechanism in THP-1 human macrophage-like cells (Valle and Guiney, 2005). The SPI-2 TTSS translocates effector proteins across the membrane of the SCV into the host cell cytoplasm. Without the intervention of specific *Salmonella* effector proteins, the p38 and NF-κB anti-apoptotic pathways dominate, and cells infected with *Salmonella* SPI-2 mutants do not undergo delayed apoptosis (Hsu et al., 2004). Two major SPI-2 TTSS-secreted effectors that induce

apoptosis have been identified: SpvB and SseL. The *spv* locus is required for the induction of apoptosis in human macrophages (Libby et al., 2000), and the ADP-ribosylating activity of SpvB is required for caspase-3 activation during human macrophage infection. However, the SpvB mutant does not reduce apoptosis to the level seen with an SPI-2 mutant that abolishes the TTSS function. This result suggests that other SPI-2 secreted effectors may also contribute to apoptosis. One of these candidates is SseL, shown to be involved in macrophage cytotoxicity (Rytkonen et al., 2007). NF-κB activation is significantly increased after infection of macrophages with an SseL mutant, and this activity can be decreased to wild-type levels by complementation with a low copy plasmid expressing SseL. These results show that the action of SseL is to decrease NF-κB signaling, thereby decreasing the anti-apoptotic and pro-inflammatory effects of this pathway. SseL deubiquitinates IκBα, the major regulator of the classical NF-κB activation pathway. In the NF-κB pathway, activation of IKK leads to phosphorylation of the inhibitor IκBα, targeting the inhibitor for ubiquitination and degradation by the proteosome. However, the deubiquitinating activity of SseL blocks degradation of IκBα and maintains the inhibition of NF-κB, preventing nuclear translocation and induction of NF-κB-dependent genes. As a result of the action of SseL in the host cell, both pro-inflammatory and anti-apoptotic pathways dependent on NF-κB are inhibited, allowing the pro-apoptotic effects through TRIF and PKR (see **Figure 2**) to dominate. Therefore, these results provide a mechanistic explanation for the cytotoxic effect of SseL previously reported (Rytkonen et al., 2007). The production of the pro-inflammatory cytokines TNF and IL-12 are decreased during infection of mice with wild-type serovar Typhimurium compared to the SseL mutant. These results support the idea that SseL may also act in concert with other effectors to decrease activation of innate immunity.

PROPOSED MODEL FOR THE VIRULENCE EFFECTS OF SpvB AND SpvC IN *SALMONELLA* INFECTION

Early in the analysis of the spv genes, genetic studies indicated that both SpvB and SpvC are required for the virulence phenotype encoded by the *spv* region (Roudier et al., 1992). A model to account for this finding is proposed in **Figure 3**. In this model, SpvB and SpvC act by different biochemical mechanisms, but they affect the same cellular pathways involved in the pathogenesis of intracellular *Salmonella* infection. Both SpvB and SpvC are translocated into the host cell cytoplasm by the SPI-2 TTSS (Browne et al., 2008; Mazurkiewicz et al., 2008). Actin depolymerization and induction of apoptosis are major effects of SpvB during intracellular *Salmonella* infection (Libby et al., 2000; Lesnick et al., 2001; Browne et al., 2002). Recent studies have shown that the NADPH oxidase associates with actin filaments during the organization and assembly of the oxidase on the membrane (elBenna et al., 1994; Allen et al., 1999; Zhan et al., 2004; Tamura et al., 2006), and wild-type *Salmonella* decreases NADPH oxidase recruitment to the phagosome by an SPI-2-dependent mechanism (Vazquez-Torres et al., 2000; Gallois et al., 2001). Therefore, SpvB-mediated actin depolymerization may decrease oxidase assembly and recruitment to the phagosome, and therefore could decrease oxidative killing of *Salmonella*. The second effect of SpvB-mediated actin depolymerization is to induce apoptosis (Libby et al., 2000; Lesnick et al., 2001). The phosphothreonine lyase activity of SpvC acts to decrease MAP kinase signaling, and inhibition of p38 has been demonstrated. As proposed in **Figure 3**, SpvC inhibition of the p38 MAP kinase isoform would block the synthesis of anti-apoptotic factors and therefore trigger apoptosis, in concert with the effects of SpvB. Macrophage apoptosis is one potential common pathway for the actions of SpvB and SpvC. In addition, SpvC may block the pro-inflammatory signaling function of the MAP kinase isoforms, leading to lower levels of key macrophage-activating cytokines such as TNF. *Salmonella* infections are known to be more severe in TNF-deficient mice (Vazquez-Torres et al., 2001). TNF has been shown promote localization of the NADPH oxidase to the phagosome of *Salmonella*-infected macrophages (Vazquez-Torres et al., 2001). Furthermore, the MAP kinase p38 has a direct effect in promoting oxidase assembly through phosphorylation of the p47 phox subunit (Laroux et al., 2005). Therefore, SpvC could act in concert with SpvB to block the recruitment of a functional NADPH oxidase to the phagosome. The SpvB and SpvC effects may be enhanced by the chromosomal sseL gene product, which further promotes apoptosis and decreases macrophage activation through blockade of NF-κB activity.

The virulence effect of decreasing NADPH oxidase activity is readily apparent, and infections in phox knockout mice have shown that oxidative killing is a major host defense mechanism against *Salmonella* (Mastroeni et al., 2000). The virulence effect of inducing apoptosis in infected macrophages has not been established. Host cell apoptosis has been described with many infectious agents (Guiney, 2005). In some systems, pathogen virulence factors appear to inhibit apoptosis, while in other systems, key virulence factors promote apoptosis. For *Salmonella*, we propose that induction of macrophage apoptosis late in the intracellular infection cycle is a specific virulence mechanism that facilitates cell-to-cell spread of the bacteria (Guiney, 2005). Apoptosis induces cell surface changes that stimulate receptor-mediated uptake of apoptotic cells by surrounding macrophages, thereby transferring infection to these naïve macrophages. Therefore, the induction of apoptosis by *Salmonella* may represent a specific mechanism promoting cell-to-cell spread and subsequent rounds of macrophage infection. If *Salmonella* produced necrosis or pyroptosis during infection, free bacteria would be released into the extracellular space. The inability of extracellular antibiotics such as gentamicin to control *Salmonella* infection is strong evidence in favor of the importance of a cell-to-cell spread mechanism (Fierer et al., 1990). The model proposes that this intracellular infection cycle of *spv*+ *Salmonella* can only be terminated by the induction of specific CD4+ T cell immunity.

The ability of *Salmonella* to cause sustained intracellular infection has been a well-established paradigm in pathogenesis, and this principle has been confirmed by numerous experimental studies indicating that CD4+ T cell immunity is required for control of the infection (reviewed in Mittrucker and Kaufmann, 2000; Mastroeni and Menager, 2003). This concept is strongly supported by the key clinical observation in humans that the CD4+ cell depletion seen in HIV infection and other immune deficiency conditions strongly

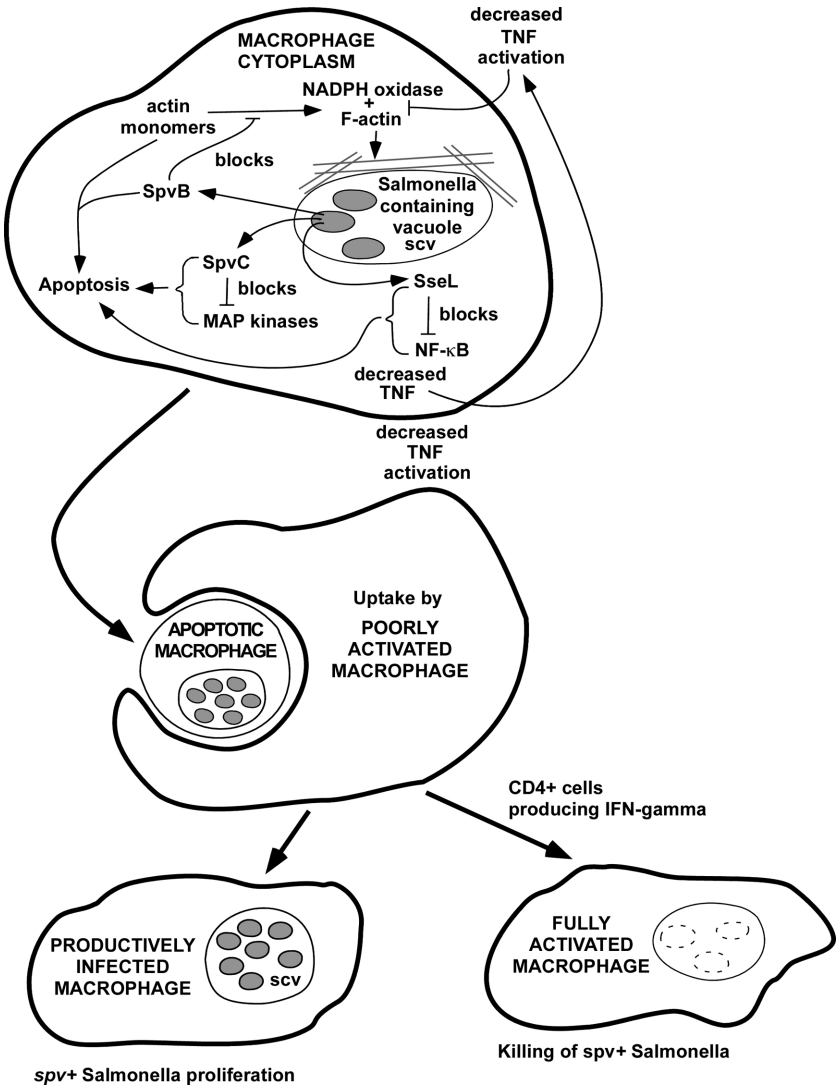

FIGURE 3 | Hypothetical model for the combined effects of SpvB, SpvC, and SseL to promote *Salmonella* virulence. In this model, SpvB, SpvC, and SseL could act by different biochemical mechanisms to inhibit NADPH oxidase recruitment to the phagosome and also to promote host cell apoptosis. The overall effect of host cell apoptosis may to be promote cell-to-cell spread of the infection, since extracellular antibiotics such as gentamicin, and phagocytosis of extracellular bacteria by neutrophils, are not able to terminate infections with *Salmonella* strains that express *spv* genes. This model would provide an explanation for the experimental and clinical evidence that CD4 T cells and IFN-gamma are required to control infections due to *spv*+ *Salmonella*, likely involving macrophage activation and killing of intracellular bacteria.

predisposes to persistent disseminated non-typhoid *Salmonella* disease (Gilks et al., 1990; Arthur et al., 2001).

ROLE OF THE spv LOCI IN HUMAN INFECTIONS WITH *SALMONELLA*

The role of the *spv* locus in the mouse model of salmonellosis is well established, but its role in human infection is less appreciated. *S. enterica* strains cause three distinct illnesses in humans. The first and most common disease is gastroenteritis; it is estimated that more than one million cases occur annually in the USA alone (Mead et al., 1999). The other two less common syndromes are typhoid or enteric fever (Tauxe, 1997), and *Salmonella* bacteremia. Of the greater than 2000 serovars of *S. enterica* that are identified using the Kauffman–White typing scheme, about 20 serovars, mostly in O antigen groups B, C, D, and E (Jones et al., 2008), cause the vast majority of cases of gastroenteritis, as these strains are widely distributed amongst domestic food animals and so enter the food chain (Foley and Lynne, 2008). Bacteremia rarely accompanies gastroenteritis, and when it does, it is usually transient (Goldberg and Rubin, 1988). Of the most commonly isolated serovars from stool cultures only Typhimurium and Enteritidis can carry virulence plasmids that encode the *spv* operon, indicating that *spv* genes are not required to cause gastroenteritis in people.

In contrast to gastroenteritis, enteric fever is caused by two serovars: Typhi and Paratyphi A (Goldberg and Rubin, 1988).

These organisms are host adapted to humans and are spread fecal-orally via contaminated food or water. Enteric fever is a sub-acute to chronic disease characterized by a transient rash, fever, weight loss, cough, and headache, but rarely diarrhea. Clinically, the infection localizes in the RES, as hepatosplenomegaly and abnormal liver function tests are common. Bacteremia is present in the first week of the illness but is either transient or very low grade in the following weeks. However, the organism can still be recovered from the bone marrow, another indication of its RES localization (Hoffman et al., 1984). Neither Typhi nor Paratyphi A possesses spv genes. Furthermore, typhoid fever does not have an increased incidence or altered pathogenesis in HIV patients, indicating that CD4+ T cell immunity is not crucial for the human host response to typhoid.

In contrast, non-typhoid *Salmonella* bacteremias are usually due to strains that encode the *spv* locus (Guiney et al., 1995), implying that *spv* products are required for bacteremia. The evidence for this is indirect, since it would be unethical to try to compare the dissemination potential of isogenic strains of *Salmonella* in humans. Therefore, the evidence for this association is obtained from molecular epidemiological studies of patients with *Salmonella* bacteremia to determine if there is a relationship between the clinical syndrome and infections with *spv*+ *Salmonella*. *Salmonella* bacteremia is distinct from enteric fever, in most cases presenting as high fever, usually without a rash or localizing signs or symptoms unless it has already caused a metastatic infection, e.g., infection of a bone or joint. Diarrhea is uncommon, and often there is no history of recent intestinal symptoms. The diagnosis of *Salmonella* bacteremia is established by positive blood cultures for non-typhoid *S. enterica*. In older immunocompetent patients *S. enterica* bacteremia in the absence of localizing signs or symptoms suggests the presence of a mycotic aneurysm (Cohen et al., 1978). With rare exceptions the aneurysms are infected with serovars known to carry virulence plasmids (Mendelowitz et al., 1979).

The syndrome of non-typhoid *Salmonella* bacteremia was first recognized as a manifestation of human infection with serovar Choleraesuis, a pig-adapted pathogen. In a study of 329 culture-proven cases of Choleraesuis in the northeastern US, only 26 had isolated gastroenteritis, while 239 isolates were from blood, and another 88 were from extra-intestinal sites of infection, such as CSF and abscesses (Saphra and Winter, 1957). Since all strains of Choleraesuis carry a virulence plasmid with the *spv* operon, this establishes an association between *spv* genes and the extra-intestinal infection syndrome, even though those particular isolates were not tested to see if they were *spv*+. This study and others (Maccready et al., 1957) showed that Choleraesuis can cause invasive infections in patients with no known immunological defects. In most developed countries *S.* Choleraesuis is now an uncommon human isolate, probably because of improvements in animal husbandry that have greatly reduced the risk of human infection.

The other *spv* bearing serovars are relatively less virulent in humans than Choleraesuis, mostly infecting patients with immunodeficiencies. More recently serovar Dublin has emerged as a cause of *Salmonella* bacteremia (Fierer, 1983; Fang and Fierer, 1991). Like Choleraesuis, Dublin infections present primarily as bacteremia without gastroenteritis (Fang and Fierer, 1991). Most infections with this bovine-adapted serovar are acquired from drinking unpasteurized milk or other dairy products (Headrick et al., 1998). Patients with T cell immune defects are over-represented in case series of Dublin infection (Werner et al., 1979; Fang and Fierer, 1991), suggesting that the functions of *spv* genes are largely controlled by a normal CD4 T cell response to infection. The most frequent serovars causing *Salmonella* bacteremia are Typhimurium and Enteritidis. Since they are also the most common causes of gastroenteritis one might assume that their prominence in the former reflects their prominence in the latter syndrome. However, if this was the whole explanation, one would expect stool and blood isolates to have the same prevalence of *spv* genes, but this is not the case (Fierer et al., 1992). In epidemiologically unrelated isolates from different states in the United States, 76% of the blood isolates carried *spv* genes, about twice the percentage of fecal isolates. Since clinical information was not available for these cases, it is not certain that all the blood isolates were from patients with the syndrome of *Salmonella* bacteremia, which may explain why nearly 25% of blood isolates did not have spv genes. Heithoff et al. (2008) examined *S.* Typhimurium isolates from Utah and they found that 10/10 blood isolates had the *spv* locus, while only 19/29 fecal isolates had spv. Others have examined the proportion of isolates of different serovars that cause bacteremia and Dublin and Choleraesuis stand out because they have such a high ratio of blood to fecal isolates (Blaser and Feldman, 1981; Jones et al., 2008).

The AIDS epidemic revealed the importance of CD4 T cells in preventing *Salmonella* bacteremia, a syndrome that was often the presenting manifestation of AIDS (Celum et al., 1987; Gruenewald et al., 1994). In those publications that included information about the serovars involved, they were nearly always strains that are known to carry virulence plasmids (Blaser and Feldman, 1981; Celum et al., 1987; Gruenewald et al., 1994). One exception was *S. enterica* serovar Arizona, an infection of patients who had AIDS and ingested folk remedies made from rattlesnakes (Noskin and Clarke, 1990). Subsequently, these isolates were shown to have the *spv* operon inserted in the chromosome (Libby et al., 2002).

Non-typhoid *Salmonella* bacteremia is a very common problem in sub-Saharan Africa, in both adults and children. Very few studies have looked for the presence of *spv* genes in the African blood culture isolates, but one large study of *S.* Typhimurium blood culture isolates from Malawi and two regions of Kenya found that all of them had virulence plasmids (Kingsley et al., 2009). Disturbingly, many of the isolates carried a pSLT virulence plasmid that had a large transposon insert encoding multiple antibiotic resistance genes. This genetic association has also been observed in *S.* Typhimurium isolates from the Asturia region of Spain, potentially linking selection for virulence with that for antibiotic resistance. Recently, analysis of 24 blood culture isolates from AIDS patients in Uganda, comprising serovars Typhimurium, Typhimurium var Copenhagen, and Enteritidis, has found that all contained the *spvB* gene (unpublished results; Fierer and Guiney). Thus, it appears likely that the *spv* operon is necessary for the syndrome of *Salmonella* bacteremia in AIDS, regardless of the serovar involved. To establish this, one would need to examine isolates for people in the same communities with gastroenteritis and show that a lower percentage had the virulence plasmid.

SUMMARY

The *spv* genes comprise an accessory virulence locus in *Salmonella* located on virulence plasmids in the subspecies I lineage and in the chromosome of certain other subspecies. Regulation of expression of the *spv* operon occurs through the SpvR transcription activator and the RpoS sigma factor, and responds physiologically to the stationary phase of growth and the intracellular environment of host cells. SpvB and SpvC have been identified as essential effector proteins for the spv virulence phenotype and their biochemical activities have been characterized. Both SpvB and SpvC are translocated by the TTSS encoded by SPI-2. SpvB is an ADP-ribosyltransferase that modifies G-actin and prevents polymerization to F-actin, thereby disrupting the actin cytoskeleton. SpvC has phosphothreoninelyase activity and inhibits MAP kinases. The mechanisms of the concerted virulence effects of SpvB and SpvC remain to be determined. SpvB has been shown to be required for apoptosis in human macrophages, and may act together with SpvC and other *Salmonella* effectors.

Evidence from experimental models and human epidemiologic data indicate that the *spv* genes promote the virulence of non-typhoid *Salmonella* serovars to cause extra-intestinal disease. The *spv* genes appear to subvert the innate immune mechanisms in the host that are able to terminate the common intestinal infections due to non-typhoid *Salmonella* manifested as self-limited gastroenteritis. Resistance to *spv*-expressing *Salmonella* causing disseminated infection appears to require acquired immunity mediated by CD4+ T cells as demonstrated experimentally in mice and by the high incidence of *spv*+ non-typhoid *Salmonella* bacteremia in HIV infection. Analysis of the distribution of the *spv* locus in *Salmonella* serovars has underscored a key difference between the pathogenesis of typhoid fever and non-typhoid *Salmonella* bacteremia in people. The typhoid serovars Typhi and Paratyphi lack the *spv* genes, and are not associated with increased infections in patients deficient in CD4+ T cells. Therefore, the mechanisms of pathogenesis and host immunity are likely to have important differences between typhoid and non-typhoid serovars of *Salmonella*.

ACKNOWLEDGMENTS

Work by the authors was supported in part by NIH grants AI032178 and AI077661.

REFERENCES

Abrahams, G. L., and Hensel, M. (2006). Manipulating cellular transport and immune responses: dynamic interactions between intracellular *Salmonella enterica* and its host cells. *Cell. Microbiol.* 8, 728–737.

Abrahams, G. L., Muller, P., and Hensel, M. (2006). Functional dissection of SseF, a type III effector protein involved in positioning the *Salmonella*-containing vacuole. *Traffic* 7, 950–965.

Allen, L. A., DeLeo, F. R., Gallois, A., Toyoshima, S., Suzuki, K., and Nauseef, W. M. (1999). Transient association of the nicotinamide adenine dinucleotide phosphate oxidase subunits p47phox and p67phox with phagosomes in neutrophils from patients with X-linked chronic granulomatous disease. *Blood* 93, 3521–3530.

Arthur, G., Nduba, V. N., Kariuki, S. M., Kimari, J., Bhatt, S. M., and Gilks, C. F. (2001). Trends in bloodstream infections among human immunodeficiency virus-infected adults admitted to a hospital in Nairobi, Kenya, during the last decade. *Clin. Infect. Dis.* 33, 248–256.

Blaser, M. J., and Feldman, R. A. (1981). From the centers for disease control. *Salmonella* bacteremia: reports to the Centers for Disease Control, 1968–1979. *J. Infect. Dis.* 143, 743–746.

Boyd, E. F., and Hartl, D. L. (1998). *Salmonella* virulence plasmid. Modular acquisition of the spv virulence region by an F-plasmid in *Salmonella enterica* subspecies I and insertion into the chromosome of subspecies II, IIIa, IV and VII isolates. *Genetics* 149, 1183–1190.

Browne, S. H., Hasegawa, P., Okamoto, S., Fierer, J., and Guiney, D. G. (2008). Identification of *Salmonella* SPI-2 secretion system components required for SpvB-mediated cytotoxicity in macrophages and virulence in mice. *FEMS Immunol. Med. Microbiol.* 52, 194–201.

Browne, S. H., Lesnick, M. L., and Guiney, D. G. (2002). Genetic requirements for *Salmonella*-induced cytopathology in human monocyte-derived macrophages. *Infect. Immun.* 70, 7126–7135.

Brumell, J. H., Goosney, D. L., and Finlay, B. B. (2002). SifA, a type III secreted effector of *Salmonella typhimurium*, directs *Salmonella*-induced filament (Sif) formation along microtubules. *Traffic* 3, 407–415.

Celum, C. L., Chaisson, R. E., Rutherford, G. W., Barnhart, J. L., and Echenberg, D. F. (1987). Incidence of salmonellosis in patients with AIDS. *J. Infect. Dis.* 156, 998–1002.

Chen, C. Y., Buchmeier, N. A., Libby, S., Fang, F. C., Krause, M., and Guiney, D. G. (1995). Central regulatory role for the RpoS sigma factor in expression of *Salmonella dublin* plasmid virulence genes. *J. Bacteriol.* 177, 5303–5309.

Chen, C. Y., Eckmann, L., Libby, S. J., Fang, F. C., Okamoto, S., Kagnoff, M. F., Fierer, J., and Guiney, D. G. (1996). Expression of *Salmonella typhimurium* rpoS and rpoS-dependent genes in the intracellular environment of eukaryotic cells. *Infect. Immun.* 64, 4739–4743.

Coburn, B., Grassl, G. A., and Finlay, B. B. (2007). *Salmonella*, the host and disease: a brief review. *Immunol. Cell Biol.* 85, 112–118.

Cohen, P. S., O'Brien, T. F., Schoenbaum, S. C., and Medeiros, A. A. (1978). The risk of endothelial infection in adults with *Salmonella* bacteremia. *Ann. Intern. Med.* 89, 931–932.

Deiwick, J., Salcedo, S. P., Boucrot, E., Gilliland, S. M., Henry, T., Petermann, N., Waterman, S. R., Gorvel, J. P., Holden, D. W., and Méresse, S. (2006). The translocated *Salmonella* effector proteins SseF and SseG interact and are required to establish an intracellular replication niche. *Infect. Immun.* 74, 6965–6972.

elBenna, J., Ruedi, J. M., and Babior, B. M. (1994). Cytosolic guanine nucleotide-binding protein Rac2 operates in vivo as a component of the neutrophil respiratory burst oxidase. Transfer of Rac2 and the cytosolic oxidase components p47phox and p67phox to the submembranous actin cytoskeleton during oxidase activation. *J. Biol. Chem.* 269, 6729–6734.

Fang, F. C., and Fierer, J. (1991). Human infection with *Salmonella dublin*. *Medicine* 70, 198–207.

Fang, F. C., Krause, M., Roudier, C., Fierer, J., and Guiney, D. G. (1991). Growth regulation of a *Salmonella* plasmid gene essential for virulence. *J. Bacteriol.* 173, 6783–6789.

Fang, F. C., Libby, S. J., Buchmeier, N. A., Loewen, P. C., Switala, J., Harwood, J., and Guiney, D. G. (1992). The alternative sigma factor katF (rpoS) regulates *Salmonella* virulence. *Proc. Natl. Acad. Sci. U.S.A.* 89, 11978–11982.

Fields, P. I., Swanson, R. V., Haidaris, C. G., and Heffron, F. (1986). Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are a virulent. *Proc. Natl. Acad. Sci. U.S.A.* 83, 5189–5193.

Fierer, J. (1983). Invasive *Salmonella dublin* infections associated with drinking raw milk. *West. J. Med.* 138, 665–669.

Fierer, J., Eckmann, L., Fang, F., Pfeifer, C., Finlay, B. B., and Guiney, D. (1993). Expression of the *Salmonella* virulence plasmid gene spvB in cultured macrophages and nonphagocytic cells. *Infect. Immun.* 61, 5231–5236.

Fierer, J., and Guiney, D. G. (2001). Diverse virulence traits underlying different clinical outcomes of *Salmonella* infection. *J. Clin. Invest.* 107, 775–780.

Fierer, J., Hatlen, L., Lin, J. P., Estrella, D., Mihalko, P., and Yau-Young, A. (1990). Successful treatment using gentamicin liposomes of *Salmonella dublin* infections in mice. *Antimicrob. Agents Chemother.* 34, 343–348.

Fierer, J., Krause, M., Tauxe, R., and Guiney, D. (1992). *Salmonella typhimurium* bacteremia: association with the virulence plasmid. *J. Infect. Dis.* 166, 639–642.

Fink, S. L., and Cookson, B. T. (2007). Pyroptosis and host cell death responses during *Salmonella* infection. *Cell. Microbiol.* 9, 2562–2570.

Foley, S. L., and Lynne, A. M. (2008). Food animal-associated *Salmonella* challenges: pathogenicity and antimicrobial resistance. *J. Anim. Sci.* 86, E173–E187.

Franchi, L., Amer, A., Body-Malapel, M., Kanneganti, T. D., Ozoren, N., Jagirdar, R., Inohara, N., Vandenabeele, P., Bertin, J., Coyle, A., Grant, E. P., and Núñez, G. (2006). Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in *Salmonella*-infected macrophages. *Nat. Immunol.* 7, 576–582.

Galan, J. E. (2001). *Salmonella* interactions with host cells: type III secretion at work. *Annu. Rev. Cell Dev. Biol.* 17, 53–86.

Gallois, A., Klein, J. R., Allen, L. A., Jones, B. D., and Nauseef, W. M. (2001). *Salmonella* pathogenicity island 2-encoded type III secretion system mediates exclusion of NADPH oxidase assembly from the phagosomal membrane. *J. Immunol.* 166, 5741–5748.

Gilks, C. F., Brindle, R. J., Newnham, R. S., Watkins, W. M., Waiyaki, P. G., Were, J. B. O., Otieno, L. S., Simani, P. M., Bhatt, S. M., Lule, G. N., Okelo, G. B. A., Brindle, R. J., Newnham, R. S., Gilks, C. F., Warrell, D. A. (1990). Life-threatening bacteraemia in HIV-1 seropositive adults admitted to hospital in Nairobi, Kenya. *Lancet* 336, 545–549.

Goldberg, M. B., and Rubin, R. H. (1988). The spectrum of *Salmonella* infection. *Infect. Dis. Clin. North Am.* 2, 571–598.

Grob, P., and Guiney, D. G. (1996). In vitro binding of the *Salmonella dublin* virulence plasmid regulatory protein SpvR to the promoter regions of spvA and spvR. *J. Bacteriol.* 178, 1813–1820.

Grob, P., Kahn, D., and Guiney, D. G. (1997). Mutational characterization of promoter regions recognized by the *Salmonella dublin* virulence plasmid regulatory protein SpvR. *J. Bacteriol.* 179, 5398–5406.

Gruenewald, R., Blum, S., and Chan, J. (1994). Relationship between human immunodeficiency virus infection and salmonellosis in 20- to 59-year-old residents of New York City. *Clin. Infect. Dis.* 18, 358–363.

Guiney, D. G. (2005). The role of host cell death in *Salmonella* infections. *Curr. Top. Microbiol. Immunol.* 289, 131–150.

Guiney, D. G., Fang, F. C., Krause, M., and Libby, S. (1994). Plasmid-mediated virulence genes in non-typhoid *Salmonella* serovars. *FEMS Microbiol. Lett.* 124, 1–9.

Guiney, D. G., Fang, F. C., Krause, M., Libby, S., Buchmeier, N. A., and Fierer, J. (1995). Biology and clinical significance of virulence plasmids in *Salmonella* serovars. *Clin. Infect. Dis.* 21(Suppl. 2), S146–S151.

Guiney, D. G., and Lesnick, M. (2005). Targeting of the actin cytoskeleton during infection by *Salmonella* strains. *Clin. Immunol.* 114, 248–255.

Headrick, M. L., Korangy, S., Bean, N. H., Angulo, F. J., Altekruse, S. F., Potter, M. E., and Klontz, K. C. (1998). The epidemiology of raw milk-associated foodborne disease outbreaks reported in the United States, 1973 through 1992. *Am. J. Public Health* 88, 1219–1221.

Heithoff, D. M., Shimp, W. R., Lau, P. W., Badie, G., Enioutina, E. Y., Daynes, R. A., Byrne, B. A., House, J. K., and Mahan, M. J. (2008). Human *Salmonella* clinical isolates distinct from those of animal origin. *Appl. Environ. Microbiol.* 74, 1757–1766.

Hochmann, H., Pust, S., von Figura, G., Aktories, K., and Barth, H. (2006). *Salmonella enterica* SpvB ADP-ribosylates actin at position arginine-177-characterization of the catalytic domain within the SpvB protein and a comparison to binary clostridial actin-ADP-ribosylating toxins. *Biochemistry* 45, 1271–1277.

Hoffman, S. L., Punjabi, N. H., Rockhill, R. C., Sutomo, A., Rivai, A. R., and Pulungsih, S. P. (1984). Duodenal string-capsule culture compared with bone-marrow, blood, and rectal-swab cultures for diagnosing typhoid and paratyphoid fever. *J. Infect. Dis.* 149, 157–161.

Hsu, L. C., Park, J. M., Zhang, K., Luo, J. L., Maeda, S., Kaufman, R. J., Eckmann, L., Guiney, D. G., and Karin, M. (2004). The protein kinase PKR is required for macrophage apoptosis after activation of Toll-like receptor 4. *Nature* 428, 341–345.

Jones, T. F., Ingram, L. A., Cieslak, P. R., Vugia, D. J., Tobin-D'Angelo, M., Hurd, S., Medus, C., Cronquist, A., and Angulo, F. J. (2008). Salmonellosis outcomes differ substantially by serotype. *J. Infect. Dis.* 198, 109–114.

Kim, J. M., Eckmann, L., Savidge, T. C., Lowe, D. C., Witthoft, T., and Kagnoff, M. F. (1998). Apoptosis of human intestinal epithelial cells after bacterial invasion. *J. Clin. Invest.* 102, 1815–1823.

Kingsley, R. A., Msefula, C. L., Thomson, N. R., Kariuki, S., Holt, K. E., Gordon, M. A., Harris, D., Clarke, L., Whitehead, S., Sangal, V., Marsh, K., Achtman, M., Molyneux, M. E., Cormican, M., Parkhill, J., MacLennan, C. A., Heyderman, R. S., and Dougan, G. (2009). Epidemic multiple drug resistant *Salmonella* Typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype. *Genome Res.* 19, 2279–2287.

Knodler, L. A., and Steele-Mortimer, O. (2003). Taking possession: biogenesis of the *Salmonella*-containing vacuole. *Traffic* 4, 587–599.

Krause, M., Fang, F. C., el-Gedaily, A., Libby, S., and Guiney, D. G. (1995). Mutational analysis of SpvR binding to DNA in the regulation of the *Salmonella* plasmid virulence operon. *Plasmid* 34, 37–47.

Krause, M., Fang, F. C., and Guiney, D. G. (1992). Regulation of plasmid virulence gene expression in *Salmonella dublin* involves an unusual operon structure. *J. Bacteriol.* 174, 4482–4489.

Krause, M., Roudier, C., Fierer, J., Harwood, J., and Guiney, D. (1991). Molecular analysis of the virulence locus of the *Salmonella dublin* plasmid pSDL2. *Mol. Microbiol.* 5, 307–316.

Lara-Tejero, M., Sutterwala, F. S., Ogura, Y., Grant, E. P., Bertin, J., Coyle, A. J., Flavell, R. A., and Galán, J. E. (2006). Role of the caspase-1 inflammasome in *Salmonella typhimurium* pathogenesis. *J. Exp. Med.* 203, 1407–1412.

Laroux, F. S., Romero, X., Wetzler, L., Engel, P., and Terhorst, C. (2005). Cutting edge: MyD88 controls phagocyte NADPH oxidase function and killing of gram-negative bacteria. *J. Immunol.* 175, 5596–5600.

Lesnick, M. L., and Guiney, D. G. (2001). The best defense is a good offense – *Salmonella* deploys an ADP-ribosylating toxin. *Trends Microbiol.* 9, 2–4; discussion 4–5.

Lesnick, M. L., Reiner, N. E., Fierer, J., and Guiney, D. G. (2001). The *Salmonella* spvB virulence gene encodes an enzyme that ADP-ribosylates actin and destabilizes the cytoskeleton of eukaryotic cells. *Mol. Microbiol.* 39, 1464–1470.

Li, H., Xu, H., Zhou, Y., Zhang, J., Long, C., Li, S., Chen, S., Zhou, J. M., and Shao, F. (2007). The phosphothreonine lyase activity of a bacterial type III effector family. *Science* 315, 1000–1003.

Libby, S. J., Adams, L. G., Ficht, T. A., Allen, C., Whitford, H. A., Buchmeier, N. A., Bossie, S., and Guiney, D. G. (1997). The spv genes on the *Salmonella dublin* virulence plasmid are required for severe enteritis and systemic infection in the natural host. *Infect. Immun.* 65, 1786–1792.

Libby, S. J., Lesnick, M., Hasegawa, P., Kurth, M., Belcher, C., Fierer, J., and Guiney, D. G. (2002). Characterization of the spv locus in *Salmonella enterica* serovar Arizona. *Infect. Immun.* 70, 3290–3294.

Libby, S. J., Lesnick, M., Hasegawa, P., Weidenhammer, E., and Guiney, D. G. (2000). The *Salmonella* virulence plasmid spv genes are required for cytopathology in human monocyte-derived macrophages. *Cell. Microbiol.* 2, 49–58.

Ly, K. T., and Casanova, J. E. (2007). Mechanisms of *Salmonella* entry into host cells. *Cell. Microbiol.* 9, 2103–2111.

Maccready, R. A., Reardon, J. P., and Saphra, I. (1957). Salmonellosis in Massachusetts; a sixteen-year experience. *N. Engl. J. Med.* 256, 1121–1128.

Mastroeni, P., and Menager, N. (2003). Development of acquired immunity to *Salmonella*. *J. Med. Microbiol.* 52, 453–459.

Mastroeni, P., Vazquez-Torres, A., Fang, F. C., Xu, Y., Khan, S., Hormaeche, C. E., and Dougan, G. (2000). Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival in vivo. *J. Exp. Med.* 192, 237–248.

Mazurkiewicz, P., Thomas, J., Thompson, J. A., Liu, M., Arbibe, L., Sansonetti, P., and Holden, D. W. (2008). SpvC is a *Salmonella* effector with phosphothreoninelyase activity on host mitogen-activated protein kinases. *Mol. Microbiol.* 67, 1371–1383.

Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M., and Tauxe, R. V. (1999). Food-related illness and death in the United States. *Emerg. Infect. Dis.* 5, 607–625.

Mendelowitz, D. S., Ramstedt, R., Yao, J. S., and Bergan, J. J. (1979). Abdominal aortic salmonellosis. *Surgery* 85, 514–519.

Miao, E. A., Alpuche-Aranda, C. M., Dors, M., Clark, A. E., Bader, M. W., Miller, S. I., and Aderem, A. (2006). Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. *Nat. Immunol.* 7, 569–575.

Miao, E. A., Mao, D. P., Yudkovsky, N., Bonneau, R., Lorang, C. G., Warren, S. E., Leaf, I. A., and Aderem, A. (2010). Innate immune detection of the type III secretion apparatus through the NLRC4 inflammasome. *Proc. Natl. Acad. Sci. U.S.A.* 107, 3076–3080.

Mittrucker, H. W., and Kaufmann, S. H. (2000). Immune response to infection with *Salmonella typhimurium* in mice. *J. Leukoc. Biol.* 67, 457–463.

Noskin, G. A., and Clarke, J. T. (1990). *Salmonella arizonae* bacteremia as the presenting manifestation of human immunodeficiency virus infection following rattlesnake meat ingestion. *Rev. Infect. Dis.* 12, 514–517.

Paesold, G., Guiney, D. G., Eckmann, L., and Kagnoff, M. F. (2002). Genes in the *Salmonella* pathogenicity island 2 and the *Salmonella* virulence plasmid are essential for *Salmonella*-induced apoptosis in intestinal epithelial cells. *Cell. Microbiol.* 4, 771–781.

Park, J. M., Greten, F. R., Li, Z. W., and Karin, M. (2002). Macrophage apoptosis by anthrax lethal factor through p38 MAP kinase inhibition. *Science* 297, 2048–2051.

Poh, J., Odendall, C., Spanos, A., Boyle, C., Liu, M., Freemont, P., and Holden, D. W. (2007). SteC is a *Salmonella* kinase required for SPI-2-dependent F-actin remodelling. *Cell. Microbiol.* 10, 20–30.

Raupach, B., Peuschel, S. K., Monack, D. M., and Zychlinsky, A. (2006). Caspase-1-mediated activation of interleukin-1beta (IL-1beta) and IL-18 contributes to innate immune defenses against *Salmonella enterica* serovar Typhimurium infection. *Infect. Immun.* 74, 4922–4926.

Richter-Dahlfors, A., Buchan, A. M., and Finlay, B. B. (1997). Murine salmonellosis studied by confocal microscopy: *Salmonella typhimurium* resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes in vivo. *J. Exp. Med.* 186, 569–580.

Roudier, C., Fierer, J., and Guiney, D. G. (1992). Characterization of translation termination mutations in the spv operon of the *Salmonella* virulence plasmid pSDL2. *J. Bacteriol.* 174, 6418–6423.

Rytkonen, A., Poh, J., Garmendia, J., Boyle, C., Thompson, A., Liu, M., Freemont, P., Hinton, J. C., and Holden, D. W. (2007). SseL, a *Salmonella* deubiquitinase required for macrophage killing and virulence. *Proc. Natl. Acad. Sci. U.S.A.* 104, 3502–3507.

Saphra, I., and Winter, J. W. (1957). Clinical manifestations of salmonellosis in man; an evaluation of 7779 human infections identified at the New York *Salmonella* Center. *N. Engl. J. Med.* 256, 1128–1134.

Tamura, M., Itoh, K., Akita, H., Takano, K., and Oku, S. (2006). Identification of an actin-binding site in p47phox an organizer protein of NADPH oxidase. *FEBS Lett.* 580, 261–267.

Tauxe, R. V. (1997). Emerging foodborne diseases: an evolving public health challenge. *Emerging Infect. Dis.* 3, 425–434.

Valle, E., and Guiney, D. G. (2005). Characterization of *Salmonella*-induced cell death in human macrophage-like THP-1 cells. *Infect. Immun.* 73, 2835–2840.

Vassiloyanakopoulos, A. P., Okamoto, S., and Fierer, J. (1998). The crucial role of polymorphonuclear leukocytes in resistance to *Salmonella dublin* infections in genetically susceptible and resistant mice. *Proc. Natl. Acad. Sci. U.S.A.* 95, 7676–7681.

Vazquez-Torres, A., Fantuzzi, G., Edwards, C. K. III, Dinarello, C. A., and Fang, F. C. (2001). Defective localization of the NADPH phagocyte oxidase to *Salmonella*-containing phagosomes in tumor necrosis factor p55 receptor-deficient macrophages. *Proc. Natl. Acad. Sci. U.S.A.* 98, 2561–2565.

Vazquez-Torres, A., Jones-Carson, J., Baumler, A. J., Falkow, S., Valdivia, R., Brown, W., Le, M., Berggren, R., Parks, W. T., and Fang, F. C. (1999). Extraintestinal dissemination of *Salmonella* by CD18-expressing phagocytes. *Nature* 401, 804–808.

Vazquez-Torres, A., Xu, Y., Jones-Carson, J., Holden, D. W., Lucia, S. M., Dinauer, M. C., Mastroeni, P., and Fang, F. C. (2000). *Salmonella* pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. *Science* 287, 1655–1658.

Waterman, S. R., and Holden, D. W. (2003). Functions and effectors of the *Salmonella* pathogenicity island 2 type III secretion system. *Cell. Microbiol.* 5, 501–511.

Werner, S. B., Humphrey, G. L., and Kamei, I. (1979). Association between raw milk and human *Salmonella dublin* infection. *Br. Med. J.* 2, 238–241.

Zhan, Y., He, D., Newburger, P. E., and Zhou, G. W. (2004). p47(phox) PX domain of NADPH oxidase targets cell membrane via moesin-mediated association with the actin cytoskeleton. *J. Cell. Biochem.* 92, 795–809.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 16 March 2011; accepted: 25 May 2011; published online: 14 June 2011.

*Citation: Guiney DG and Fierer J (2011) The role of the spv genes in Salmonella pathogenesis. Front. Microbio. **2**:129. doi: 10.3389/fmicb.2011.00129*

This article was submitted to Frontiers in Cellular and Infection Microbiology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

GENERAL COMMENTARY
published: 16 March 2011
doi: 10.3389/fmicb.2011.00050

Type three secretion system effector translocation: one step or two?

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A commentary on

Translocation of surface-localized effectors in type III secretion

by Akopyan, K., Edgren, T., Wang-Edgren, H., Rosqvist, R., Fahlgren, A., Wolf-Watz, H., and Fallman, M. (2011). Proc. Natl. Acad. Sci. U.S.A. 108, 1639–1644.

Type three secretion systems (T3SSs) are macromolecular structures utilized by a number of bacterial pathogens to transfer proteins from the bacterial cytosol, across bacterial and host membranes, into host cells (Cornelis, 2006). The process of crossing the bacterial membranes is termed secretion with the steps of crossing the host membranes being called translocation. Once inside the host cells, the bacterial proteins subvert normal cellular processes to benefit the bacterium; the specific effect dependent on the infectious cycle of the bacterium. For example, *Salmonella enterica* uses effector proteins to remodel the host cell actin cytoskeleton and plasma membrane, mediating its uptake into non-phagocytic cells (Ly and Casanova, 2007).

Type three secretion systems are highly conserved and consist of approximately 20 proteins that assemble the basal body and needle components of a functional T3SS. The basal body is believed to carry out secretion and the hollow channel inside the needle structure is presumed to act as a conduit through which bacterial proteins enter the host cell (Kubori et al., 1998; Marlovits et al., 2004; Cornelis, 2006). Although various models have been proposed for the secretion apparatus, there is very little evidence detailing the translocation mechanism. The current prevailing model is that the effectors transport from the needle tip across the host cell membrane using bacterial proteins that create a translocon pore in the host cell membrane (Hakansson et al., 1996; Scherer et al., 2000), thus completing secretion and translocation in a "one-step" process. In a recent publication, Akopyan et al. (2011) provide evidence challenging this canonical "one-step" model for the transport of effector proteins, proposing that in *Yersinia pseudotuberculosis* at least, a "two-step" model of T3SS-dependent protein translocation may be possible. In this "two-step" process, bacterial effectors are first secreted to the surface of the bacterium and then translocated across the host cell membrane in a T3SS-dependent manner.

Immunoelectron microscopy showed that *Yersinia* T3SS effectors YopE and YopH, and the translocator YopD are evenly distributed across the *Yersinia* surface prior to contact with host cells (Akopyan et al., 2011). It is not uncommon for translocator proteins to be found on the bacterial surface (Ménard et al., 1994; Watarai et al., 1996; Lara-Tejero and Galan, 2009), particularly since the hydrophilic translocator protein forms a tip at the end of the T3SS needle (Mueller et al., 2008). However, previous data employing immunofluorescent microscopy indicated that effector proteins accumulated in the bacterial cytoplasm awaiting exit through the T3SS, and often exhibited polarized localization toward active T3SS needles (Schlumberger et al., 2005; Jaumouille et al., 2008; Winnen et al., 2008). Such data fits with a one-step T3SS translocation model where T3SS substrates are synthesized and stored in the bacterial cytoplasm ready to be fed to the T3SS basal body upon T3SS activation, with subsequent transport through the interior of the needle. While there is no direct evidence for effector transport through the needle, experiments have shown the need for effectors to unfold prior to secretion – indicative of transport though the needle core (Akeda and Galan, 2005). A one-step model would also explain how effectors can be translocated in a hierarchial manner as has been shown for SipA, SopE, and SptP in *Salmonella* (Winnen et al., 2008; Lara-Tejero et al., 2011). Perhaps the most compelling evidence to support the current "one-step" model is that most T3SS pathogens cannot be cross-complemented, that is, a mixed infection with mutants deficient in secretion and translocation separately will not restore the wild-type phenotype. Thus, the finding that functional effectors are present on the *Yersinia* surface and can be translocated to induce physiological responses in host cells is surprising and requires reappraisal of the current data.

Akopyan et al. (2011) presented evidence that coating a *Y. pseudotuberculosis yopH* mutant with YopH *in vitro* restores the mutant to inducing a similar physiological response to wild-type bacteria. Furthermore, use of YopH-Bla or YopK-Bla hybrid proteins to coat various *Yersinia* mutants led to the rapid, specific T3SS-specific translocation of the surface effectors into host cells. It is intriguing that surface effectors appear to be translocated in a manner similar to secreted effectors. It is still unclear how these surface effectors are transferred to the host cell membrane but it is clear that the translocon remains important for their transfer inside the host cell; a *Yersinia* mutant lacking YopB and YopD failed to promote translocation of a YopH-Bla coat, and the surface effectors require a translocation domain for transport inside the host cell (Akopyan et al., 2011). This led the authors to propose that the translocon recognizes surface effectors via this domain and can then feed them into the host cell. This also explains why effectors possess such a domain. Hitherto, it was unclear why the translocon domain was required in addition to a secretion signal if the translocon acted as a continuation of the T3SS needle. This does not rule out the potential for the translocon to act as a checkpoint in T3SS secretion and ensure the smooth transition of effectors across the host cell membrane in both a one-step and two-step model. Indeed, relatively little is known as to how exactly the translocon functions, which could shed further light on the necessity of translocation domains in effector proteins. The additional finding that

the YopH secretion signal is not required for translocation supports the ability for T3SS substrates to be located on the bacterial surface prior to their translocation in a T3SS manner (Akopyan et al., 2011). The possession of both secretion and translocation sequences by T3SS effectors may indicate a flexibility in T3S hitherto unknown, and may suggest one-step and two-step translocation occur at the same time, although it is not clear yet what the advantage may be during infection.

One of the major questions this latest research poses is how universal two-step translocation is to other bacteria possessing T3SSs. Initial experiments coating *Salmonella* Typhimurium with YopH-Bla constructs showed *Salmonella* was able to translocate YopH, in a SPI-1 T3SS-dependent manner (Akopyan et al., 2011). A more definitive experiment would be to demonstrate whether such translocation could restore a biological phenotype, such as ruffling conferred by SopB or SopE during *Salmonella* infection. Given the homology that exists between components and effectors of the *Yersinia*, *Salmonella*, and *Shigella* T3SSs (Rosqvist et al., 1995) it would not be surprising if the two-step translocation mechanism was used by each. Indeed, the *Salmonella* effector SptP has homology with both YopE and YopH (Kaniga et al., 1996) which would make it a likely candidate for two-step translocation. However, homology alone will not dictate that this translocation method is universal. The interactions of *Yersinia*, *Salmonella*, and *Shigella* with host cells leads to different outcomes, reflecting the different lifestyles led by these bacteria that have ultimately shaped their effector repertoire and may therefore potentially have individualized the function of their T3SSs. It will be necessary now to determine whether two-step translocation is universal to some or all T3SS-possessing bacteria in addition to elucidating the role of this process in infection. Hints may come from determining what proportion of effectors are targeted to the bacterial surface, whether all effectors are represented on the surface and of course how these proteins are transported to the surface. With the advancement in imaging technology, it is not impossible to speculate that we might be able to visualize both the secretion and the translocation process in the near future and to be able to demonstrate the "one-step" and/or "two-step" translocation beyond reasonable doubt.

REFERENCES

Akeda, Y., and Galan, J. E. (2005). Chaperone release and unfolding of substrates in type III secretion. *Nature* 437, 911–915.

Akopyan, K., Edgren, T., Wang-Edgren, H., Rosqvist, R., Fahlgren, A., Wolf-Watz, H., and Fallman, M. (2011). Translocation of surface-localized effectors in type III secretion. *Proc. Natl. Acad. Sci. U.S.A.* 108, 1639–1644.

Cornelis, G. R. (2006). The type III secretion injectisome. *Nat. Rev. Microbiol.* 4, 811–825.

Hakansson, S., Schesser, K., Persson, C., Galyov, E. E., Rosqvist, R., Homble, F., and Wolf-Watz, H. (1996). The YopB protein of *Yersinia pseudotuberculosis* is essential for the translocation of Yop effector proteins across the target cell plasma membrane and displays a contact-dependent membrane disrupting activity. *EMBO J.* 15, 5812–5823.

Jaumouille, V., Francetic, O., Sansonetti, P. J., and Tran Van Nhieu, G. (2008). Cytoplasmic targeting of IpaC to the bacterial pole directs polar type III secretion in *Shigella*. *EMBO J.* 27, 447–457.

Kaniga, K., Uralil, J., Bliska, J. B., and Galán, J. E. (1996). A secreted protein tyrosine phosphatase with modular effector domains in the bacterial pathogen *Salmonella typhimurium*. *Mol. Microbiol.* 21, 633–641.

Kubori, T., Matsushima, Y., Nakamura, D., Uralil, J., Lara-Tejero, M., Sukhan, A., Galán, J. E., and Aizawa, S. I. (1998). Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. *Science* 280, 602–605.

Lara-Tejero, M., and Galan, J. E. (2009). *Salmonella enterica serovar typhimurium* pathogenicity island 1-encoded type III secretion system translocases mediate intimate attachment to nonphagocytic cells. *Infect. Immun.* 77, 2635–2642.

Lara-Tejero, M., Kato, J., Wagner, S., Liu, X., and Galan, J. E. (2011). A sorting platform determines the order of protein secretion in bacterial type III systems. *Science* 331, 1188–1191.

Ly, K. T., and Casanova, J. E. (2007). Mechanisms of *Salmonella* entry into host cells. Cell. Microbiol. 9, 2103–2111.

Marlovits, T. C., Kubori, T., Sukhan, A., Thomas, D. R., Galán, J. E., and Unger, V. M. (2004). Structural insights into the assembly of the type III secretion needle complex. *Science* 306, 1040–1042.

Ménard, R., Sansonetti, P., Parsot, C., and Vasselon, T. (1994). Extracellular association and cytoplasmic partitioning of the IpaB and IpaC invasins of *S. flexneri*. *Cell* 79, 515–525.

Mueller, C. A., Broz, P., and Cornelis, G. R. (2008). The type III secretion system tip complex and translocon. *Mol. Microbiol.* 68, 1085–1095.

Rosqvist, R., Persson, C., Hakansson, S., Nordfeldt, R., and Wolf-Watz, H. (1995). Translocation of the *Yersinia* YopE and YopH virulence proteins into target cells is mediated by YopB and YopD. *Contrib. Microbiol. Immunol.* 13, 230–234.

Scherer, C. A., Cooper, E., and Miller, S. I. (2000). The *Salmonella* type III secretion translocon protein SspC is inserted into the epithelial cell plasma membrane upon infection. *Mol. Microbiol.* 37, 1133–1145.

Schlumberger, M. C., Muller, A. J., Ehrbar, K., Winnen, B., Duss, I., Stecher, B., and Hardt, W.-D. (2005). Real-time imaging of type III secretion: *Salmonella* SipA injection into host cells. *Proc. Natl. Acad. Sci. U.S.A.* 102, 12548–12553.

Watarai, M., Funato, S., and Sasakawa, C. (1996). Interaction of Ipa proteins of *Shigella flexneri* with alpha5beta1 integrin promotes entry of the bacteria into mammalian cells. *J. Exp. Med.* 183, 991–999.

Winnen, B., Schlumberger, M. C., Sturm, A., Schupbach, K., Siebenmann, S., Jenny, P., and Hardt, W. D. (2008). Hierarchical effector protein transport by the *Salmonella* Typhimurium SPI-1 type III secretion system. *PLoS ONE* 3, e2178. doi: 10.1371/journal.pone.0002178

Received: 28 February 2011; accepted: 04 March 2011; published online: 16 March 2011.

Citation: Perrett CA and Zhou D (2011) Type three secretion system effector translocation: one step or two? Front. Microbio. ***2****:50. doi: 10.3389/fmicb.2011.00050*

This article was submitted to Frontiers in Cellular and Infection Microbiology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

OPINION ARTICLE
published: 18 March 2011
doi: 10.3389/fmicb.2011.00048

Salmonella exploits suicidal behavior of epithelial cells

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Infection with enteric bacterial pathogens such as *Salmonella* leads to tissue invasion and intracellular replication in the intestinal mucosa. Bacterial invasion is detected by the host innate immune system and triggers a stereotypical, inflammatory response characterized by recruitment of immune cells to the infected tissue. In a remarkable paper, Knodler et al. (2010) propose a novel mechanism by which the intestinal epithelium can clear *Salmonella*-infected cells through a form of proinflammatory cell death and how *Salmonella* in fact may exploit this mechanism.

TAKING ONE FOR THE TEAM: PYROPTOTIC CELL DEATH OF INFECTED EPITHELIAL CELLS AS A PROINFLAMMATORY SIGNAL

Using a polarized epithelial cell culture model, Knodler et al. (2010) observed that in a fraction of infected cells, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) replicated with an astonishing doubling time of only 20 min. Although conventional wisdom holds that intracellular *Salmonella* typically reside in a vacuolar compartment, this population had escaped the membrane bound compartment by unknown mechanisms and was replicating rapidly inside the cytoplasm. Epithelial cells harboring hyperreplicating bacteria were expelled from the monolayer into the luminal space. This process resembled the homeostatic turnover of epithelial cells in the intestinal tract. However, during infection with a pathogen, extrusion of dying, infected cells was accompanied by activation of caspase-1, and secretion of the caspase-1 substrate interleukin (IL)-18, a proinflammatory cytokine (**Figure 1**). This observation suggests that a proinflammatory programmed (pyroptotic) cell death of epithelial cells may contribute to the induction of inflammatory responses *in vivo*. Indeed, in a murine model of *Salmonella*-induced colitis, Muller et al. (2009) have shown recently that SopE, a translocated effector of the invasion-associated type three secretion system, triggers intestinal inflammation by activating caspase-1 in intestinal stromal cells, presumably enterocytes. IL-18 signaling downstream of caspase-1 was required for SopE-mediated induction of inflammation. Taken together, these findings support the idea that pyroptotic cell death contributes to the initiation of the inflammatory host response in the gut (Bergsbaken et al., 2009) and suggest that intestinal epithelial cells serve as sentinels for bacterial invasion.

EXPLOITATION OF HOST IMMUNE RESPONSES BY *SALMONELLA*

The inflammatory response induced by *S.* Typhimurium leads to profound changes in the intestinal tract, turning the gut lumen into a nutritional niche in which *S.* Typhimurium efficiently outcompetes the endogenous microbiota (Stecher et al., 2007; Winter et al., 2010). Interestingly, Knodler et al. (2010) also observed that *S.* Typhimurium might exploit the pyroptotic cell death of bacteria-laden epithelial cells for its own benefit. *S.* Typhimurium rapidly replicating inside the cytosol of extruded epithelial cells expressed flagella and the invasion-associated type III secretion system, two key virulence factors for invasion of non-phagocytic host cells. Expression of these virulence factors might prime a subpopulation of released bacteria for re-entry into other epithelial cells, thereby prolonging the duration of disease. Furthermore, epithelial pyroptosis might aid in reseeding the intestinal lumen, thereby contributing to transmission by the fecal oral route.

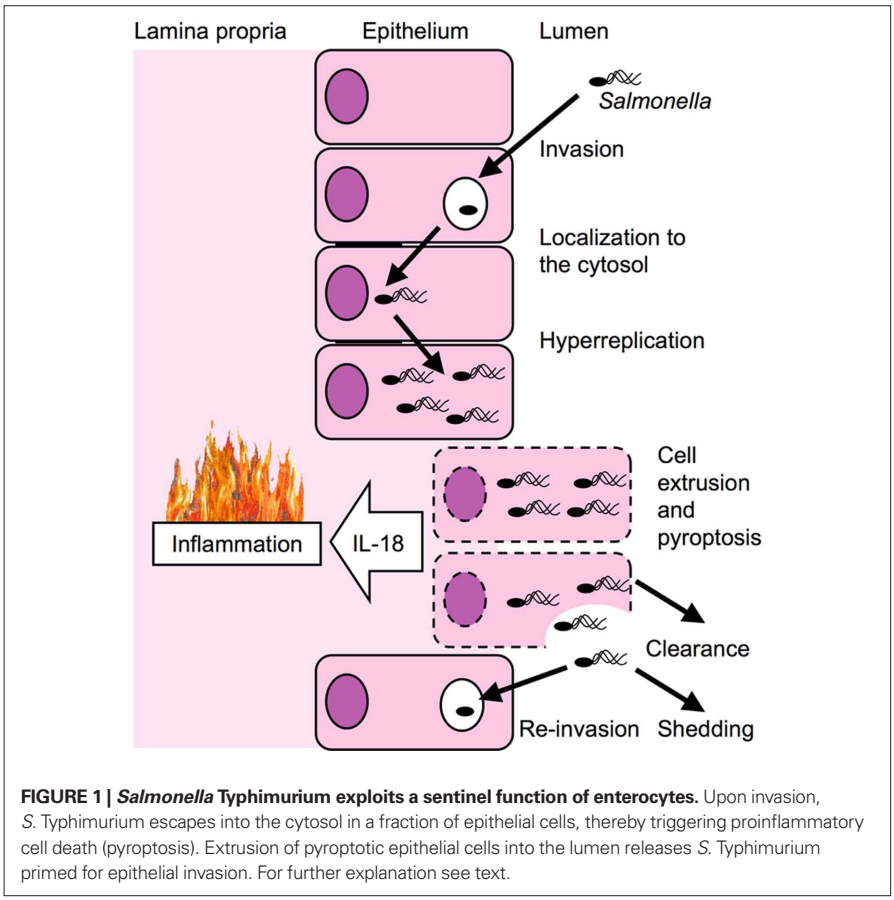

FIGURE 1 | *Salmonella* Typhimurium exploits a sentinel function of enterocytes. Upon invasion, *S.* Typhimurium escapes into the cytosol in a fraction of epithelial cells, thereby triggering proinflammatory cell death (pyroptosis). Extrusion of pyroptotic epithelial cells into the lumen releases *S.* Typhimurium primed for epithelial invasion. For further explanation see text.

This work by Knodler et al. (2010) is pioneering since it sheds light on a new mechanism of host response aimed at clearing invasive pathogens from the intestinal epithelium and initiating intestinal inflammation. At the same time, the experiments suggest that *S.* Typhimurium may take advantage of this pathway to enhance its transmission success.

REFERENCES

Bergsbaken, T., Fink, S. L., and Cookson, B. T. (2009). Pyroptosis: host cell death and inflammation. *Nat. Rev. Microbiol.* 7, 99–109.

Knodler, L. A., Vallance, B. A., Celli, J., Winfree, S., Hansen, B., Montero, M., and Steele-Mortimer, O. (2010). Dissemination of invasive *Salmonella* via bacterial-induced extrusion of mucosal epithelia. *Proc. Natl. Acad. Sci. U.S.A.* 107, 17733–17738.

Muller, A. J., Hoffmann, C., Galle, M., Van Den Broeke, A., Heikenwalder, M., Falter, L., Misselwitz, B., Kremer, M., Beyaert, R., and Hardt, W. D. (2009). The *S. Typhimurium* effector SopE induces caspase-1 activation in stromal cells to initiate gut inflammation. *Cell Host Microbe* 6, 125–136.

Stecher, B., Robbiani, R., Walker, A. W., Westendorf, A. M., Barthel, M., Kremer, M., Chaffron, S., Macpherson, A. J., Buer, J., Parkhill, J., Dougan, G., von Mering, C., and Hardt, W. D. (2007). *Salmonella enterica* serovar typhimurium exploits inflammation to compete with the intestinal microbiota. *PLoS Biol.* 5, 2177–2189. doi: 10.1371/journal.pbio.0050244

Winter, S. E., Thiennimitr, P., Winter, M. G., Butler, B. P., Huseby, D. L., Crawford, R. W., Russell, J. M., Bevins, C. L., Adams, L. G., Tsolis, R. M., Roth, J. R., and Baumler, A. J. (2010). Gut inflammation provides a respiratory electron acceptor for *Salmonella*. *Nature* 467, 426–429.

Received: 25 February 2011; accepted: 03 March 2011; published online: 18 March 2011.

*Citation: Winter SE and Bäumler AJ (2011) Salmonella exploits suicidal behavior of epithelial cells. Front. Microbio. **2**:48. doi: 10.3389/fmicb.2011.00048*

This article was submitted to Frontiers in Cellular and Infection Microbiology, a specialty of Frontiers in Microbiology.