



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 (O₂) or iron and low-molecular weight thiols, are preferentially bacteriostatic against *Salmonella*, while reaction of NO and superoxide (O₂⁻) 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

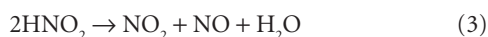
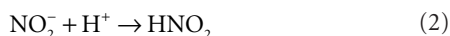
Salmonella outbreaks are often associated with contaminated produce. This suggests that *Salmonella* must establish relationships with plants to ensure persistence in the environment while in transit between animal hosts. The plant surface is for the most part a

hostile environment in which bacteria can suffer the adverse effects of ultraviolet light and desiccation. Stomata (small openings in leaves involved in H₂O 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 non-specific 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 (NO₃⁻). NO₃⁻ 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 NO₃⁻ is reduced to nitrite (NO₂⁻) by resident microbiota populating the posterior, anaerobic regions of the tongue (reaction 1; Duncan et al., 1995). In the low pH of the stomach, NO₂⁻ is protonated to its nitrous acid (HNO₂) conjugate, which serves as a precursor for various RNS such as NO, nitrogen dioxide (NO₂), and dinitrogen trioxide (N₂O₃; reactions 2–4).



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 NO₃⁻ 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 mod-

erate 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 SPII-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. NO₃⁻ can arise in the gut as either an auto-oxidative product or through enzymatic detoxification of NO or nitrification reactions (Tannenbaum et al., 1978). NO₃⁻ could be used as alternative electron acceptor to maintain electron transport chain function in the O₂-limited environment of the gut (Jones et al., 2007). Failure of *Salmonella* to use O₂ 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 O₂-depleted environment of the gastrointestinal lumen, *Salmonella* likely utilizes available alternative electron acceptors. In favor of this idea, the *Salmonella* NO₂⁻ 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 NO₃⁻ as a respiratory substrate (Prior et al., 2009). However, the role of these nitrate reductases to *Salmonella* pathogenesis awaits investigation. In contrast to NO₃⁻, 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 NO₃⁻ 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 SPII 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 O₂⁻ and NO, also contributes to the early killing of *Salmonella* by IFN γ -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 IFN γ -DEPENDENT STIMULATION OF NO SYNTHESIS

The expression of iNOS is controlled at the transcriptional level. Both innate and IFN γ -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 NF κ B signaling that stimulates IFN β 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 NF κ B 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 TNF α for the induction of iNOS expression (Ables et al., 2001). However, *Salmonella* can induce iNOS enzymatic activity in the absence of signaling through the TNF α 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 IFN γ 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 IFN γ is optimal in the presence of a triggering signal. Findings in macrophages deficient in TLR4 indicate that the expression of iNOS seen in IFN γ -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 IFN γ in the activation of iNOS transcription (Vitiello et al., 2008). The elevated NO fluxes of IFN γ -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 IFN γ 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 IFN γ -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 O₂, O₂⁻, 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

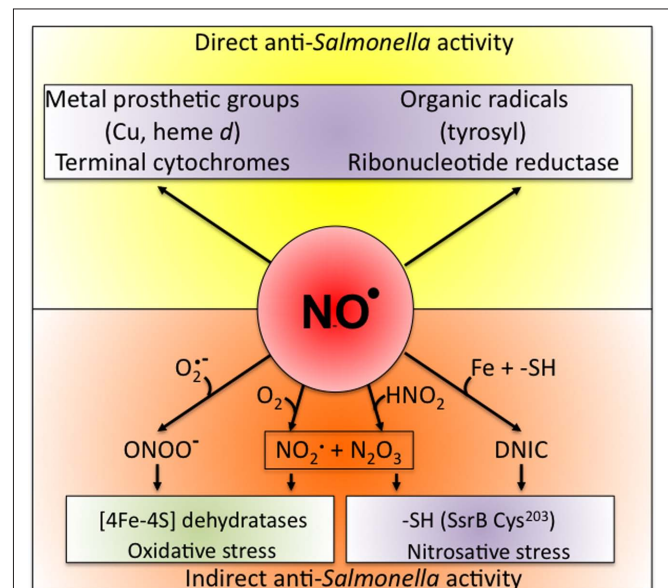


FIGURE 1 | Biological chemistry of NO and *Salmonella* targets. NO can react directly (yellow box) with metal prosthetic groups or other radicals. Binuclear centers containing copper (Cu) or iron heme d in terminal quinol cytochrome oxidases of the electron transport chain are directly nitrosylated by NO. The covalent attachment of NO to molecular targets causes nitrosylated 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 (O₂⁻), molecular oxygen (O₂), or iron and low-molecular weight thiols (-SH). Nitrogen dioxide (NO₂) and dinitrogen trioxide (N₂O₃) can independently arise from the condensation of acidified nitrite (HNO₂) or the auto-oxidation of NO in the presence of O₂. Peroxynitrite (ONOO⁻), NO₂[•], and N₂O₃ are strong oxidants. [4Fe-4S] clusters of dehydratases are frequent targets of ONOO⁻ (green box). N₂O₃ and dinitrosyl-iron complexes (DNIC), which frequently target thiol-containing proteins, are common sources of nitrosative stress. The redox active thiol of Cys²⁰³ 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 O₂ to H₂O (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 O₂. 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 O₂-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).

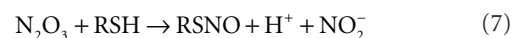
In *E. coli*, NO forms iron–nitrosyl complexes with the ferric uptake regulatory protein Fur, thereby derepressing iron-regulated gene transcription (D’Autreaux et al., 2002). In addition to affecting iron metabolism, the nitrosylation of Fur could have broad implications in *Salmonella* gene expression. 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 O₂ or O₂⁻. 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:



Since reaction (5) is second order for NO, it follows that N₂O₃ 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, N₂O₃ 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, N₂O₃ can independently be generated in the phagosomal lumen through the condensation of HNO₂. In fact, about a third of the N₂O₃ generated in IFNγ-primed macrophages appears to form via HNO₂ (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 N₂O₃. 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 O₂⁻ 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 O₂⁻ 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 O₂⁻ generated adventitiously from the reduction of O₂ 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 cytoplasm (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; Vázquez-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 Cys²⁰³ 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 Cys²⁰³ 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 Cys²⁰³ 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 (Chakravorty 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 $ONOO^-$ (Chakravorty et al., 2002), which being an anion does not cross freely through membranes. IFN γ -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 IFN γ -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 IFN γ -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 IFN γ -primed macrophages (McCollister et al., 2005).

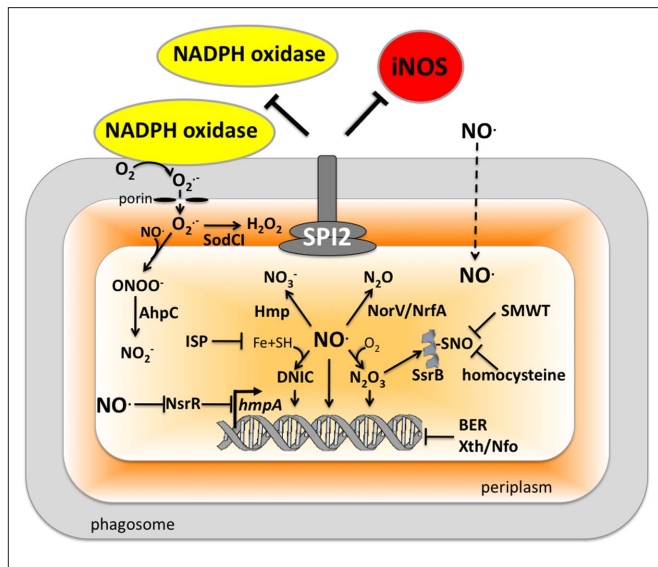


FIGURE 2 | Antinitrosative defenses of *Salmonella*. Effectors secreted by the type III secretion system encoded within SPI2 actively avoid NADPH oxidase- and iNOS-containing vesicles. Superoxide (O_2^-) produced by the enzymatic activity of NADPH oxidase enters the periplasmic space of *Salmonella* 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 environ-

ment 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 Nrp1⁺ 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

O₂⁻ and NO produced by NADPH oxidase and iNOS hemoproteins (De Groote et al., 1997; Sansone et al., 2002). Alternatively, ONOO⁻ can be detoxified to NO₂⁻ 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/aprimidinic 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 O₂-depleted environment of the gut. The ability of *Salmonella* to utilize NO₃⁻ 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 Cys²⁰³ 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.

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