THE STRINGENT RESPONSE OF *SALMONELLA* TYPHIMURIUM

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree

Doctor of Philosophy

McMaster University Doctor of Philosophy (2021) Hamilton, Ontario
TITLE: The Stringent Response of *Salmonella* Typhimurium
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SUPERVISOR: Dr. Brian K. Coombes, Ph.D.
NUMBER OF PAGES: 184

Abstract

Bacteria inhabit diverse environmental niches and consequently, must modulate their metabolism to adapt to stress. The nucleotide second messengers guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) (collectively referred to as (p)ppGpp) are essential for survival during nutrient starvation. (p)ppGpp is synthesized by the RelA-SpoT homologue (RSH) protein family and coordinates the control of cellular metabolism through its combined effect on over 50 proteins. While the role of (p)ppGpp has largely been associated with nutrient limitation, recent studies have shown that (p)ppGpp and related nucleotides have a previously underappreciated effect on different aspects of bacterial physiology, such as regulating bacterial interactions with its host. This thesis focuses on the coordination of virulence gene expression and evasion of host immunity by (p)ppGpp in *Salmonella enterica* serovar Typhimurium. In the first data chapter, I describe the role of (p)ppGpp in mediating bacterial resistance to killing by the human complement system. I identified that (p)ppGpp activates *ppnN*, a nucleotide metabolism associated enzyme, and the biosynthesis of lipopolysaccharide O-antigen to protect *Salmonella* from cell lysis by complement. The second data chapter compares and contrasts the stringent response of an invasive clinical isolate of *Salmonella* Typhimurium to a strain of *Salmonella* Typhimurium that causes acute gastroenteritis using RNA-sequencing. Critical analysis of our transcriptomics dataset showed that flagellar-based motility is differentially regulated by (p)ppGpp in the two strains of *Salmonella*. Together, these findings demonstrate that (p)ppGpp has significant functional roles beyond mediating adaptation to nutrient limitation.

Acknowledgements

This thesis reflects on the work from my Ph.D. and was made possible with the support of my mentors, peers, and above all, my family.

I begin with gratitude for my supervisor, Dr. Brian Coombes. I started my graduate journey armed with nothing more than a drive for continuing my education and a love for science. In earnest, I lacked a solid foundation in basic biology and the ability to think critically, yet you showed me your utmost patience. In my Ph.D., I developed skills beyond the scientific method as I tried to emulate your professionalism, leadership, and initiative towards learning. These are qualities I wish to carry with me on the road ahead. I also wish to acknowledge my committee members, Drs. Lori Burrows and Dawn Bowdish for their support throughout graduate school and their unwavering faith in me to achieve my career goals. You have taught me to be confident in the face of any challenges that come my way. I also give thanks to Dr. John Whitney for the many candid conversations we had about science. I always found your advice to be very thoughtful.

I reserve a heartfelt thank you to members of the Coombes lab whom I have had the privilege to work with and learn from. I am in debt to Dr. Wael Elhenawy who showed great care in teaching me and I must say that I am lucky to have absorbed some of it. Your thought-provoking questions, methodical approach to science, and passion is unmatched and truly admirable. We also shared many laughs along the way, which helped me stay afloat as I trekked the hardships of a Ph.D. To Caressa Tsai whom I have

shared this road with for 5 years, it has been a pleasure to watch your talent unfold at the bench. I could not imagine having someone else to talk science, share stories, and humor with. I must also mention Kara Tsang, Timothy Klein, and Shehryar Ahmad who are some of my dearest friends I have met in the biochemistry department. Thank you for loving me for who I am and always rooting for me. You saved me from some of the most troubling times in my Ph.D.

Finally, to my parents, Tam Chau and Dao Ngo, and my brother, Edward Chau, words cannot describe the place that you hold in my heart. You have seen me at my best and you have held me close when I am at my lowest. Thank you for loving me unconditionally and investing your time and energy to support me. This doctoral degree is yours as much as it is mine.

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List of Abbreviations

AMP	Adenosine 5′-Monophosphate
AMPs	Antimicrobial peptides
ATP	Adenosine Triphosphate
bp	Basepair
CI	Competitive Index
CDS	Coding Sequence
CFU	Colony Forming Units
CTD	C-terminal Domain
CVF	Cobra Venom Factor
DNA	Deoxyribonucleic Acid
EMSA	Electrophoretic Mobility Shift Assay
g	Gravity
GMP	Guanosine 5′-Monophosphate
HILIC	Hydrophilic Chromatography
HK	Histidine Kinase
HPLC	High Performance Liquid Chromatography
iNTS	Invasive Non-typhoidal *Salmonella*
IPTG	Isopropyl β-D-1-thiogalactopyranoside
MAC	Membrane Attack Complex
MIC	Minimal Inhibitory Concentration
MS	Mass Spectrometry
mRNA	Messenger RNA
NHS	Normal Human Serum
NMS	Normal Mouse Serum
NTD	N-terminal Domain
OD	Optical Density
OM	Outer Membrane
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
ppGpp	Guanosine 3′ 5′-bisphosphate or guanosine tetraphosphate
pppGpp	Guanosine 3′-diposphate 5′-triphosphate or guanosine pentaphosphate
LB	Lysogeny/Luria Broth
LOG	Lonely Guy
LPM	Low phosphate, low magnesium medium
LPS	Lipopolysaccharide
TCS	Two-Component Regulatory System
TLC	Thin Layer Chromatography
TLR	Toll-like Receptor
tRNA	Transfer RNA
T3SS	Type III Secretion System

RNA	Ribonucleic Acid
RNAP	RNA Polymerase
rRNA	Ribosomal RNA
RLU	Relative Light Unit
RR	Response Regulator
RSH	RelA-SpoT Homologue
SCV	*Salmonella* Containing Vacuole
SDS	Sodium Dodecyl Sulfate
SGD-Kn	*Salmonella* Gene Deletion Library- Kanamycin Collection
SIFs	*Salmonella*-Induced Filaments
SNPs	Single Nucleotide Polymorphisms
SPI	*Salmonella* Pathogenicity Island
UMP	Uridine 5´-Monophosphate

Chapter I – Introduction

Chapter I – Co-authorship Statement

Parts of Chapter I were adapted from the following publication:

Chau, N. Y E., Ahmad, S., Whitney, J. C., and Coombes, B. K. (2021). Emerging and divergent roles of pyrophosphorylated nucleotides in bacterial physiology and pathogenesis. PLoS Pathogens. 17(5):e1009532.

Manuscript was written by N. Y E. C. with contributions from S. A., and reviewed by J. C. W. and B. K. C.

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Chapter I – Introduction

Salmonella Disease and Prevalence

Salmonella enterica is a pathogenic species of bacteria with a broad host range for animals and humans, and infections tend to vary in disease severity. Infections by non-typhoidal serovars such as Typhimurium and Enteriditis are typically acquired from the consumption of contaminated food or water and result in a self-limiting gastrointestinal disease that presents with vomiting and diarrhea. Bloodstream infections by non-typhoidal *Salmonella* are rare and occur in 5% of cases particularly in children, the elderly, or immunocompromised individuals (1). By contrast, *Salmonella* Typhi, Paratyphi A, Paratyphi B, and Paratyphi C are collectively referred to as typhoidal *Salmonella* and cause bona fide systemic disease. Infections with typhoidal strains of *Salmonella* are typified by episodes of fever and complications in the intestine, spleen, and liver (2). The risk of infection in low- and middle-income countries is high due to poor sanitation and inaccessibility to safe food and water. However, this is less of a concern in developed countries and typhoidal *Salmonella* infections are usually acquired abroad. In many cases, patients are able to clear the infection, while others may have long-term asymptomatic carriage and infrequent fecal shedding of the bacteria (2).

In sub-Saharan Africa and other developing parts of the world, the emergence of highly virulent, invasive non-typhoidal *Salmonella* (iNTS) has been linked to systemic bacteremia and a high case fatality rate of 20-25% (3). Adults and children with iNTS

infections commonly exhibit fever, enlargement of the spleen and liver, and respiratory symptoms, while colitis is often absent. The primary risk factors include co-morbidities with human immunodeficiency virus (HIV) or malaria, as well as malnutrition and poor sanitation (3). Invasive non-typhoidal *Salmonella* are typically resistant to multiple antibiotics including chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole making infections difficult to treat (4, 5). The multi-drug resistance of ST313 contributes to the overall disease burden, as Africa is also underequipped with medical supplies and drugs (3).

Evolution of the Salmonella Genome

At the molecular level, alterations in bacterial genomes account for many virulence-associated traits that allow pathogenic bacteria to cause disease. A major driver in the evolution of bacteria is the acquisition of new genetic elements, which can immediately confer fitness advantages within a host (6). *Salmonella* is a member of the *Enterobacteriaceae* family that diverged from *E. coli* as a result of a major genetic acquisition called *Salmonella* Pathogenicity Island-1 (SPI-1) (7). More recently, the *Salmonella* genus evolved into two species, *S. enterica* and *S. bongori*, when *S. enterica* acquired SPI-2 (7, 8). Both SPI-1 and SPI-2 encode a type III secretion system (T3SS) and effector proteins that are critical for *S. enterica* to successfully infect mammalian hosts. SPI-1 is required for the invasion of intestinal epithelial cells, whereas, SPI-2 conferred *S. enterica* with the ability to exploit the intracellular environment of immune cells. In *S. bongori*, it is unclear whether SPI-2 was lost or never acquired due to the lack

of a third comparator *Salmonella* species (9). Moreover, ectopic expression of SPI-2 in *S. bongori* is not sufficient to cause disease in mice suggesting that the co-regulation of SPI-2 with other genes in *S. enterica* is required for pathogenesis (10). Indeed, following the acquisition of SPI-2, mutations occurring in the non-coding regions of DNA have fine-tuned gene expression and led to the integration of other gene targets across the *S. enterica* genome that are required for evasion of host immunity and virulence (11-16). SPI-3, SPI-4, and SPI-5 encode additional virulence factors that modulate *Salmonella* adhesion and interactions with host cells in the gut (17-19). A total of 22 SPIs have been identified in the *Salmonella* genus thus far, each with a unique role in bacterial pathogenesis (8).

Among the strains of *S. enterica*, the typhoidal serovars, *S*. Typhi and Paratyphi are restricted to infecting humans and cause a systemic disease. Unique to *S*. Typhi is SPI-7, which encodes for the virulence (Vi) capsular polysaccharide. Capsule formation allows strains like *S*. Typhi to evade deposition of complement on its cell surface and neutrophil phagocytosis, facilitating the systemic spread of the bacteria (20, 21). Moreover, consistent with its narrow host range, typhoidal *Salmonella* displays significant genome degradation and carries approximately 200 pseudogenes compared to non-typhoidal *Salmonella* (22). The pseudogenes reflect the loss of genes that are no longer required in the early phases of gut infection and support a persistent, systemic lifestyle. For example, *sivH*, *ratB*, and *shdA* are pseudogenes in *S*. Typhi, whereas these genes are required for *S*. Typhimurium to colonize the intestine (22). The emergence of highly virulent non-

typhoidal serovars in Africa results in invasive disease similar to the typhoidal strains. *S.* Typhimurium isolate D23580 of sequence type ST313 is frequently associated with iNTS and is distinct from *S.* Typhimurium ST19 strains such as SL1344 that cause acute gastroenteritis (3, 4). Genetic comparisons between the core genomes of *S.* Typhimurium ST19 and ST313 show that they differ by about 1,000 single nucleotide polymorphisms (SNPs), but otherwise share a high degree of conservation in gene content and synteny (23). ST313 also carries 77 pseudogenes indicating partial genome degradation similar to *S.* Typhi and Paratyphi (23, 24). Additionally, ST313 has a unique repertoire of virulence plasmids including pSLT-BT, pBT1, pBT2, and pBT3, and the prophages BTP1 and BTP5. In contrast, ST19 carries the pSLT, pColB9, and pRSF1010 plasmids, and the SopEϕ prophage (24-26). The distinct genetic differences of ST313 likely reflect the evolving signatures of host adaptation.

Salmonella Interactions with Host Processes

Oral infection of C57BL/6 or BALB/c mice with *S.* Typhimurium causes severe intestinal inflammation, systemic spread to the spleen and liver, and ultimately death resulting from high bacterial burdens (27, 28). C57BL/6 and BALB/c mice are genetically susceptible to infection due to the lack of the natural resistance associated-macrophage protein 1 (*Nramp*/*SLC11A1*$^{-/-}$), a divalent cation transporter that is crucial for host control of infection (29). The mice are typically pre-treated with streptomycin to facilitate *Salmonella* infection by lowering colonization resistance from the resident microbiota (28). These models have been widely used to understand how *Salmonella* colonizes the

intestine and the pathological consequences that occur during the systemic phase of infection.

Salmonella is transmitted through the fecal-oral route and preferentially colonizes the lumen of the small intestine. In response to a *Salmonella* infection, detection of surface proteins such as flagellin and lipopolysaccharide (LPS) by host pattern recognition receptors (PRRs) triggers inflammation in the gut environment (30). Intestinal inflammation is in part characterized by the influx of neutrophils and the production of harmful substances such as reactive oxygen species (ROS) that target bacteria for killing (**Fig. 1.1**) (31). In contrast to the resident microbiota, *S*. Typhimurium is able to thrive in the inflamed environment as it expresses multiple ROS detoxifying enzymes including the superoxide dismutases, SodCI and SodCII (32). Neutrophils also produce lipocalin-2, which helps to protect against bacterial infections by sequestering the iron-scavenging siderophore, enterobactin. However, *Salmonella* has evolved to express salmochelin, a glycosylated variant of enterobactin that is not recognized by lipocalin-2 (33, 34). The production of tetrathionate during inflammation also provides an alternative energy source that *Salmonella* uses to outcompete commensal microbes (35). Other host defenses that *Salmonella* encounters in the intestine include antimicrobial peptides (AMPs) secreted by Paneth cells in the intestinal crypts and complement proteins (36, 37). Mechanisms of outer membrane modification such as the palmitoylation of lipid A by the PhoP-PhoQ regulated protein, PagP, confers resistance to AMPs (38). In addition, *S*. Typhimurium expression of the protease, PtgE allows it to evade host autophagy

triggered by complement deposited on its cell surface in the gut (39). The successful subversion of luminal host defenses is crucial for *S.* Typhimurium to transit and adhere to the underlying epithelium.

Once the bacteria reach the epithelial lining, *S.* Typhimurium invades host cells by inducing membrane ruffling and endocytosis (**Fig. 1.1**). This process is mediated by the concerted action of the SPI-1 T3SS translocated effectors, SopE, SopE2, and SopB, which activate the host Rho GTPases Cdc42, Rac1, and RhoG, leading to actin cytoskeletal rearrangement and remodeling of the plasma membrane (19, 40, 41). SipA and SipC contribute by helping to nucleate and stabilize the actin bundles (42, 43). The SPI-1 effectors also induce the release of IL-8, IL-1β, and IL-18, demonstrating that *S.* Typhimurium actively contributes to creating an inflammatory environment (30). Following invasion, the actin cytoskeleton reverts to its normal architecture and the release of pro-inflammatory signals is suppressed. The reversal process occurs via proteasome-mediated degradation of the early SPI-1 effectors, and by the action of later effectors such as SptP and SspH1 (44, 45). In the intracellular environment, *S.* Typhimurium typically resides in a replicative niche called the *Salmonella* containing vacuole (SCV), however, in non-phagocytic cells, a subpopulation of the bacteria replicate in the cytosolic compartment (46). Bacterial replication in the cytosol can induce cellular pyroptosis and extrusion of bacteria into the extracellular space where they can infect other enterocytes or be taken up by phagocytes (46-48).

Following invasion of the intestinal barrier, *S.* Typhimurium is thought to predominantly reside in neutrophils and macrophages. Mutant strains of *Salmonella* that are unable to survive in host cells are avirulent in a mouse model (49). However, mutants that are unable to invade intestinal epithelial cells can still cause lethal infections when mice are infected via the peritoneal cavity (50). Once intracellular, the bacteria express the SPI-2 T3SS, which contributes to the development of the SCV. The SPI-2 effectors, SifA, SseF, SseG, SopD2, and PipB2 have a central role in maintaining the integrity and proper positioning of the SCV (51-55). In addition, SifA and PipB2 contribute to the formation of *Salmonella*-induced filaments (SIF), which are tubule-like extensions from the SCV that are thought to facilitate the acquisition of nutrients (56). The intracellular lifestyle of *Salmonella* is essential for the systemic spread of the bacteria to the spleen and liver as macrophages and other phagocytes tend to migrate through the lymphatic system or bloodstream (**Fig. 1.1**). However, immune cells are armed with antimicrobial defenses and pose various challenges to the bacteria. The SCV must avoid fusion with lysosomes containing hydrolytic enzymes, and *Salmonella* must survive in an acidic environment with low nutrients, antimicrobial peptides (AMPs), and reactive oxygen and nitrogen species (57). Indeed, SifA subverts the transport of hydrolytic enzymes to lysosomes by mannose-6-phosphate receptors, thereby attenuating lysosome function (58). In the intracellular environment, *S.* Typhimurium also exploits the low pH and low nutrient environment as chemical cues to activate the expression of virulence genes that in turn promotes bacterial resistance to AMPs, and reactive oxygen and nitrogen intermediates (59, 60). The interplay between *Salmonella* and host cells represent an evolutionary arms

race where *Salmonella* continues to develop immune evasion strategies to establish an infection.

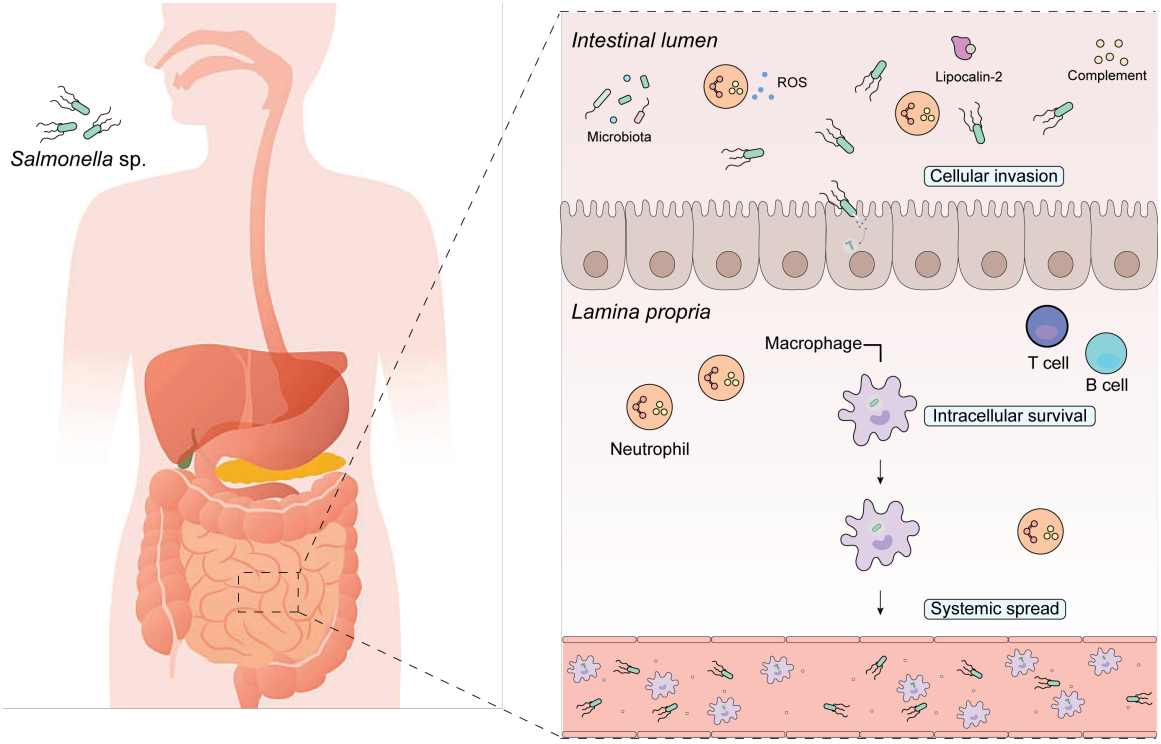

Figure 1.1. *Salmonella* disseminates systemically during host infection. *Salmonella* sp. is transmitted via the oral route of infection. Upon entry into the intestine, *Salmonella* must compete with the resident microbiota and evade various host defenses including nutrient limitation by lipocalin-2, reactive oxygen species (ROS), and complement proteins. Adherence to the intestinal epithelium triggers bacterial invasion into host cells where *Salmonella* resides in a *Salmonella* containing vacuole (SCV). Following invasion, *Salmonella* are taken up by underlying phagocytes such as macrophages that facilitate the systemic spread of the bacteria through the lymphatic system or bloodstream.

Host Adaptation by Invasive Non-typhoidal Salmonella

Genomic analyses of *S*. Typhimurium ST313 isolates from Africa suggest that the strains have begun to adapt to facilitate invasive disease in humans. To better understand the infection biology of ST313, comparisons of virulence-associated traits have been made to ST19.

The ability of ST313 strains to spread systemically indicates that the extracellular virulence program of ST313 differs from ST19. For example, ST313 invades epithelial cells less efficiently than ST19 in part due to a lower level of expression of the SPI-1 effector, *sopE2* (61). ST313 also contains unique genetic variants of the *macAB* efflux pump that may represent an important adaptation for systemic infection. A strain of D23580 containing the ST19 *macAB* genotype showed improved fitness in the intestine relative to wild type D23580, but it was significantly outcompeted in the spleen and liver (62). However, the mechanism of MacAB mediated adaptation is unclear as the ST313 genetic variants result in impaired resistance to antimicrobial peptides. It is possible that MacAB is involved in improving the efflux of other substrates that have not been tested (62). Consistent with ST313 shifting towards a systemic lifestyle, it has evolved mechanisms to evade killing by extracellular host defenses, particularly by the complement system (63, 64). This is in part attributable to a SNP in the promoter region of the outer membrane protease, *ptgE* that increases its expression and consequently, increases degradation of complement factor B (64).

In the intracellular environment, ST313 infection of macrophages resulted in a lower level of apoptosis and pro-inflammatory cytokines such as IL-8 and TNF-α compared to ST19 (65). ST313 infection also stimulates a decreased level of IL-1β production suggesting dampened activation of the inflammasome, which could be explained by a lower level of flagellin production (61). Consistent with ST313 expressing less flagellin, ST313 is less motile and forms less biofilms compared to ST19 (24, 66, 67). Another possible explanation for the differences in macrophage survival could be due to a SNP in the signaling domain of the diguanylate cyclase, STM1987 (68). Diguanylate cyclases catalyze the formation of c-di-GMP, which is important for activating cellulose production and biofilm formation. The SNP identified in *STM1987* resulted in significantly less cellulose, increased survival in macrophages, and increased fitness in a mouse model of acute infection (68). In addition to SNPs, the accumulation of pseudogenes in ST313 is another signature of bacterial adaptation to the host environment. One possible explanation for pseudogenization is that it promotes a decrease in virulence to promote host survival and pathogen transmission. For example, the loss of function of *sseI*, a SPI-2 encoded gene in ST313 enhances its ability to spread to the spleen and liver compared to ST19 (69). These data demonstrate that the distinct genetic changes in ST313 account for important adaptive traits that promote the systemic dissemination and survival of ST313 within a host.

Environmental Sensing via Two-Component Regulatory Systems

Two-component regulatory systems (TCS) are critical to the successful adaptation and survival of bacteria in their environment as they allow them to process surrounding information through the detection of chemical cues. The prototypical architecture of a TCS in Gram-negative bacteria contains an inner membrane sensor kinase and a cytosolic response regulator that is activated by a phosphorylation cascade (70). Transcriptional regulation is a common output of two-component signaling pathways that is achieved through the interaction of the activated response regulator and regulatory motifs upstream of genes (70). Moreover, the stringent coupling of input signals to the desired cellular output requires that these signaling pathways are properly insulated to prevent unwanted cross-talk. Specificity is primarily enforced by the ability of the sensor kinase to recognize its cognate partner while excluding other response regulators (71). This molecular recognition occurs through a unique set of amino acid residues that co-vary between partner proteins (72, 73). As well, most histidine sensor kinases are bi-functional and also act as phosphatases to dephosphorylate the corresponding response regulator. The phosphatase activity serves to modulate pathway output and inhibit the signaling pathway in the absence of the activating signal (74). In sum, two-component systems are highly prevalent in bacteria and represent a reliable way to couple information from the external environment to the appropriate transcriptional programs.

In *Salmonella*, two-component systems are crucial in the regulation of virulence gene expression as the bacteria reside in multiple niches in a host and must tightly regulate the

spatiotemporal expression of its gene products (75). In the intestinal lumen, several cues such as osmolarity and bile salts activate the expression of genes encoded within SPI-1. The regulatory hierarchy of SPI-1 begins with the TCSs, OmpR-EnvZ and BarA-SirA, which directs transcriptional input to the transcription factors, HilD, HilC, and RtsA (76, 77). Each transcription factor is capable of feeding into the activation of the master SPI-1 regulator, HilA, which then directly regulates the *prg*/*org* and *inv*/*spa* operon leading to the assembly of the SPI-1 encoded T3SS and its cognate effector proteins that are required for invading the gut epithelium (**Fig. 1.2**) (78). Similarly, the genes within SPI-2 are also under the control of TCSs including OmpR-EnvZ and PhoP-PhoQ, which are involved in the sensing of low osmolarity and low magnesium, respectively (79, 80). Signaling by OmpR-EnvZ and PhoP-PhoQ ultimately results in the downstream activation of the master SPI-2 TCS, SsrA-SsrB. SsrA is capable of sensing low pH through the protonation of histidine residues in its periplasmic domain (59). The activation of SsrB allows it to co-regulate genes both within and outside of SPI-2 through the recognition of an evolved palindromic motif upstream of target genes (**Fig. 1.2**) (12, 14). Together, the integration of signaling from these regulators and others leads to the expression of T3SS-2 and various virulence factors required for *S.* Typhimurium survival within the SCV.

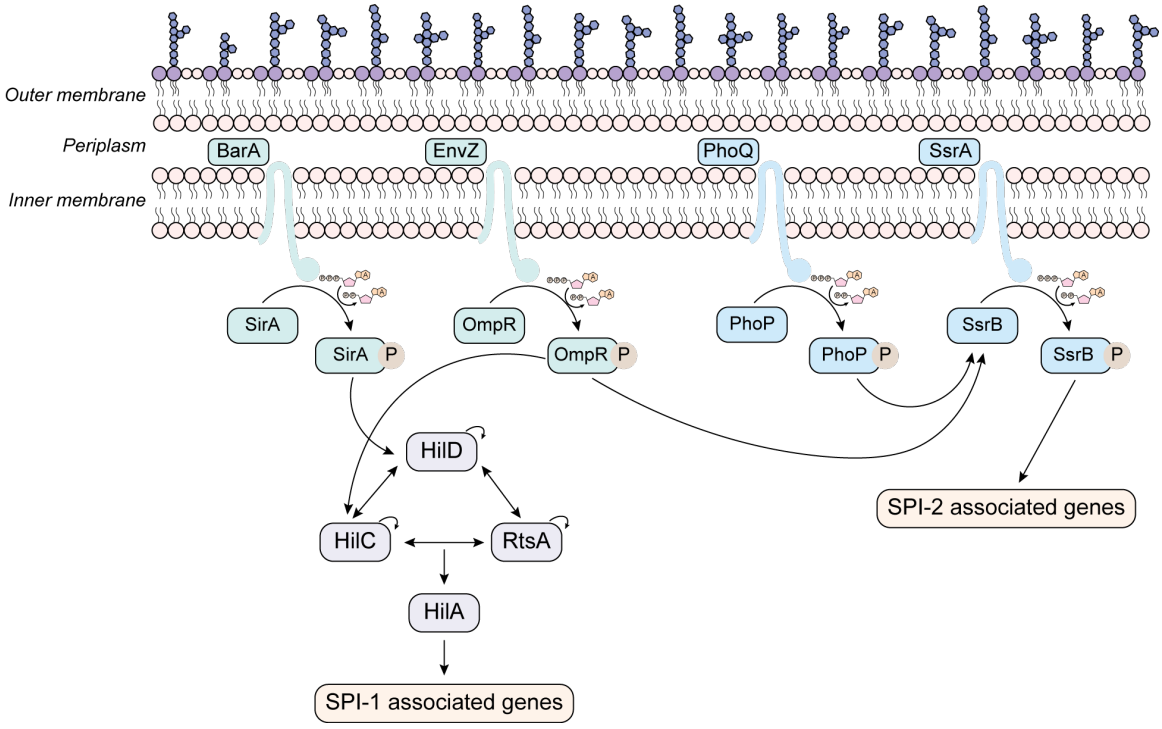

Figure 1.2. Transcriptional regulation of genes involved in *Salmonella* invasion and intracellular virulence. The activation of genes encoded within Salmonella pathogenicity island-1 (SPI-1) required for host cell invasion occurs via a positive feed forward loop comprised of HilD, HilC, RtsA, and the master regulator, HilA. In contrast, genes associated with SPI-2 that are required for the intracellular lifestyle of *Salmonella* are primarily activated by the SsrA-SsrB two-component system. The regulatory cascade of both SPI-1 and SPI-2 are under the control of the two-component systems OmpR-EnvZ, PhoP-PhoQ, or BarA-SirA.

Environmental Sensing via the Stringent Response

The use of nucleotide second messengers such as cyclic AMP, cyclic di-AMP, cyclic di-GMP, and (p)ppGpp to reprogram cellular physiology is another strategy that allows bacteria to respond and adapt to changes in their surroundings. Under nutrient limiting conditions, energetically costly synthesis pathways for DNA, rRNAs and ribosomal proteins are rapidly inhibited, and cellular resources are redirected towards the synthesis of stress resistance factors, amino acids, and carbon metabolism (81, 82). These physiological changes are accomplished during the stringent response by guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp); referred to herein as (p)ppGpp.

The cellular pool of (p)ppGpp is regulated by members of the RelA-SpoT homologue (RSH) protein family, which is comprised of small alarmone synthetases (SASs) and small alarmone hydrolases (SAHs), and multi-domain proteins containing both synthase and hydrolase domains. Three multi-domain proteins called Rel, RelA, and SpoT are capable of synthesizing (p)ppGpp. (p)ppGpp is produced by the transfer of a pyrophosphate moiety from ATP to the 3′ position of GDP or GTP (81-83). In contrast to RelA, Rel and SpoT have a hydrolase domain that degrades (p)ppGpp to pyrophosphate and either GDP or GTP (81-83). Since the discovery of (p)ppGpp by Cashel and Gallant in 1969, extensive follow up work has shown that (p)ppGpp coordinates adaptation to nutrient starvation through global transcriptomic reprogramming by directly interacting

with RNA polymerase (RNAP) and several other downstream protein targets (84-88) (**Fig. 1.3**).

In addition to coping with nutrient related stresses, pathogenic bacteria employ (p)ppGpp signaling to regulate virulence gene expression. Evidence to support this includes the inability of bacteria devoid of (p)ppGpp ((p)ppGpp^0) to swim, form biofilms, invade host cells, and resist innate immunity (82, 89). As a result, ppGpp^0 strains are significantly attenuated for fitness in animal models of infection (89). Similarly, commensal microbes that are deficient in (p)ppGpp are also unable to survive and persist within the host environment (90). These findings demonstrate the pervasive role of (p)ppGpp signaling in bacteria and highlights the stringent response as an attractive target for therapeutic inhibition.

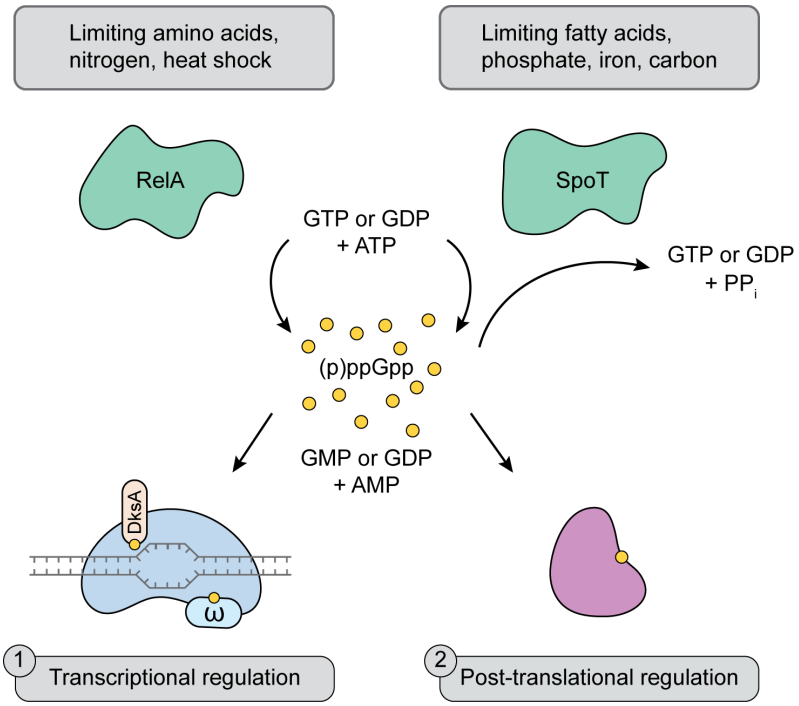

Figure 1.3. (p)ppGpp reprograms cell metabolism by transcriptional and post-translational regulatory mechanisms. In response to environmental stress, RelA and SpoT in β- and γ-Proteobacteria synthesize ppGpp and pppGpp by catalyzing the transfer of pyrophosphate from ATP to GDP or GTP, respectively, and generating AMP and GMP or GDP as byproducts. In contrast to RelA, SpoT is also able to hydrolyze (p)ppGpp to produce GTP or GDP and inorganic phosphate. ppGpp binds to RNA polymerase with DnaK suppressor A (DksA) to modulate transcription, and to effector proteins to regulate their biochemical activity.

Distribution and Regulation of RSH proteins

The genes encoding RSH proteins are broadly conserved in most species of bacteria with the exception of the phyla Planctomycetes, Verrucomicrobia, and Chlamydiae, and certain species of obligate intracellular endosymbionts (81, 91). Some eukaryotic species also contain RSH homologs. For example, *Drosophila melanogaster* expresses metazoan SpoT homologue 1 (MESH1) that functions as a ppGpp hydrolase and is likely involved in insect development and responses to nutrient starvation, whereas human MESH1 is a cytosolic NADPH phosphatase important for regulating ferroptosis (92, 93). Interestingly, although ppGpp has been detected in eukaryotic cell lines, a cognate synthetase has not been identified (94).

The superfamily of enzymes that synthesize and degrade (p)ppGpp are named after RelA and SpoT and the genes encoding both of these proteins are found in many species of β-

and γ-Proteobacteria. RelA and SpoT likely originated from a gene duplication event from the ancestral, long multi-domain protein, Rel (81, 91). The N-terminus of Rel, RelA, and SpoT is comprised of a (p)ppGpp hydrolase domain (HD) and a (p)ppGpp synthetase domain (SYN) whereas the C-terminus consists of a threonyl-tRNA synthetase (ThrRS), GTPase, and SpoT domain (TGS), a helical domain, a conserved cysteine domain (CC), and an aspartokinase, chorismate mutase, and TyrA domain (ACT) (81, 83) (**Fig. 1.4A**). The functional role of the TGS, helical domain, CC, and ACT domains are not fully understood, but they have been suggested to regulate the enzymatic activities of the N-terminus via conformational changes, oligomerization, or interactions with other protein partners (95-105).

Bifunctional Rel

In species outside of the β- and γ-Proteobacteria, (p)ppGpp is produced by the ancestral RSH protein, Rel, which maintains a close interaction with the ribosome to survey nutrient availability. (p)ppGpp synthesis occurs upon Rel detecting deacylated tRNAs in the ribosomal acceptor site (A-site). However, while this is a general mechanism, other cues can also stimulate Rel-mediated (p)ppGpp production. In *Caulobacter crescentus*, Rel requires carbon or nitrogen starvation in addition to amino acid deprivation to initiate the stringent response (106). Biochemical and genetic analyses of the C-terminal domains (CTD) of Rel enzymes have revealed molecular level insight into how the opposing synthetase and hydrolase functions of the N-terminal domain (NTD) are controlled (95). In the absence of the ribosome, interaction of the TGS-helical domain of Rel with the

NTD in *Bacillus subtilis* represses the synthetase domain while leaving the hydrolase domain active (96). Recent structural studies on the NTD of Rel from *Thermus thermophilus* revealed an allosteric control mechanism mediated by (p)ppGpp and GTP. Binding of (p)ppGpp leads to a closed, inactive conformation, permitting only hydrolase activity, whereas GTP binding activates the synthetase domain (97). In this way, the allosteric regulation of Rel allows for only one catalytic function to be active at a time, which prevents a futile cycle of (p)ppGpp synthesis and degradation. Other metabolites such as small molecules like branched chain amino acids have also been shown to allosterically mediate negative feedback regulation of Rel activity in *Rhodobacter capsulatus* (98). Valine and isoleucine are downstream products of the stringent response and their binding to the ACT domain of Rel increases its (p)ppGpp hydrolase activity. Interestingly, RelA from *E. coli* also has a strong binding affinity for valine, but it lacks hydrolytic function, suggesting that this may be an evolutionary relic following the duplication and divergence of Rel into RelA and SpoT (98).

Monofunctional RelA

Unlike Rel and SpoT, RelA has an inactive hydrolase domain and is the main enzyme involved in (p)ppGpp synthesis in *E. coli* and other β- and γ-Proteobacteria. During unstressed growth, it is proposed that the synthetase function of RelA is kept inactive through the formation of RelA homodimers that form via intermolecular disulfide bonds between adjacent CTDs (99-101). However, this regulatory mechanism has been challenged by work showing that the expression of the CTD or full-length RelA does not

compromise the ability of *E. coli* to respond to nutrient limitation nor does it result in a reduction in (p)ppGpp levels as would be expected if inactive dimers were formed (102). Alternatively, it is possible that RelA remains as a monomer and is regulated by intramolecular interactions between the CTD and NTD (102). The synthetase activity of RelA is activated upon exposure to various stresses including amino acid starvation, heat shock, and nitrogen stress (107-113). Similar to Rel, RelA responds to the accumulation of deacylated tRNAs in the ribosomal A-site (107-110) (**Fig. 1.4B**). One model suggests that (p)ppGpp synthesis occurs in a ribosome-independent manner and that RelA "hops" between ribosomes to monitor the translational status of the cell (114, 115). An extension of this model is that RelA retains "memory" of the starvation state and can continue to synthesize (p)ppGpp upon dissociating from the ribosome (116). More recent studies suggest that RelA and deacylated tRNAs bind to the ribosome as a pre-formed complex, which, in turn, activates RelA-mediated (p)ppGpp synthesis. These findings are supported by a cryo-EM structure that shows the C-terminal domain (CTD) of RelA interacting with the ribosome, whereas the N-terminal catalytic domain is flexible and protrudes away from the complex (117). This is an area of ongoing investigation and more work examining the order and timing of RelA binding to the ribosome is necessary to fully understand how it is activated. Additionally, RelA is regulated by (p)ppGpp through a positive feedback mechanism, which results in a transition to a ribosome-independent stimulation of enzyme function (118).

SpoT

SpoT functions primarily as a hydrolase for (p)ppGpp, however, it is also capable of mediating (p)ppGpp synthesis in response to signals distinct from those that activate RelA. Proteobacteria that contain RelA also have a hydrolytically active SpoT to prevent the accumulation of toxic levels of (p)ppGpp (119). While the hydrolase activity of SpoT is essential for survival, mutations in regulatory domains that abrogate its synthetase function, particularly at the C-terminus are well tolerated (120). SpoT synthesizes (p)ppGpp in response to diverse stress signals including carbon, iron, phosphate, and fatty acid starvation (103, 121, 122). The mechanisms by which SpoT senses different stress cues are not fully understood, but studies suggest that direct interactions with other cytosolic proteins plays an important role in modulating the switch between its synthetase and hydrolase activities. Under nutrient-rich conditions, binding of the GTPase Obg to SpoT is thought to repress its (p)ppGpp-synthetic activity, whereas during fatty acid starvation, SpoT interacts with acyl carrier protein (ACP) and YtfK to activate (p)ppGpp synthesis (104, 105). During carbon source downshift, Rsd binds to the TGS domain of SpoT to stimulate its hydrolase activity thus preventing toxic accumulation of (p)ppGpp (103).

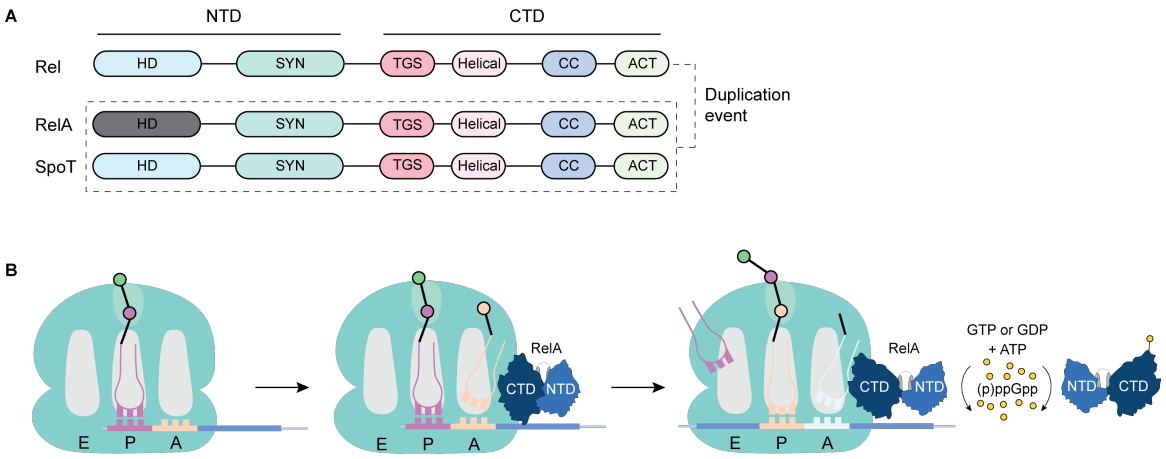

Figure 1.4. (p)ppGpp levels are regulated by RelA-SpoT homologue (RSH) proteins. (**A**) Rel, RelA, and SpoT are multi-domain RSH enzymes that synthesize (p)ppGpp. Rel and SpoT also have an active hydrolase domain that degrades (p)ppGpp. The domains of each RSH enzyme are depicted with the N-terminus comprised of the catalytic synthetase and hydrolase domains, and the C-terminus consisting of a threonyl-tRNA synthetase (ThrRS), GTPase, and SpoT domain [(TGS)], a helical domain, a conserved cysteine domain (CC), and an aspartokinase, chorismate mutase, and TyrA domain (ACT). (**B**) RelA monitors the translational status of the cell by directly associating with the ribosome. Accumulation of uncharged tRNAs into the ribosomal acceptor site (A-site) triggers (p)ppGpp production. (p)ppGpp mediates positive feedback regulation of RelA-stimulating ribosome-independent (p)ppGpp synthesis.

Transcriptional Regulation by (p)ppGpp

One of the main consequences of a rise in (p)ppGpp concentrations upon starvation is a change in the transcriptional profile of the cell. The transcriptional repression of energetically costly pathways such as rRNA synthesis and DNA metabolism, and the concurrent increase in nutrient biosynthesis pathways is essential to promoting bacterial survival during environmental stress (82).

In species of Proteobacteria such as *E. coli*, transcriptional changes are mediated through direct binding of RNAP by (p)ppGpp. (p)ppGpp was shown to bind RNAP at the interface of the β' pincer and the ω domain, and at the interface between RNAP and the transcription initiation factor, DnaK suppressor A (DksA) (123-125). Site 1 is likely important for (p)ppGpp-binding under non-starving conditions where (p)ppGpp concentrations are low, as the binding affinity of this site for (p)ppGpp is higher compared to site 2. However, binding of (p)ppGpp at site 2 enhances the inhibitory effects of DksA on transcription, and thus, likely occurs under starvation conditions to promote physiological changes in the cell. Transcriptional reprogramming in *E. coli* is also achieved by (p)ppGpp inhibiting RNAP from binding to the housekeeping sigma factor, σ^{70}. As a result, RNAP is available to bind to the alternative sigma factors, σ^{32} and σ^{38}, that are involved in activating genes required for adaptation to heat shock or nutrient limitation, respectively (126). Consistent with the role of (p)ppGpp in modulating transcription by interacting with RNAP, a suppressor screen conducted on minimal

medium with *E. coli* unable to produce (p)ppGpp selected for mutations in RNAP that rescued growth (127). Moreover, transcriptional profiling of *E. coli* expressing RNAP unable to bind ppGpp revealed that little to no changes in transcription occur following the accumulation of ppGpp (86). These data demonstrate that the stringent response is mainly coordinated in an RNAP-dependent manner in *E. coli* and other Gram-negative bacteria.

In contrast to *E. coli*, transcriptional changes during the stringent response in Gram-positive bacteria occurs through (p)ppGpp mediated regulation of GTP concentrations. At basal levels, (p)ppGpp maintains homeostatic control of purine metabolism to prevent the toxic accumulation of GTP. Indeed, *B. subtilis* that are unable to produce (p)ppGpp display gross metabolic imbalances and a reduction in cell viability even in nutrient rich conditions (128). However, at high (p)ppGpp concentrations, GTP levels decrease due to the synthesis of pppGpp and the direct inhibition of GTP biosynthesis enzymes by (p)ppGpp (128-130). Consequently, a decline in GTP relieves repression of the transcriptional regulator, CodY, and allows for the activation of genes involved in amino acid biosynthesis and transport (131). The decrease in GTP levels also reduces the transcription of ribosomal genes that require GTP for initiation (132). Together, these studies show that transcriptional changes in Gram-positive bacteria during the stringent response occur indirectly through the regulation of purine nucleotide metabolism.

(p)ppGpp at the interface between Salmonella and the host

The stringent response is commonly described as the rapid accumulation of (p)ppGpp during nutrient stress resulting in the inhibition of bacterial growth and conservation of key metabolic resources. Beyond facilitating adaptation to nutrient limitation, (p)ppGpp signaling also plays a prominent role in the pathogenesis of many clinically relevant pathogens. The stringent response regulates various virulence-associated traits during infection including invasion, immune evasion, and biofilm formation primarily by coordinating changes in transcription.

In *S*. Typhimurium, it is well-established that (p)ppGpp regulates the genes required for the invasion of intestinal epithelial cells and intracellular survival (112, 120, 133-137). Complementing these findings, recent work shows that the exposure of *S*. Typhimurium to nitric oxide (NO) induces amino acid auxotrophies that can be rescued by RelA-dependent activation of amino acid biosynthesis as well as the expression of the flavohemoglobin Hmp to relieve NO stress (112). Another component of innate immunity that bacterial (p)ppGpp functions to subvert is the complement system, a collection of proteins that target bacteria for phagocytic uptake or cell lysis (138). In *Salmonella enterica* serovar Typhi, (p)ppGpp regulates extracellular capsule formation to promote serum survival (137). *S*. Typhimurium lacks a capsule and (p)ppGpp instead regulates a nucleotide hydrolase called PpnN, and the biosynthesis of lipopolysaccharide O-antigen to confer complement resistance (88, 139-141). These data demonstrate that (p)ppGpp

signaling is crucial in coordinating the evasion of extracellular host defenses by *S.* Typhimurium.

To persist within a host, *S.* Typhimurium has also developed the ability to reside in the intracellular environment of immune cells such as macrophages (37). A recent transposon mutagenesis screen in J774 macrophages found that mutations at the 3'-end of *spoT*, which disrupt the C-terminal regulatory domain, affect the ability of *Salmonella* to synthesize (p)ppGpp in response to acid stress (120). (p)ppGpp activates multiple two-component systems including PhoP-PhoQ and SsrA-SsrB following phagocytosis that are required for intracellular survival and replication (14, 142). Downstream of PhoP-PhoQ signaling, it was found that (p)ppGpp facilitates dimerization of the SlyA transcription factor that contributes to coordinating resistance to low pH and cationic antimicrobial peptides (143). However, this was recently challenged by conflicting evidence where the addition of (p)ppGpp did not induce SlyA dimerization nor affect its ability to bind DNA (144). (p)ppGpp also directly activates the transcription of *ssrAB* by relieving the negative repression on its promoter (136). We and others have shown that SsrA-SsrB represses flagellar-based motility genes to facilitate evasion of inflammasome-mediated killing (13, 14, 75). Consistent with these data, *S.* Typhimurium develops a metabolically active, but slow- to non-growing state intracellularly and delivers effector proteins via its type III secretion system to reprogram macrophages into an anti-inflammatory state to evade immune killing (145, 146). In addition, it has been suggested that (p)ppGpp is dispensable for inducing slow growth in *S.* Typhimurium and that environmental

fluctuations such as low Mg^{2+} is sufficient (147). These studies highlight the complexity of virulence gene regulation and that it likely involves multiple factors including (p)ppGpp.

Purpose and Aims of the Present Study

The purpose of this study was to investigate the roles of (p)ppGpp signaling in *S.* Typhimurium virulence. It has been well described in the literature that (p)ppGpp regulates genes associated with SPI-1 and SPI-2, required for host cell invasion and intracellular survival, respectively. There have also been recent studies suggesting that (p)ppGpp contributes to *Salmonella* evasion of host immunity. However, the full inventory of genes coordinated by (p)ppGpp in *S.* Typhimurium to mediate successful host infection has been understudied. I hypothesize that the regulatory control of diverse virulence factors by (p)ppGpp is required to optimize the pathogenicity of *S.* Typhimurium.

The specific goals and findings are highlighted in the following two chapters:

1. (p)ppGpp-dependent regulation of the nucleotide hydrolase PpnN confers complement resistance in *Salmonella enterica* serovar Typhimurium.
    - This study characterized the function and regulation of PpnN and LPS biosynthesis by (p)ppGpp and how it contributes to *S.* Typhimurium survival against the complement system.
2. Genome-wide effects of (p)ppGpp signaling in invasive *Salmonella enterica* serovar Typhimurium.

- This study used RNA-sequencing to explore the stringent response of a clinical isolate of *S.* Typhimurium that causes invasive disease in comparison to a strain of *S.* Typhimurium that causes acute gastroenteritis.

Chapter II – (p)ppGpp-dependent regulation of the nucleotide hydrolase PpnN confers complement resistance in *Salmonella enterica* serovar Typhimurium

Chapter II – Co-authorship Statement

Chapter II consists of the following publication:

Chau, N. Y E, Pérez-Morales, D., Elhenawy, W., Bustamante V. H., Zhang Y. E., and Coombes, B. K. (2021). (p)ppGpp-dependent regulation of the nucleotide hydrolase PpnN confers complement resistance in *Salmonella enterica* serovar Typhimurium. Infect Immun. 89(2):e00639-20.

The following experiments were performed by authors other than myself:

(1) Outer membrane protein composition profiling was performed by W. E.

(2) Thin layer chromatography was performed by Y. E. Z.

- Unpublished supplementary data included in Appendix I.

(3) Electrophoretic mobility shift assays were performed by D. P.-M. And V. H. B.

- Unpublished supplementary data included in Appendix I.

(4) Manuscript was written and edited by N. Y E. C. and B. K. C.

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(p)ppGpp-dependent regulation of the nucleotide hydrolase PpnN confers complement resistance in *Salmonella enterica* serovar Typhimurium

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Running title: PpnN confers complement resistance

Abstract

The stringent response is an essential mechanism of metabolic reprogramming during environmental stress that is mediated by the nucleotide alarmones, guanosine tetraphosphate and pentaphosphate ((p)ppGpp). In addition to physiological adaptations, (p)ppGpp also regulates virulence programs in pathogenic bacteria including *Salmonella enterica* serovar Typhimurium. *S.* Typhimurium is a common cause of acute gastroenteritis, but it may also spread to systemic tissues resulting in severe clinical outcomes. During infection, *S.* Typhimurium encounters a broad repertoire of immune defenses that it must evade for successful host infection. Here, we examined the role of the stringent response in *S.* Typhimurium resistance to complement-mediated killing and found that the (p)ppGpp synthetase-hydrolase, SpoT, is required for bacterial survival in human serum. We identified the nucleotide hydrolase, PpnN, as a target of the stringent response that is required to promote bacterial fitness in serum. Using chromatography and mass spectrometry, we show that PpnN hydrolyzes purine and pyrimidine monophosphates to generate free nucleobases and ribose 5′-phosphate, and that this metabolic activity is required for conferring resistance to complement killing. In addition to PpnN, we show that (p)ppGpp is required for the biosynthesis of the very long and long O-antigen in the outer membrane known to be important for complement resistance. Our results provide new insights into the role of the stringent response in mediating evasion of the innate immune system by pathogenic bacteria.

Introduction

Salmonella enterica is a bacterial pathogen with a broad host range for both animals and humans. Non-typhoidal strains of *Salmonella* such as serovar Typhimurium are acquired through contaminated food or water and cause gastrointestinal disease that is usually self-limiting (57). However, in developing countries, invasive, non-typhoidal serovars have been linked to severe clinical complications due to systemic bacteremia (3). One of the hallmarks of *S.* Typhimurium pathogenesis is its capacity to transition between the extracellular and intracellular environment due, in part, to the evolved ability of the bacteria to evade innate host defenses (14, 148).

A prominent arm of innate immunity that *S.* Typhimurium can encounter during infection is the complement system. Complement is composed of more than 30 proteins that are present in the circulation and at mucosal surfaces where frequent contact with microorganisms is made (39, 138). Activation of complement results in a proteolytic cascade that leads to the coating of target surfaces for phagocytic uptake or perturbation of bacterial membranes by the membrane attack complex (MAC) (138, 149, 150). Mechanisms of complement resistance by pathogenic bacteria have been described as proteolytic inactivation of complement proteins or the expression of cell surface structures to prevent complement deposition (151). For example, *S.* Typhimurium can express long variants of LPS O-antigen and this prevents the integration of the MAC onto the outer membrane (152, 153). Bacteria also undergo metabolic changes to ensure

optimal use of resources upon exposure to serum (154-156). However, the role of these metabolic adaptations in promoting survival in serum is largely underexplored.

The stringent response is a mechanism of cellular reprogramming that allows bacteria to survive environmental stressors. It is characterized by the generation of the nucleotide alarmones, ppGpp and pppGpp, collectively referred to as (p)ppGpp, and leads to rapid alterations in bacterial physiology (81, 89). The synthetase RelA produces (p)ppGpp during amino acid starvation through the sensing of uncharged tRNAs in the ribosomal A site (107-109). (p)ppGpp is also synthesized by SpoT in response to fatty acid, phosphate, iron, or carbon source limitation (103, 121, 122). In contrast to RelA, SpoT also has a hydrolase domain to balance cellular (p)ppGpp levels (157). Accumulation of (p)ppGpp inhibits the synthesis of ribosomal proteins, rRNA, and tRNA, and activates stress-specific genes (81, 89). (p)ppGpp also regulates various metabolic pathways including nucleotide biosynthesis, which occurs through allosteric regulation of enzymes such as PurF and PpnN in *Escherichia coli* (87, 88, 139, 158). Bacterial pathogens often couple (p)ppGpp signaling to virulence gene expression (81, 89). In *S.* Typhimurium, (p)ppGpp helps activate genes required for invasion of the intestinal epithelium and intracellular survival (120, 134-136). Thus, the stringent response mediates both physiological changes and expression of virulence factors in bacteria for successful host infection.

In this study, we examined the crosstalk between the stringent response, metabolic reprogramming, and evasion of innate immunity. We found that SpoT is required for the

survival of *S.* Typhimurium against human complement. Furthermore, we show that (p)ppGpp-mediated regulation of a nucleosidase called PpnN (formerly SL1344_2949) and biosynthesis of the LPS O-antigen promotes the survival of *S.* Typhimurium in serum. Our data provides insight into the role of nucleotide metabolism in bacterial resistance against complement.

Results

The stringent response is required for complement resistance in *S.* Typhimurium

The stringent response is activated in response to environmental insults and is characterized by the production of (p)ppGpp. Mutants lacking the regulators, *relA* and *spoT*, are devoid of (p)ppGpp and have pleiotropic phenotypes including the inability to grow in defined minimal media due to amino acid auxotrophies (85, 159). Consistent with this, we found that the growth of the Δ*relA* Δ*spoT* mutant was severely attenuated in M9-glucose minimal medium compared to nutrient rich LB broth, whereas, the Δ*relA* mutant grew similarly to wild type under both conditions (**Fig. 2.S1**). We were unable to generate a single Δ*spoT* mutant because the (p)ppGpp hydrolase domain of SpoT is essential for bacterial viability in the presence of RelA (119). Given the role of the stringent response in enabling bacteria to adapt to changes in the environment, we next tested whether the stringent response was required for resistance against complement-mediated killing. We grew *S.* Typhimurium strains lacking *relA* or both *relA* and *spoT* in LB until stationary phase to mimic stringent response conditions, and tested their survival in pooled human

serum. The Δ*relA* mutant survived similarly to the wild type strain, whereas the Δ*relA* Δ*spoT* mutant was highly susceptible to killing in normal human serum (**Fig. 2.1A**). Consistent with these data, complementation of the Δ*relA* Δ*spoT* mutant with SpoT expressed *in trans* from its native promoter completely rescued bacterial viability to wild type levels. In contrast, the double mutant remained highly susceptible to serum mediated killing when it was complemented with $spoT^{G955C}$, which encodes for a catalytically inactive SpoT mutant containing an E319Q point mutation that abrogates (p)ppGpp synthase activity (160). These data confirmed that the (p)ppGpp synthase activity of SpoT is important for *S.* Typhimurium survival in normal human serum. Moreover, we could recover bacterial survival of the wild type and mutant strains following pre-treatment of the serum with cobra venom factor (CVF), which depletes the serum of human C3 and prevents downstream activation of the membrane attack complex (**Fig. 2.1A**) (161). These data demonstrate that killing was due to the activation of the complement system (161, 162).

It is well established that (p)ppGpp signaling results in global regulatory changes in bacteria, including nucleotide metabolism (81). Recent work in *Escherichia coli* has shown that (p)ppGpp can allosterically activate the nucleoside hydrolase, PpnN, to degrade nucleotide 5′-monophosphates to the corresponding free bases and ribose 5′-phosphate (88, 139, 163, 164). PpnN is part of the Lonely Guy (LOG) protein family (Pfam PF03641), which are mainly single-domain phosphohydrolases that share a conserved "$PGG_xGT_{xx}E$" motif that is important for the biosynthesis of cytokinins in

plant growth and development (164-168) (**Fig. 2.S2A**). Furthermore, an amino acid sequence alignment between PpnN from *E. coli* and SL1344_2949 (PpnN hereafter) in *S.* Typhimurium showed that these proteins share 94% pairwise identity in amino acids (**Fig. 2.S2B**). We also found that *S.* Typhimurium *ppnN* is co-regulated with genes such as *pagP* and *pgtE* that are involved in bacterial resistance against innate immunity (14, 38, 64, 169). To study the role of nucleotide metabolism in promoting serum resistance, we deleted *ppnN* in *S.* Typhimurium and tested the viability of the mutant in human serum. Although the Δ*ppnN* mutant grew similarly to the wild type strain in both LB and M9-Glucose medium (**Fig. 2.S1**), it showed a significant ~30% reduction in viability upon exposure to human serum compared to wild type *S.* Typhimurium. Expression of PpnN *in trans* or pre-treatment of the serum with CVF restored bacterial survival back to wild type levels, indicating a direct connection of this phenotype to complement (**Fig. 2.1B**). To determine whether the "$PGG_xGT_{xx}E$" motif was required for PpnN-mediated complement resistance in *S.* Typhimurium, we generated a $ppnN^{G790C}$ variant using site-directed mutagenesis, which mutates residue E264 to glutamine. A Δ*ppnN* mutant expressing $PpnN^{E264Q}$ remained susceptible to killing in human serum confirming that the function of PpnN is required for complement resistance (**Fig. 2.1B**).

PpnN is regulated in a (p)ppGpp-dependent manner

Based on our findings that the Δ*relA* Δ*spoT* and Δ*ppnN* mutants were compromised for serum survival, we hypothesized that (p)ppGpp-mediated regulation of PpnN is required

for complement resistance. To test this, we generated a deletion of *ppnN* in the Δ*relA* Δ*spoT* background and tested the survival of the Δ*relA* Δ*spoT ppnN*::*cat* mutant in human serum. Consistent with our previous data with the Δ*relA* Δ*spoT* mutant, the Δ*relA* Δ*spoT ppnN*::*cat* mutant was highly susceptible to complement killing (**Fig. 2.1A and 2.1C**). Complementation of Δ*relA* Δ*spoT ppnN*::*cat* with *spoT* partially recovered *S.* Typhimurium survival in human serum compared to wild type, whereas, expression of *spoT* fully restored the survival of the Δ*relA* Δ*spoT* double mutant (**Fig. 2.1A and 2.1C**). Similarly to the Δ*relA* Δ*spoT* mutant, expression of $SpoT^{E319Q}$ was unable to rescue the viability of Δ*relA* Δ*spoT ppnN*::*cat* (**Fig. 2.1A and 2.1C**). In contrast, pre-treatment of the serum with CVF rescued the survival of both mutants to wild type levels (**Fig. 2.1A and 2.1C**). Taken together, these data support that (p)ppGpp production by SpoT acts through *ppnN* and other unknown factor(s) to confer resistance to complement killing by *S.* Typhimurium.

Previously it was shown that PpnN in *E. coli* K-12 is allosterically regulated by (p)ppGpp (88, 139, 164). This prompted us to investigate the regulation of PpnN by (p)ppGpp in *S.* Typhimurium. First, we tested whether the activation of the *ppnN* promoter was dependent on (p)ppGpp. *ppnN* was activated to similar levels in wild type *S.* Typhimurium and the Δ*relA* mutant, but its expression was significantly reduced in the Δ*relA* Δ*spoT* mutant that is defective for (p)ppGpp synthesis (**Fig. 2.2A**). Complementation with *spoT* was sufficient to recover activation of *ppnN*, whereas complementation with $spoT^{G955C}$ was unable to restore activity of the *ppnN* promoter.

Notably, the activation of the *ppnN* promoter between wild type and the Δ*relA* Δ*spoT* mutant was significantly different at 5 h of growth in LB when the bacteria are in stationary phase and (p)ppGpp levels increase (170, 171). As a control, we also tested the expression of a known (p)ppGpp-regulated target, *argI*, which is involved in L-arginine biosynthesis (172). The *argI* promoter was partially repressed in the *relA* mutant compared to wild type, which is consistent with the role of RelA in responding to amino acid stress during bacterial growth in stationary phase (**Fig. 2.2B**) (170). Furthermore, *argI* promoter activity was completely abolished in the Δ*relA* Δ*spoT* mutant and complementation with *spoT*, but not *spoT*^{G955C}, was sufficient to restore *argI* activation to wild type levels (**Fig. 2.2B**). The amino acid sequence alignment of PpnN in *E. coli* and *S.* Typhimurium also show that the (p)ppGpp binding residues, R68, R70, K73, R341, and Y347 are conserved between the two homologues strongly suggesting that (p)ppGpp regulates PpnN in *S.* Typhimurium post-translationally (**Fig. 2.S2B)** (88).

PpnN is a pyrimidine/purine nucleosidase

Our data indicated that PpnN promotes the survival of *S.* Typhimurium in human serum. In *E. coli*, PpnN is a cytoplasmic hydrolase that degrades purine and pyrimidine nucleotide 5′-monophosphates to the corresponding free bases and ribose 5′-phosphate (88, 139, 163, 164). To compare PpnN in *S.* Typhimurium to its *E. coli* homologue, we tested their enzymatic activity by purifying PpnN from *S.* Typhimurium and *E. coli* using affinity chromatography and incubated it with the nucleotide substrate, uridine 5′-

monophosphate (UMP) (163). Hydrophilic interaction chromatography combined with mass spectrometry (HILIC-MS) was used to monitor the presence of the substrate and the potential products. Similar to the *E. coli* homologue, PpnN from *S.* Typhimurium degraded UMP to uracil and ribose 5′-phosphate (**Fig. 2.3A**, **2.3B**, and **2.S3**). As a control, we also purified and incubated a catalytic inactive variant of PpnN containing the E264Q point mutation with UMP and as expected, the function of the protein was abrogated **(Fig. 2.3B).** To determine the nucleotide specificity of PpnN, we tested its ability to hydrolyze purines such as guanosine 5′-monophosphate (GMP). Similar to UMP, PpnN hydrolyzed GMP to guanine and ribose 5′-phosphate (**Fig. 2.S4**). UMP and GMP were used as representative substrates as the detection of the reaction standards were optimal using HILIC-MS. Together, these data confirm that PpnN in *S.* Typhimurium is a cytosolic nucleotide 5′-phosphate nucleosidase with broad substrate specificity.

PpnN does not confer complement resistance by contributing to cell wall biogenesis

The cytosolic localization and function of PpnN suggested that it mediates complement resistance through its metabolic activity. A common metabolite that PpnN produces from the hydrolysis of purine and pyrimidine monophosphates is ribose 5′-phosphate. We hypothesized that ribose 5′-phosphate could be entering carbon metabolism and conferring complement resistance by contributing to LPS biosynthesis (**Fig. 2.4A**) (173, 174). To investigate whether PpnN contributes to maintaining the integrity of the cell wall, we determined the minimal inhibitory concentration (MIC) of ampicillin and

colistin, which are antibiotics that target peptidoglycan biosynthesis and LPS, respectively, for wild type *S.* Typhimurium and the Δ*ppnN* and Δ*relA* Δ*spoT* mutants (175). As negative controls, we also tested rifampicin and ciprofloxacin, which affect the synthesis of RNA and DNA, respectively (150, 176). Δ*ppnN* did not display an increase in susceptibility to any of the above antibiotics, whereas, the Δ*relA* Δ*spoT* mutant showed a 8-fold increase in susceptibility to colistin (**Fig. 2.4B**). These data suggest that the lack of (p)ppGpp production compromises the ability of the bacteria to synthesize the full-length variants of LPS. To investigate this possibility, we extracted the whole LPS from wild type and mutant strains of *S.* Typhimurium and used gel electrophoresis to examine the O-antigen polymers produced by each strain. The Δ*relA* Δ*spoT* mutant showed a decreased production of the very long and long O-antigen relative to the wild type strain and expression of SpoT *in trans* completely restored its production (**Fig. 2.4C**). However, complementation of Δ*relA* Δ*spoT* with $SpoT^{E319Q}$ was unable to restore the O-antigen demonstrating that the (p)ppGpp synthetase activity of SpoT is required for regulating its biosynthesis (**Fig. 2.4C**). Next, we engineered bioluminescence reporters to confirm the regulatory input of (p)ppGpp in the production of the LPS O-antigen. Using *lux*-transcriptional fusions for *wzy* and wzz^{ST}, which encode for the O-antigen polymerase and the long O-antigen chain length regulator, respectively, we found that both genes are expressed in a (p)ppGpp-dependent manner (**Fig. 2.4D and 2.4E**) (177). In contrast to Δ*relA* Δ*spoT*, the O-antigen profile of the Δ*ppnN* mutant was similar to wild type (**Fig. 2.4C**). Consistent with our previous data, we also found that the outer membrane protein composition of the Δ*ppnN* mutant was similar to wild type, whereas, the Δ*relA* Δ*spoT*

mutant showed significant differences (**Fig. 2.S5**). These findings demonstrate that PpnN promotes the survival of *S.* Typhimurium in human serum in a manner independent of cell wall integrity and suggests that the regulation of LPS O-antigen biosynthesis by (p)ppGpp is another mechanism contributing to complement resistance during the stringent response.

Discussion

Here, we showed that the stringent response is required for *S.* Typhimurium survival against the complement system, a component of innate immunity that bacteria encounter during host infection. By using bioinformatics to guide mechanistic predictions, we identified the nucleotide monophosphate nucleosidase PpnN as important for complement resistance through the SpoT-dependent second messenger, (p)ppGpp.

Our findings share some parallels with SpoT in *Helicobacter pylori* and *Borrelia burgdorferi*, where SpoT is required for optimal growth in the presence of serum (178). An *H. pylori* Δ*spoT* mutant exhibits relaxed growth over wild type bacteria in serum free media suggesting that SpoT is able to sense serum starvation. There have also been other studies relating the stringent response to bacterial survival either in whole blood or in serum (137, 179-181). For example, a (p)ppGpp^0 mutant in *Enterococcus faecalis* is attenuated in serum due to dysregulated metal homeostasis. Supplementation of the serum with iron or manganese was able to restore bacterial viability (181). In *Salmonella* Typhi, (p)ppGpp regulates the expression of the (Vi) capsular polysaccharide, which prevents

complement deposition and formation of the MAC on the cell surface (20, 137). In contrast to *S.* Typhi, *S.* Typhimurium does not express a capsule, which suggests that (p)ppGpp mediates complement resistance through a different mechanism.

The role of a LOG protein in mammalian pathogens has so far only been investigated in *Mycobacterium tuberculosis*. In *M. tuberculosis*, the LOG protein, Rv1205, produces cytokinins that are maintained at basal levels by a proteasome system. The function of the cytokinins is unclear, but it has been shown that proteasome-deficient *M. tuberculosis* are susceptible to killing due to synergy between host nitric oxide and cytokinins that accumulate (166, 182). To our knowledge, our study is the first to report that a LOG-like protein aids in resistance to complement killing. Previously it has been shown that the biosynthesis of purines and pyrimidines are needed for bacterial growth in serum (183). However, this study used heat-inactivated serum where complement was presumably inactive, suggesting that nucleotides were not serving to resist the bactericidal activity of complement. Several biochemical screens have identified that nucleotide metabolism is a key output of the stringent response (87, 88). For example, (p)ppGpp inhibits the purine biosynthesis enzymes, Gpt, Hpt, GuaB, PurA, and PurF (87, 184). It has also been shown that (p)ppGpp enhances the activity of PpnN in *E. coli* to degrade nucleotides (88, 139, 163, 164). Deletion of *ppnN* in *S.* Typhimurium did not affect the integrity of the cell wall suggesting that PpnN promotes complement resistance through its metabolic roles in the cytoplasm. It is possible that the generation of nucleotide precursors by PpnN allows for the reallocation of resources to other pathways relevant for survival in serum. In addition,

PpnN may facilitate the transition from serum to more favorable conditions by increasing the cellular pool of nucleotide metabolites (139, 141, 185). This is consistent with the finding that a Δ*ppnN* mutant is outcompeted by wild type *E. coli* when the bacteria transition between nutrient rich and poor media (139). Although the mechanism of PpnN mediated complement resistance is not fully understood, it is unlikely that it interacts directly with complement proteins, as it is not secreted (186). Exploring the contributions of PpnN to metabolism and how this influences complement resistance in *S.* Typhimurium will be the focus of future research. A functional genomics approach such as RNA-sequencing of wild type and the Δ*ppnN* mutant exposed to human serum may reveal insight into the potential pathways affected by PpnN. This may be followed by the systematic deletion of metabolic enzymes in a Δ*ppnN* mutant to test which pathways enhance complement resistance.

Our data suggests that there are other genetic targets in addition to *ppnN* that contribute to (p)ppGpp mediated resistance to complement killing. Another possible mechanism is through the regulation of cell wall biogenesis genes by the alternative sigma factor, RpoS, and (p)ppGpp during bacterial growth in stationary phase (85, 86, 170). In particular, bacteria exhibit an increase in LPS, crosslinking of outer membrane lipoproteins, and an increase in thickness of the peptidoglycan layer under nutrient limited conditions (152, 170, 187). This is supported by our findings that an *S.* Typhimurium Δ*relA* Δ*spoT* mutant displays significantly lower levels of very long and long O-antigen and increased susceptibility to complement. The decreased production of O-antigen by the Δ*relA* Δ*spoT*

mutant was corroborated by lower expression levels of *wzy* and wzz^{ST}. Our LPS silver staining also suggests that wzz^{fepE} from the O-antigen biosynthesis pathway is regulated in a (p)ppGpp-dependent manner (177). We and others have also shown that the LPS O-antigen of *S*. Typhimurium is involved in colistin resistance (175). Furthermore, RNA-sequencing of *S*. Typhimurium grown to stationary phase in LB showed that genes such as *pagC* and *pgtE* are activated by (p)ppGpp (135). PagC and PgtE are outer membrane proteins that have been directly implicated in complement resistance in *Salmonella* (64, 169, 188). Together, these data suggest that the stringent response coordinates resistance to complement in *S*. Typhimurium by remodeling the cell membrane and reprogramming metabolism.

In summary, our findings highlight the role of nucleotide metabolism and the biosynthesis of the LPS O-antigen during the stringent response in mediating evasion of innate immunity by pathogenic bacteria. (p)ppGpp signaling is an essential mechanism of the bacterial stress response that involves the regulation of virulence gene expression for successful host infection. Identifying the genetic factors that allow bacteria to evade the immune system will reveal new therapeutic targets that could be integral in informing drug discovery efforts.

Experimental Procedures

Bacterial Strains and Growth Conditions

Salmonella enterica serovar Typhimurium (SL1344) and isogenic derivatives were used in the study and listed in **Table 2.1**. Bacteria were grown in LB or M9 minimal medium supplemented with 1% glucose and 0.135 mM L-histidine. Antibiotics were added where appropriate (streptomycin, 100 μg/mL; kanamycin, 50 μg/mL; ampicillin, 200 μg/mL; chloramphenicol, 34 μg/mL) and strains were grown at 37°C with shaking.

Cloning and Mutant Generation

ppnN::*aph* from the *Salmonella* single-gene deletion (SGD) library was transduced to *S.* Typhimurium str. SL1344 as previously described with modifications (189, 190). The donor mutant strain was grown overnight in LB supplemented with 0.5 X E-Salts and 0.2% D-glucose and mixed with ~1.5 x 10^5 pfu of P22 HT phage at 37°C. Next, 1 mL of the donor strain was pelleted and the supernatant was mixed with 100 μL of chloroform to lyse any remaining live bacteria. Two μL of the donor lysate was then mixed with 200 μL of a wild type *S.* Typhimurium str. SL1344 overnight culture and incubated at 37°C for 1 h before plating on LB supplemented with 50 μg/mL of kanamycin. Colonies were re-streaked onto green indicator agar to screen for the absence of phage. To ensure that the colonies were free of phage lysogen, white colonies were streaked across a line of P22 H5 phage on green agar and incubated overnight at 37°C. Successful transduction of the mutant was verified by PCR. The *aph* cassette was removed using pCP20 resulting in Δ*ppnN*::FRT in *S.* Typhimurium str. SL1344.

Lambda Red recombination was used to generate an in-frame, marked mutant of *S.* Typhimurium *relA*::*cat* (191). Wild type *S.* Typhimurium carrying pKD46 was transformed with linear PCR products amplified using Phire Hot Start II DNA Polymerase (Thermo Fisher) and primers (Sigma Aldrich) containing gene specific regions of homology and flanking the *cat* cassette encoded on pKD3. Transformants were selected on LB agar supplemented with chloramphenicol (34 μg/mL) and knockouts were verified by PCR. The *cat* cassette was removed using pCP20 resulting in Δ*relA*::FRT and confirmed by PCR. Lambda Red was repeated in the Δ*relA*::FRT background to generate the Δr*elA*::FRT Δ*spoT*::FRT double mutant, and in the Δr*elA*::FRT Δ*spoT*::FRT double mutant background to generate the Δr*elA*::FRT Δ*spoT*::FRT *ppnN*::*cat* triple mutant.

For the cloning of *spoT*, primers EC215F (GTA TCA TAT GGC ACG CGT AAC TGT TCA GGA CGC TG) and EC215R (AGT CCC ATG GCT AGT TTC GGT TAC GGG TGA CTT TA) or EC224F (GTA TGA GCT CGC ACG CGT AAC TGT TCA GGA CGC TG) and EC224R (AGT CGC GGC CGC CTA GTT TCG GTT ACG GGT GAC TTT A) were used to PCR amplify the coding sequence of *spoT* and 291 bp upstream of the start codon. PCR amplified products were then cloned into pGEN-MCS or pWSK129 after digestion with NdeI/NcoI (Thermo Fisher) or SacI/NotI (Thermo Fisher), respectively. For the cloning of *ppnN*, the coding sequence and 800 bp upstream of the start codon of *ppnN* in SL1344 was PCR amplified using primers EC100F (ATG CGA ATT CGG ATA TCT GGA CGT TGT ATG AAC TT) and EC100R (GTA TCA TAT GTT AAG CGC AGA TCT CGT AAC AGG GG) and then cloned as a EcoRI/NdeI

(Thermo Fisher) DNA product into pGEN-MCS. pGEN-MCS-*ppnN*^{G790C} (encoding PpnN^{E264Q}) and pGEN-MCS-*spoT*^{G955C} (encoding SpoT^{E319Q}) were generated using the Q5® Site-Directed Mutagenesis Kit (NEB) and primers EC186F (TAC GGC GGA ACA GCT GCT TTA TTT GCT G) and EC186R (CCC ACG CCG CCC GGG AAG ATG ATG ATA C) or EC320F (GGC GTT CCT GTT CAA GTC CAG ATC CGT A) and EC320R (GGC GTT CCT GTT CAA GTC CAG ATC CGT A), respectively. pWSK129-*spoT*^{G955C} was cloned by PCR amplifying EC224F (GTA TGA GCT CGC ACG CGT AAC TGT TCA GGA CGC TG)/EC325R (ACG GAT CTG GAC TTG AAC AGG AAC G) and EC326F (GGC GTT CCT GTT CAA GTC CAG ATC C)/EC224R (AGT CGC GGC CGC CTA GTT TCG GTT ACG GGT GAC TTT A) then performing Splicing by Overlap Extension (SOE) PCR using EC224F and EC224R. The product of the SOE PCR was digested with SacI/NotI (Thermo Fisher) and cloned into pWSK129.

Bioluminescence reporters were generated using primers EC97F (ATG CGG ATC CGG ATA TCT GGA CGT TGT ATG AAC TT) and EC97R (GCC ATA CGT AGT AAA CTC CTT ATG GGA CGC AAC AC) to PCR amplify 800 bp upstream of the start codon of *ppnN*, EC188F (ATG CGG ATC CCA ATG GTG GCT TTC GCC AGG) and EC188R (GCC ATA CGT AAT AGA GCC TTT AGA AAA AAT GCT TA) to amplify 1000 bp upstream of the start codon of *wzy*, EC212F (AGT CGG ATC CGT GAC GCA CGC CGT CGT CAT) and EC212R (CGG CTA CGT AAC TTC CCT CAC ATG GCT TAG GCC TC) to PCR amplify 579 bp upstream of the start codon of *argI*, and EC321F (ATG CGG ATC CGT GAT CAG CAT CAA CCC CGC) and EC321R (GCC ATA CGT AAG

ATA CCC TAA CTA AAA AAA GGA TG) to PCR amplify 1000 bp upstream of the start codon of wzz^{ST}. PCR amplified products were then cloned into pGEN-*luxCDABE* after digestion with SnaBI/BamHI (Thermo Fisher) (192).

All plasmid constructs were sequence verified by Sanger sequencing (GENEWIZ®) then transformed by electroporation (BioRad) into the appropriate strain backgrounds for downstream experiments. Plasmids used in the study are listed in **Table 2.2** and primers (Sigma Aldrich) are listed in **Table 2.3**.

Bacterial Growth Curves

Bacteria grown overnight in LB medium were harvested and normalized to an OD_{600} of 0.5. Cells were washed and resuspended in phosphate buffered saline (PBS) and diluted to an OD_{600} of 0.05 in fresh LB or M9 minimal medium supplemented with 1% glucose and 0.135 mM L-histidine. Bacteria were then grown in 96 well flat, clear bottom polystyrene plates (Corning) at 37°C with shaking, and A_{600} was measured every 1 h using the BioTek Epoch™ 2 plate reader.

Serum Bactericidal Assay

Bacteria grown overnight in LB medium were harvested and normalized to an OD_{600} of 0.5. Cells were washed and resuspended in PBS and diluted (1:10) further for the assay. The equivalent OD_{600} of 0.005 of each strain was incubated in 90% pooled normal human serum (Innovative Research) at 37°C. Serum treated with 5 units/mL of cobra venom

factor (Quidel) for 30 min at 37°C was used as a negative control for each strain. The number of viable bacteria was determined by plating on LB agar supplemented with 200 μg/mL of ampicillin to select for strains carrying the pGEN-MCS empty vector control or complementation plasmid and percent survival was calculated as the cfu/mL at 30 min relative to 0 min.

Bioluminescence Reporter Assay

S. Typhimurium strains containing *lux*-transcriptional fusions were sub-cultured (1:50) and grown to mid-exponential phase (OD_{600} = 0.4 to 0.5) in LB then sub-cultured (1:50) again into LB in black 96-well flat, clear bottom polystyrene plates (Corning). Plates were incubated at 37°C with shaking, and luminescence and A_{600} were measured every 1 h up to 5 h using the PerkinElmer® Plate Reader. Luminescence was normalized to A_{600}.

Protein Purification

BL21 (DE3) *E. coli* harbouring pET-24a-*ppnN*-6HIS or pET-24a-*ppnN*^{G790C}-6HIS was sub-cultured (1:50) into LB broth at 37°C with shaking and grown until OD_{600} = 0.2. Cells were induced with 0.5 mM IPTG until the culture reached mid-logarithmic phase (OD_{600} = 0.5) after which the culture was left to incubate overnight at 18°C. Cells were pelleted by centrifugation for 10 min at 5,000 *g* and resuspended in lysis buffer (100 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM imidazole, 1 mM 2-mercaptoethanol, 300 mM NaCl, 4 mM phenylmethanesulfonyl fluoride, pH 7.5). Cells were lysed using the Continuous Cell Disruptor (Constant Systems Ltd.) operated at 20 K PSI and then centrifuged at 30,000 *g*

for 30 min to pellet cellular debris. Lysates were applied to a Ni-NTA affinity column and washed with a gradient buffer (100 mM Tris-HCl, 5 mM $MgCl_2$, 500 mM NaCl, pH 7.5) containing 20 mM, 40 mM, and 60 mM imidazole. Proteins were eluted in 100 mM Tris-HCl, 5 mM $MgCl_2$, 500 mM NaCl, 500 mM imidazole, pH 7.5. Eluted fractions were run on a 12% SDS-PAGE gel and stained with coomassie brilliant blue to verify isolation of the target protein. In a second purification step, elution fractions were combined and applied to a HiLoad 16/60 Superdex 200 pg filtration column (GE Healthcare). Proteins were eluted in 1 mL fractions in buffer containing 10 mM Tris-HCl, 1 mM $MgCl_2$, pH 7.5. Fractions containing the target proteins (as determined by SDS-PAGE) were pooled and stored at -80°C with 20% glycerol.

Enzymatic Assay

Purified proteins (50 μg/mL) were incubated with 0.2 mM of each substrate at 37°C in buffer (10 mM Tris-HCl, 1 mM $MgCl_2$, pH 7.5) for 1.5 h. At 0 h and 1.5 h, 150 μL of each reaction was inactivated with 150 μL of methanol cooled at -80°C. For negative control reactions, a catalytic inactive protein variant was used or wild type protein was heat-inactivated at 95°C for 60 min. Relative abundance of reactant compounds and products were measured using hydrophilic chromatography and mass spectrometry (HILIC-MS) on the LTQ Orbitrap XL (Thermo Fisher).

Antibiotic Susceptibility Testing

MIC determination of ampicillin, colistin, rifampicin, and ciprofloxacin (BioShop Canada) were performed using broth microdilution in 96 well flat, clear bottom polystyrene plates (Corning) (193). Antibiotics were two-fold serial diluted in LB medium from 512 µg/mL down to twenty concentrations. Bacterial cultures grown overnight were diluted to 10^6 cfu/mL in fresh LB medium then diluted further to 10^5 cfu/mL in the microplates containing the antibiotics. Plates were incubated at 37°C in sealed plastic bags. A_{600} was read after overnight incubation using the PerkinElmer® Plate Reader.

LPS Analysis

Bacteria grown overnight in LB medium were harvested and normalized to an OD_{600} of 3.0. Two mL of each strain under investigation was pelleted by centrifugation at 16,000 *g* for 2 min and the LPS was extracted as previously described (194) with some modifications. Cells were resuspended in 200 µL of TRIzol™ Reagent (Thermo Fisher) then incubated for 10 min at room temperature. Next, 20 µL of chloroform was added for every mL of culture. The resulting TRIzol™-chloroform mixture was vortexed vigorously and incubated at room temperature for an additional 10 min followed by centrifugation at 16,000 *g* for 10 min to separate the aqueous and organic phases. The aqueous phase was transferred to a new microfuge tube and 100 µL of distilled water was added to the organic phase, vortexed briefly, then incubated for an additional 10 min and centrifuged again for 10 min at 16,000 *g* to create phase separation. Two additional water extractions

were performed to ensure complete removal of the LPS. The combined aqueous phases were then dried at 45°C for ~2 h using a Vacufuge™ (Eppendorf). Dried pellets were resuspended in 500 µL of 0.375 M $MgCl_2$ dissolved in 95% ethanol that had been cooled at -20°C then centrifuged for 15 min at 16,000 *g* (195). The final pellets were normalized by weight/volume (g/µL) and resuspended in distilled water. LPS was run on 16% SDS-PAGE gels and stained with silver nitrate as previously described (196) and imaged using the ChemiDoc MP Imaging System (BioRad).

Outer Membrane Profiling

Outer membrane of different *S.* Typhimurium strains was isolated as previously described (197) with some modifications. Bacterial cell pellets were harvested from overnight cultures and resuspended in 50 mM Tris-HCl (pH 8.0), 50 mM $MgCl_2$, 150 mM NaCl then lysed by sonication. Lysates were centrifuged at 8000 *g* for 10 min then filtered through 0.2 µM low protein-binding filters to remove insoluble debris. Total membranes were harvested from cell-free lysates by ultracentrifugation at 100,000 *g* for 1 h. To isolate the outer membrane, total membrane pellets were solubilized in the above buffer supplemented with 1.5% Triton X-100 and EDTA-free protease inhibitor cocktail (Roche Applied Science) for 24 h at 4°C. Outer membranes were pelleted by ultracentrifugation at 100,000 *g* for 1 h then resuspended in sterile 1 X PBS buffer. Outer membrane preparations were normalized by protein content following quantification using the 2-D Quant Kit (GE Healthcare), and then separated on 10% SDS-PAGE gels. Coomassie

Brilliant Blue R-250 dye (BioShop) was used to stain the gels, followed by imaging using the ChemiDoc MP Imaging System (BioRad).

Statistical Analysis

Data were analyzed using GraphPad Prism 5.0a software (GraphPad Inc., San Diego, CA) using one-way ANOVA. *P* values of <0.05 were considered significant.

Acknowledgements

We thank Nicola Henriquez in the Centre for Microbial Chemical Biology for technical assistance with the HILIC-MS experiments. This work was supported by a grant to B.K.C. from the Canadian Institutes of Health Research (CIHR) (FRN 156361), a grant to Y.E.Z. from the Novo Nordisk Foundation (NNF19OC0058331), and grants to V.H.B. from Consejo Nacional de Ciencia y Tecnología (254531)-Mexico and Dirección General de Asuntos del Personal Académico de la UNAM (IN202418)-Mexico. B.K.C. holds the Canada Research Chair in Infectious Disease Pathogenesis. N.Y.E.C. is a recipient of an Ontario Graduate Scholarship. W.E. was supported by a CIHR Postdoctoral Fellowship.

Conflict of Interest: The authors declare they have no conflicts of interest with the contents of this article.

Table 2.1. Strains used in the study.

Strain	**Source or reference**
Wild type *S.* Typhimurium str. SL1344	Coombes *et al*. 2003 (198)
Δ*ppnN S.* Typhimurium str. SL1344	This study
ppnN::*aph S*. Typhimurium str. 14028s	Porwollik *et al*. (2014) (189)
Δ*relA S*. Typhimurium str. SL1344	This study
Δ*relA* Δ*spoT S*. Typhimurium str. SL1344	This study
Δ*relA* Δ*spoT ppnN*::*cat S*. Typhimurium str. SL1344	This study
Wild type *E. coli* str. K-12	Baba *et al.* 2006 (199)
BL21 (DE3) *E. coli*	Agilent Technologies
TOP10 *E. coli*	Invitrogen

Table 2.2. Plasmids used in the study.

Plasmid	Description	Source or reference
pGEN-MCS	Low-copy number cloning vector	Lane *et al.* 2007 (192)
pGEN-*luxCDABE*	Lux transcriptional reporter plasmid	Lane *et al.* 2007 (192)
pWSK129	Low-copy number cloning vector	Wang and Kushner, 1991 (200)
pEXT20	Expression vector	Dykxhoorn *et al.* 1996 (201)
pET-24a	Expression vector	Novagen
pKD3	Template plasmid for Lambda Red recombination	Datsenko and Wanner, 2000 (191)
pKD46	Lambda Red recombinase expression plasmid	Datsenko and Wanner, 2000 (191)
pCP20	Lambda Red flippase expression plasmid	Datsenko and Wanner, 2000 (191)
pGEN-MCS-*ppnN*ST	*ppnN*ST with 800 bp upstream of coding sequence cloned into pGEN-MCS for complementation experiments	This study
pGEN-MCS-*ppnN*$^{ST\ (G790C)}$	*ppnN*ST containing G790C point mutation with 800 bp upstream of coding sequence cloned into pGEN-MCS for complementation experiments	This study
pGEN-MCS-*spoT*	*spoT* with 291 bp upstream of coding sequence cloned into pGEN-MCS for complementation experiments	This study
pGEN-MCS-*spoT*^{G955C}	*spoT* containing G955C point mutation with 291 bp upstream of coding sequence cloned into pGEN-MCS for complementation experiments	This study
pWSK129-*spoT*	*spoT* with 291 bp upstream of coding sequence cloned into pGEN-MCS for complementation experiments	This study
pWSK129-*spoT*^{G955C}	*spoT* containing G955C point mutation with 291 bp upstream of coding sequence cloned into pWSK129 for complementation experiments	This study
pEXT20-*ppnN*ST-6HIS	PpnNST expression plasmid, C-terminal His-6 tagged	This study
pEXT20-*ppnN*^{EC}-6HIS	PpnN^{EC} expression plasmid, C-terminal His-6 tagged	This study
pET-24a-*ppnN*ST	PpnNST expression plasmid, C-terminal His-6 tagged	This study
pET-24a-*ppnN*$^{ST\ (G790C)}$	PpnNST expression plasmid, C-terminal His-6 tagged containing G790C point mutation in *ppnN*ST coding sequence	This study
pGEN-*luxCDABE*-*ppnN*ST	Lux transcriptional reporter for *ppnN*ST promoter	This study
pGEN-*luxCDABE*-*argI*	Lux transcriptional reporter for *argI* promoter	This study
pGEN-*luxCDABE*-*wzy*	Lux transcriptional reporter for *wzy* promoter	This study
pGEN-*luxCDABE*-*wzz*ST	Lux transcriptional reporter for *wzz*ST promoter	This study

ST and EC denote *ppnN* from *S.* Typhimurium str. SL1344 and *E. coli* str. K-12, respectively.

Table 2.3. Primers used in the study.

Primer	Gene	Direction	Destination	Sequence (5´-3´)
EC85	*ppnN*ST	F	pET24-a	CGAACATATGTTGATTACACATATTAGCCC GCTTG
EC85	*ppnN*ST	R	pET24-a	GTATCTCGAGAGCGCAGATCTCGTAACAG GGGATG
EC96	*ppnN*ST	F	pEXT20	CGAAGGATCCTTGATTACACATATTAGCCC GCTTG
EC98	*ppnN*ST	R	pEXT20	GTATAAGCTTTTAATGGTGGTGGTGATGAT GAGCG CAGATCTCGTAACAGGGG
EC97	*ppnN*ST	F	pGEN-*luxCDABE*	ATGCGGATCCGGATATCTGGACGTTGTAT GAACTT
EC97	*ppnN*ST	R	pGEN-*luxCDABE*	GCCATACGTAGTAAACTCCTTATGGGACG CAACAC
EC100	*ppnN*ST	F	pGEN-MCS	ATGCGAATTCGGATATCTGGACGTTGTATG AACTT
EC100	*ppnN*ST	R	pGEN-MCS	GTATCATATGTTAAGCGCAGATCTCGTAAC AGGGG
EC167	*ppnN*^{EC}	F	pEXT20	CGAAGAATTCTTGATTACACATATTAGCCC GCTTG
EC167	*ppnN*^{EC}	R	pEXT20	GTATGGTACCTTAATGGTGGTGGTGATGAT GCGTGCAGATTTCGTAGCAAGGG
EC186	*ppnN*ST	F	*ppnN*ST	TACGGCGGAACAGCTGCTTTATTTGCTG
EC186	*ppnN*ST	R	*ppnN*ST	CCCACGCCGCCCGGGAAGATGATGATAC
EC188	*wzy*	F	pGEN-*luxCDABE*	ATGCGGATCCCAATGGTGGCTTTCGCCAG G
EC188	*wzy*	R	pGEN-*luxCDABE*	GCCATACGTAATAGAGCCTTTAGAAAAAA TGCTTA
EC200	*relA*	F	Δ*relA*	CGCATGTAATGATTACCGGCTTACCGACTT CGGTAGGCCTGGTCCCTTAAGGAGAGGAC GATGGTCGCGGTAAGAAGTGCACATATTA ATGTGTAGGCTGGAGCTGCTTCG
EC200	*relA*	R	Δ*relA*	GTTGCTAATGCGGCTTTGCTGAACGAGTA GCAAAGCCGCTACATGATTACTGTCTGGG GTTTACCCCCCGTGCAGTCGCCGTGCATCA ATCATATGAATATCCTCCTTAG
EC204	*spoT*	F	Δ*spoT*	GAATTACAAGCCGTTACCGCTATTGCTGA AGGTCGTCGTTAATCACAAAGCGGGTCGC CCTTGTATCTGTTTGAAAGCCTGAATCAAC TGGTGTAGGCTGGAGCTGCTTCG
EC204	*spoT*	R	Δ*spoT*	TCAGGCTGACGCCTGGCGAGCATTTCGCA TATACGCGCATAACGTTTTGGATTCATAGC GCTAGTTTCGGTTACGGGTGACTTTAATGA CCATATGAATATCCTCCTTA
EC212	*argI*	F	pGEN-*luxCDABE*	AGTCGGATCCGTGACGCACGCCGTCGTCA T
EC212	*argI*	R	pGEN-*luxCDABE*	CGGCTACGTAACTTCCCTCACATGGCTTAG GCCTC

EC215	*spoT*	F	pGEN-MCS	GTATCATATGGCACGCGTAACTGTTCAGG ACGCTG
EC215	*spoT*	R	pGEN-MCS	AGTCCCATGGCTAGTTTCGGTTACGGGTGA CTTTA
EC224	*spoT*	F	pWSK129	GTATGAGCTCGCACGCGTAACTGTTCAGG ACGCTG
EC224	*spoT*	R	pWSK129	AGTCGCGGCCGCCTAGTTTCGGTTACGGGT GACTTTA
EC245	$ppnN^{ST}$	F	$\Delta ppnN^{ST}$	ATCAGCCAGGGCTATTGTAATCAACAGGG AATGGCGTGTTGCGTCCCATAAGGAGTTT ACTTGATTACACATATTAGCCCGCTTGGCT CAGTGTAGGCTGGAGCTGCTTCG
EC245	$ppnN^{ST}$	R	$\Delta ppnN^{ST}$	ATACCGCAATGAAAGGAATGGGAGAAGC GCCCGGCTGCTGGCGGCAACCGGGCATAA GCGTTAAGCGCAGATCTCGTAACAGGGGA TGTACATATGAATATCCTCCTTAG
EC320	*spoT*	F	pGEN-MCS	GGCGTTCCTGTTCAAGTCCAGATCCGTA
EC320	*spoT*	R	pGEN-MCS	GTGCGGGCCGATCATTGAGGTGTGCAAA
EC321	wzz^{ST}	F	pGEN-*luxCDABE*	ATGCGGATCCGTGATCAGCATCAACCCCG C
EC321	wzz^{ST}	R	pGEN-*luxCDABE*	GCCATACGTAAGATACCCTAACTAAAAAA AGGATG
EC325	*spoT*	R	pWSK129	ACGGATCTGGACTTGAACAGGAACG
EC326	*spoT*	F	pWSK129	GGCGTTCCTGTTCAAGTCCAGATCC

ST and EC denote *ppnN* from *S.* Typhimurium str. SL1344 and *E. coli* str. K-12, respectively.

Main Figures

Figure 2.1. The stringent response is required for complement resistance in *S.* Typhimurium. (**A**) Δ*relA* Δ*spoT* is susceptible to killing in pooled normal human serum (NHS) and complementation with *spoT* or inhibition of complement with cobra venom factor (CVF) is sufficient to rescue bacterial survival. (**B**) Δ*ppnN* is susceptible to killing in pooled NHS. Complementation with *ppnN* or inhibition of complement with CVF is sufficient to rescue bacterial viability, whereas, complementation with $ppnN^{G790C}$ does not recover the survival of the bacteria (**C**) Δ*relA* Δ*spoT ppnN*::*cat* complemented with *spoT* or $spoT^{G955C}$ is susceptible to killing in pooled NHS and inhibition of complement with CVF is sufficient to rescue bacterial viability. Data are the means ± SEM (error bars) of at least three independent experiments. Strains are carrying the empty pGEN-MCS vector control unless otherwise specified. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$.

Figure 2.1

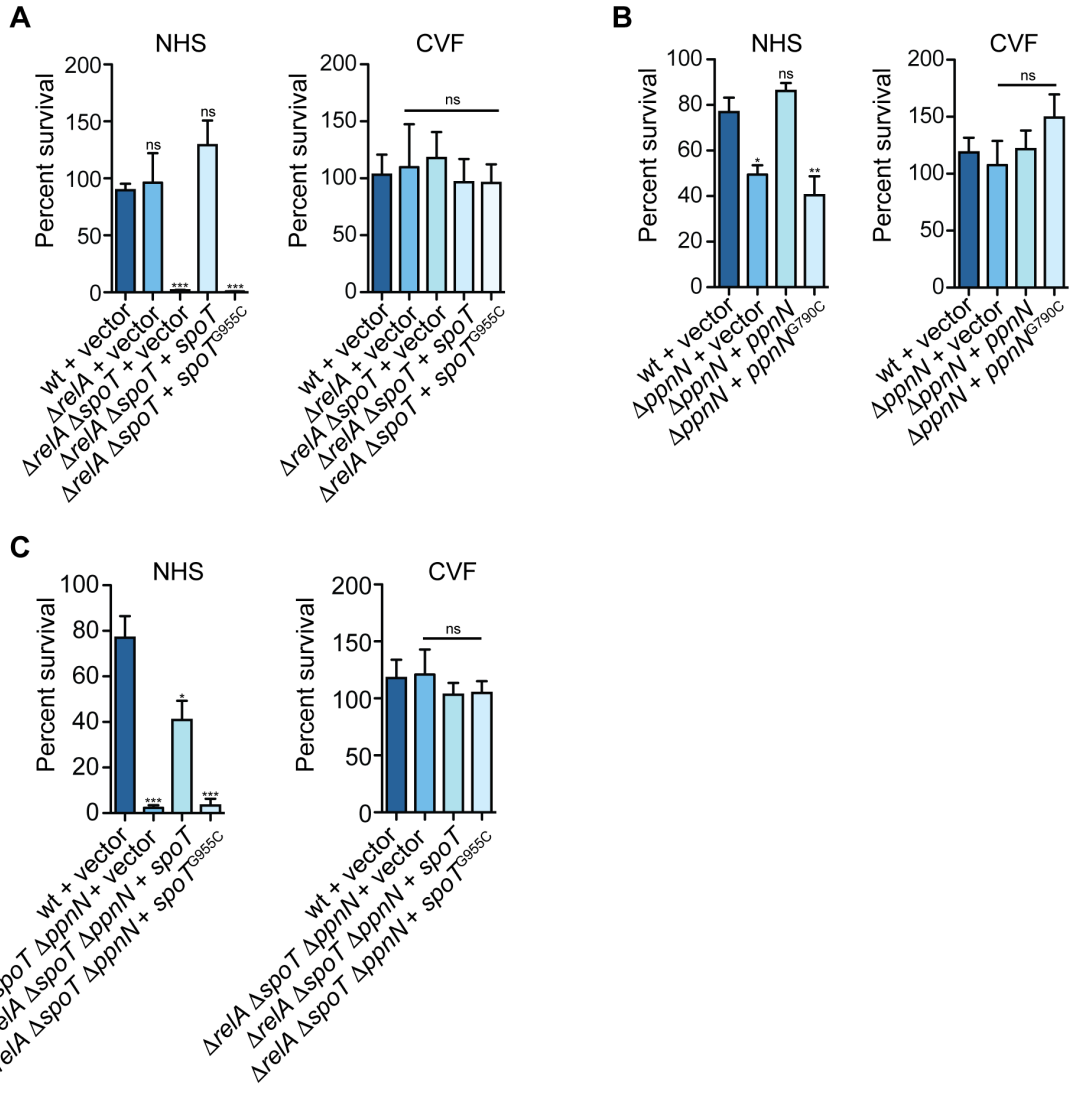

A
NHS
CVF
Percent survival
wt + vector
ΔrelA + vector
ΔrelA ΔspoT + vector
ΔrelA ΔspoT + spoT
ΔrelA ΔspoT + spoT G955C
ns
***
B
NHS
CVF
Percent survival
wt + vector
ΔppnN + vector
ΔppnN + ppnN
ΔppnN + ppnN G790C
ns
*
**
C
NHS
CVF
Percent survival
wt + vector
ΔrelA ΔspoT ΔppnN + vector
ΔrelA ΔspoT ΔppnN + spoT
ΔrelA ΔspoT ΔppnN + spoT G955C
ns
*
***

Figure 2.2. PpnN is regulated in a (p)ppGpp-dependent manner. Transcriptional reporter of the full-length (**A**) *ppnN* promoter and (**B**) *argI* promoter, a canonical gene target regulated during the stringent response, showed (p)ppGpp-dependent up-regulation. Data are the means ± SEM (error bars) of three independent experiments. Strains are carrying the empty pWSK129 vector control unless otherwise specified. ns, not significant; *, $P < 0.05$; ***, $P < 0.0001$.

Figure 2.2

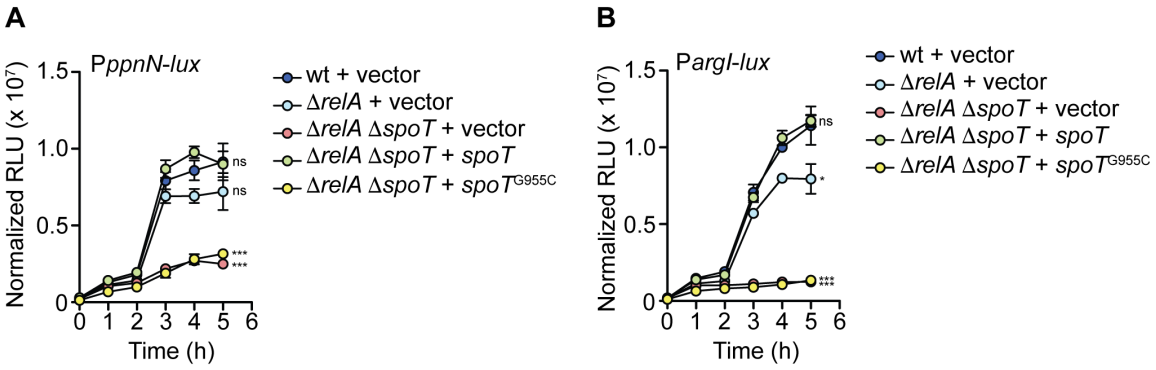

A
PppnN-lux
Normalized RLU (x 10^7)
1.5
1.0
0.5
0
0 1 2 3 4 5 6
Time (h)
ns
ns
***
***
wt + vector
ΔrelA + vector
ΔrelA ΔspoT + vector
ΔrelA ΔspoT + spoT
ΔrelA ΔspoT + spoT^G955C
B
PargI-lux
Normalized RLU (x 10^7)
1.5
1.0
0.5
0
0 1 2 3 4 5 6
Time (h)
ns
*
***
wt + vector
ΔrelA + vector
ΔrelA ΔspoT + vector
ΔrelA ΔspoT + spoT
ΔrelA ΔspoT + spoT^G955C

Figure 2.3. PpnN from *S*. Typhimurium str. SL1344 is a pyrimidine/purine nucleosidase. (**A** and **B**) PpnN hydrolyzes uridine 5´-monophosphate (UMP) to uracil and ribose 5´-phosphate. Introduction of an E264Q catalytic site mutation in PpnN abrogates protein function. Data are representative of two replicates.

Figure 2.3

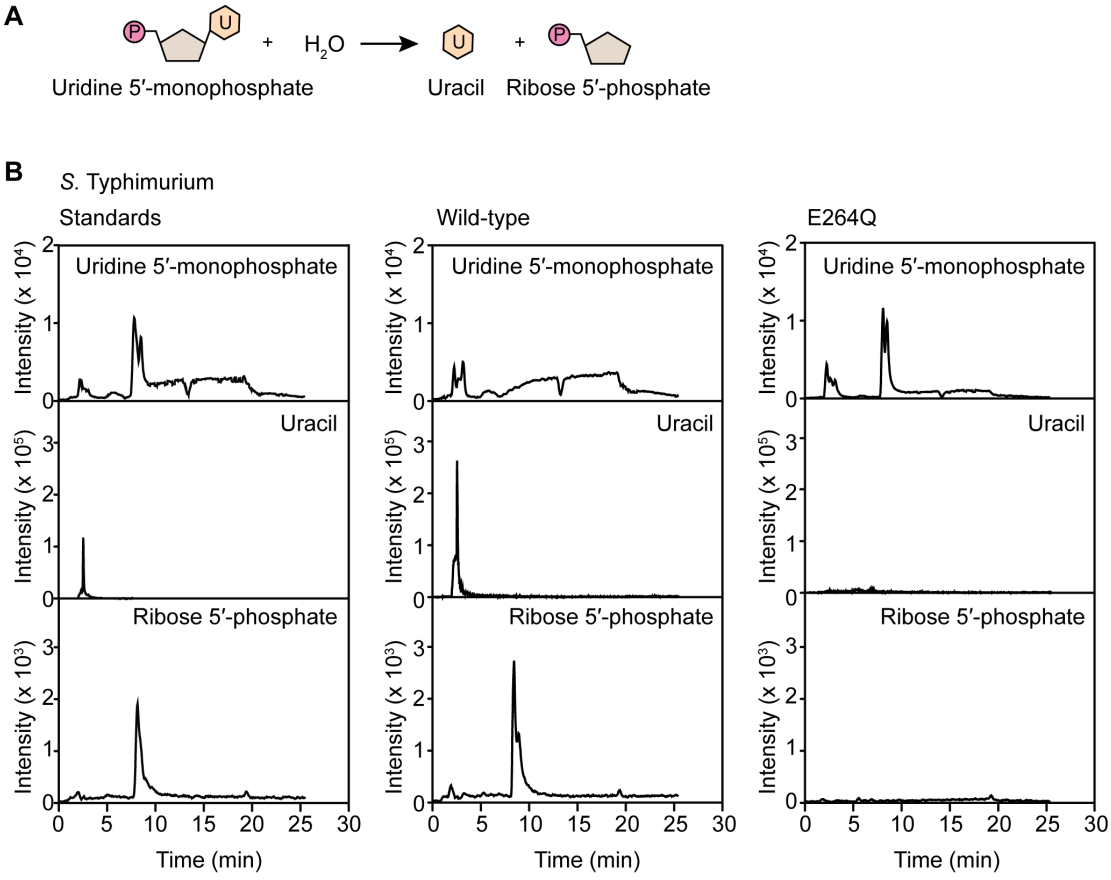
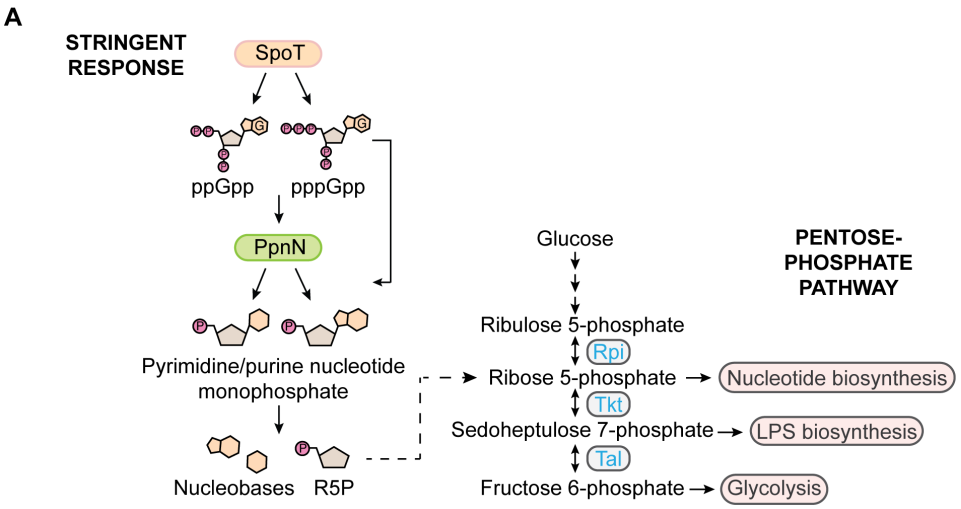

A
Uridine 5′-monophosphate
Uracil
Ribose 5′-phosphate
B
S. Typhimurium
Standards
Wild-type
E264Q
Uridine 5′-monophosphate
Uracil
Ribose 5′-phosphate
Intensity (x 10⁴)
Intensity (x 10⁵)
Intensity (x 10³)
Time (min)

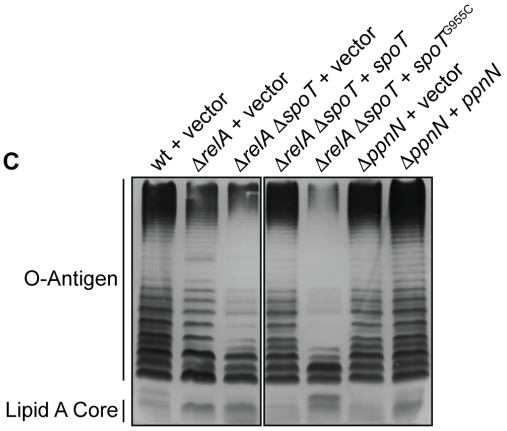
Figure 2.4. PpnN does not confer complement resistance by contributing to cell wall biogenesis. (**A**) Model showing (p)ppGpp mediated regulation of *ppnN*. PpnN produces free nucleobases and ribose 5´-phosphate (R5P), the latter of which could enter the pentose phosphate pathway and regulate carbon metabolism. (**B**) Minimal inhibitory concentration of ampicillin, ciprofloxacin, rifampicin, and colistin for wild type *S.* Typhimurium str. SL1344, Δ*ppnN*, and Δ*relA* Δ*spoT*. Δ*ppnN* does not show increased susceptibility to any of the tested antibiotics relative to wild type *S.* Typhimurium, whereas, Δ*relA* Δ*spoT* is 8-fold more susceptible to colistin. Data are representative of three replicates. (**C**) LPS analysis of the Δ*relA* Δ*spoT* and Δ*ppnN* mutants compared to wild type *S.* Typhimurium. The Δ*relA* Δ*spoT* mutant expresses significantly lower levels of very long and long O-antigen, whereas, the LPS O-antigen of Δ*ppnN* is similar to wild type. Data are representative of two replicates. Strains are carrying the empty pGEN-MCS vector control unless otherwise specified. Transcriptional reporter of the full-length (**D**) *wzy* promoter and (**E**) wzz^{ST} promoter showed (p)ppGpp-dependent up-regulation. Data are the means ± SEM (error bars) of three independent experiments. Strains are carrying the empty pWSK129 vector control unless otherwise specified. ns, not significant; *, $P < 0.05$; ***, $P < 0.0001$.

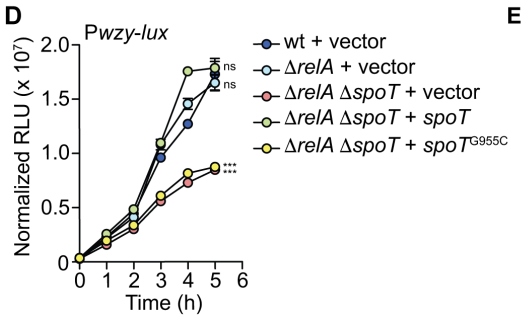
Figure 2.4

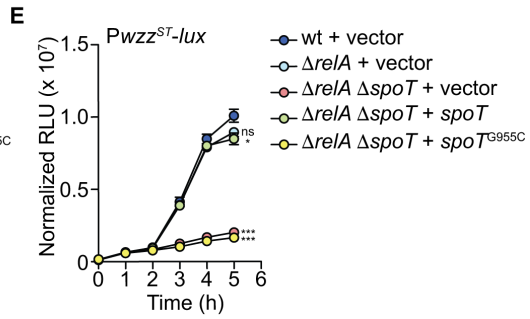


B

Antibiotic	MIC (μg/ml) Wild type	*ΔppnN*	*ΔrelA ΔspoT*
Ampicilin	4	4	4
Ciprofloxacin	0.15	0.15	0.15
Rifampicin	8	8	8
Colistin	2	2	0.25







Supplementary Figures

Figure 2.S1. RelA-SpoT is required for growth in minimal medium. Growth curves of wild type *S.* Typhimurium, Δ*relA,* Δ*relA* Δ*spoT*, and Δ*ppnN* in (**A**) LB and (**B**) M9-glucose media. Data are the means ± SEM (error bars) of three independent experiments.

Figure 2.S1

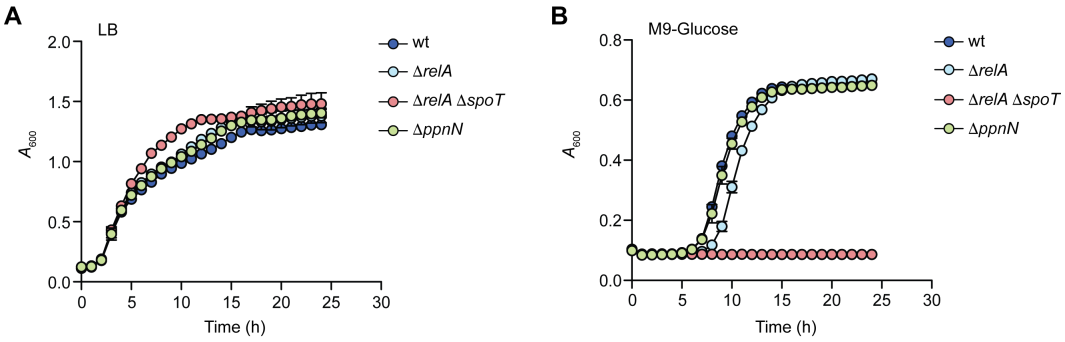

A
LB
wt
ΔrelA
ΔrelA ΔspoT
ΔppnN
A600
Time (h)
B
M9-Glucose
wt
ΔrelA
ΔrelA ΔspoT
ΔppnN
A600
Time (h)

Figure 2.S2. PpnN from *E. coli* str. K-12 and *S.* Typhimurium str. SL1344 share significant amino acid sequence homology. (**A**) PpnN has two domains of unknown function, DUF4478 and DUF3412, and a lysine decarboxylase (LDC)-like domain. The LDC-like domain contains a conserved "$PGG_xGT_{xx}E$" motif characteristic of the Lonely Guy (LOG) protein family. (**B**) Amino acid sequence alignment of PpnN from *E. coli* str. K-12 and *S.* Typhimurium str. SL1344. Protein sequences share 94% pairwise identity in amino acid sequences. DUF4478, DUF3412, and LDC-like domains are highlighted. The conserved "$PGG_xGT_{xx}E$" motif found in LOG homologues is underlined in red. Asterisks indicate the amino acid residues involved in (p)ppGpp binding.

Figure 2.S2


A
PpnN
DUF4478
LDC-like domain
DUF3412
PGGVGTXEE
256
264
1
454
B
E. coli 1 MITHISPLGSMDMLSQLEVDMLKRTASSDLYQLFRNCSLAVLNSGSLTDNSKELLSRFENFDINVLRRERG
S. Typhimurium 1 MITHISPLGSMDMLSQLEVDMLKRTASSDLYQLFRNCSLAVLNSGSLTDNSKELLSRFENFDINVLRRERG
E. coli 72 VKLELINPPEEAFVDGRIIRALQANLFAVLRDILFVYGQIHNTVRFPNLNLDNSVHITNLVFSILRNARAL
S. Typhimurium 72 VKLELINPPEDAFVDGRIIRALQANLFAVLRDILFVNGQIHNAGRFQHLDLESSTHITNLVFSILRNARAL
E. coli 143 HVGEAPNMVVCWGGHSINENEYLYARRVGNQLGLRELNICTGCGPGAMEAPMKGAAVGHAQQRYKDSRFIG
S. Typhimurium 143 HVGEAPNMVVCWGGHSINENEYLYARRVGTQLGLRELNICTGCGPGAMEAPMKGAAVGHAQQRYKDSRFIG
E. coli 214 MTEPSIIAAEPPNPLVNELIIMPDIEKRLEAFVRIAHGIIIFPGGVGTAEELLYLLGILMNPANKDQVLPL
S. Typhimurium 214 MTEPSIIAAEPPNPLVNELIIMPDIEKRLEAFVRIAHGIIIFPGGVGTAEELLYLLGILMNPANKNQVLPL
E. coli 285 ILTGPKESADYFRVLDEFVVHTLGENARRHYRIIIDDAAEVARQMKKSMPLVKENRRDTGDAYSFNWSMRI
S. Typhimurium 285 ILTGPKESADYFRVLDEFITHTLGEAARRHYRIIIDDAAEVARLMKKAMPQVKENRRDTGDAYSFNWSMRI
E. coli 356 APDLQMPFEPSHENMANLKLYPDQPVEVLAADLRRAFSGIVAGNVKEVGIRAIEEFGPYKINGDKEIMRRM
S. Typhimurium 356 APDLQVPFEPSHENMANLKLYPDQPVEILAADLRRAFSGIVAGNVKEVGIRAIEANGPYKIHGDREMMRRM
E. coli 427 DDLLQGFVAQHRMKLPGSAYIPCYEICT
S. Typhimurium 427 DDLLQGFVAQHRMKLPGSAYIPCYEICA
DUF4478
LDC-like domain
DUF3412

Figure 2.S3. PpnN from *E. coli* str. K-12 is a pyrimidine/purine nucleosidase. PpnN hydrolyzes uridine 5′-monophosphate (UMP) to uracil and ribose 5′-phosphate. Heat-inactivation of PpnN abrogates protein function. Data are representative of two replicates.

Figure 2.S3

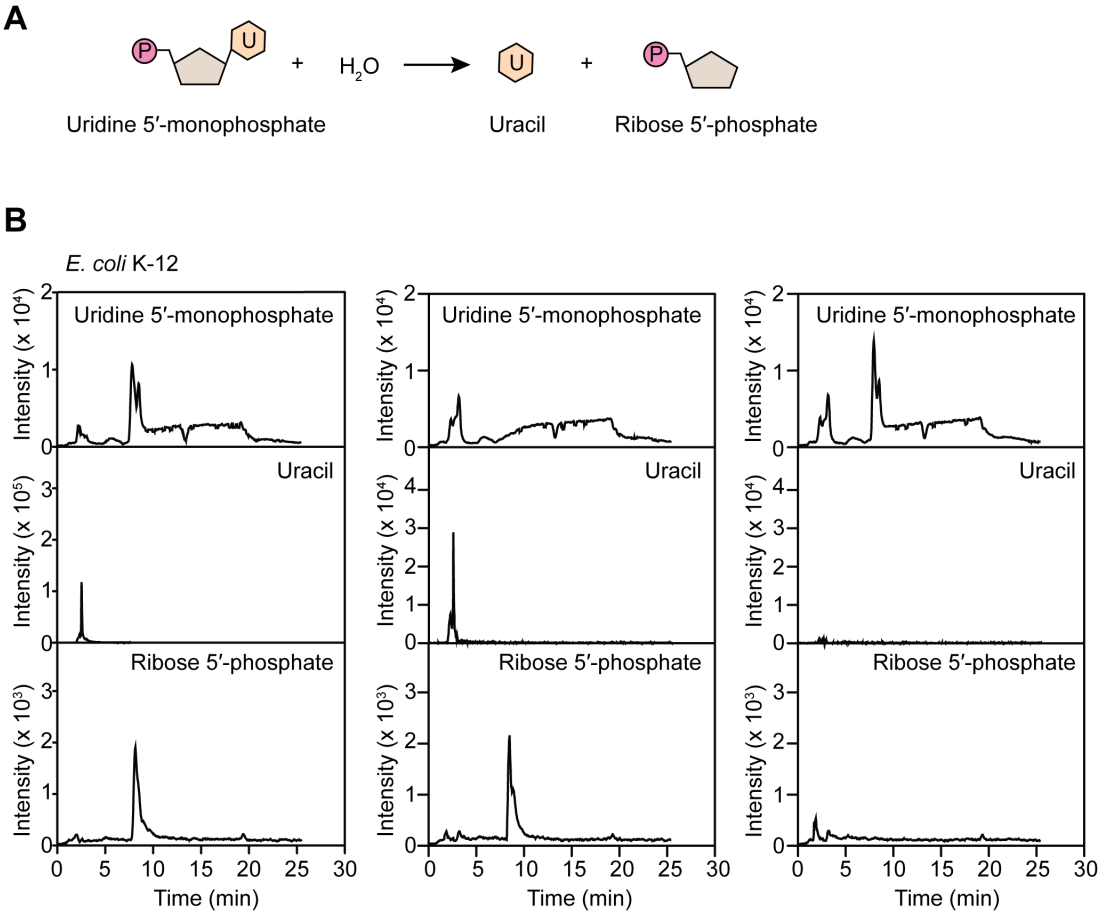

A
P
U
+
H2O
U
+
P
Uridine 5′-monophosphate
Uracil
Ribose 5′-phosphate
B
E. coli K-12
Uridine 5′-monophosphate
Uracil
Ribose 5′-phosphate
Intensity (x 10⁴)
Intensity (x 10⁵)
Intensity (x 10³)
Time (min)

Figure 2.S4. PpnN from *S.* Typhimurium str. SL1344 is a pyrimidine/purine nucleosidase. PpnN hydrolyzes guanosine 5′-monophosphate (GMP) to guanine and ribose 5′-phosphate. Introduction of an E264Q mutation in PpnN abrogates protein function. Data are representative of two replicates.

Figure 2.S4

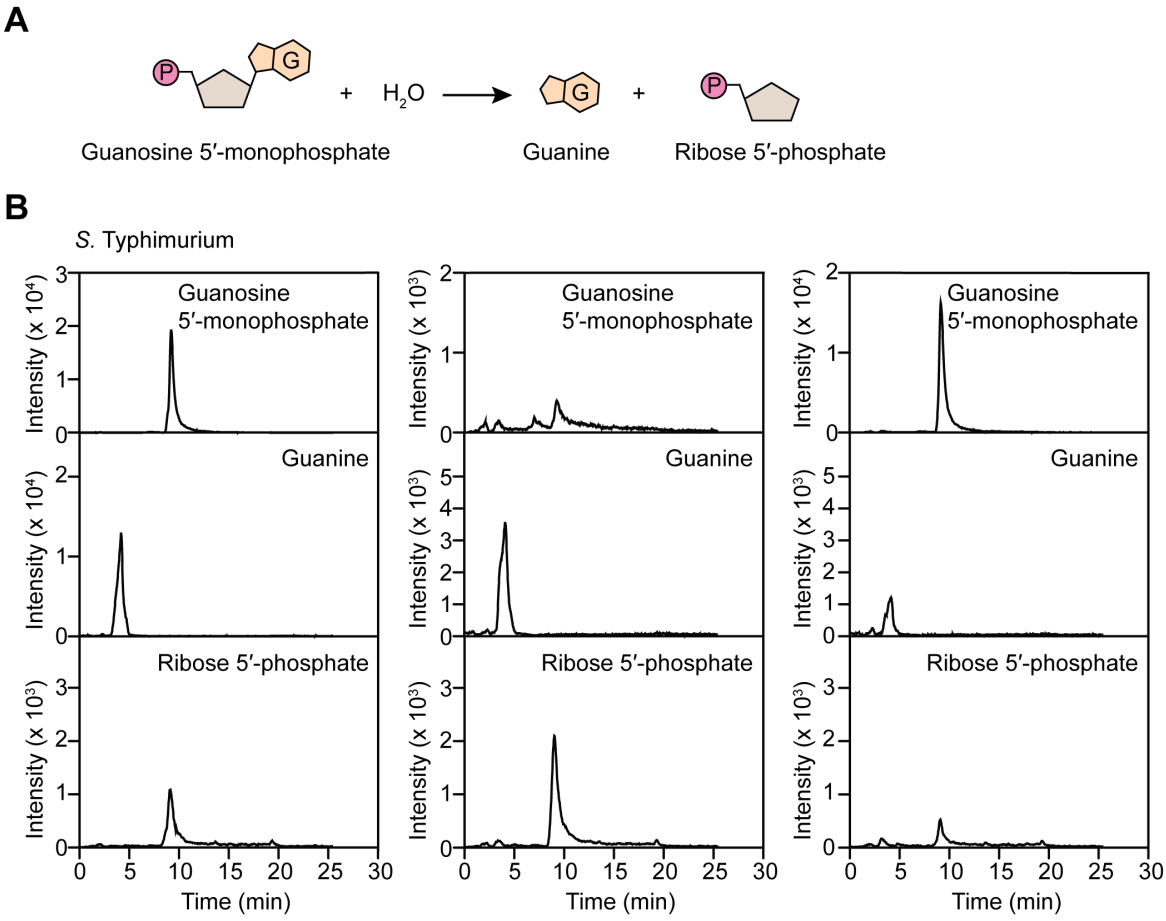
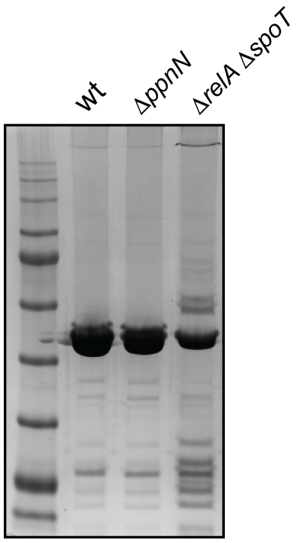

A
P
G
+
H2O
G
+
P
Guanosine 5′-monophosphate
Guanine
Ribose 5′-phosphate
B
S. Typhimurium
Guanosine 5′-monophosphate
Guanine
Ribose 5′-phosphate
Intensity (x 10⁴)
Intensity (x 10³)
Time (min)

Figure 2.S5. PpnN does not contribute to the protein composition of the outer membrane. Outer membrane protein composition of the Δ*ppnN* mutant is similar to wild type *S*. Typhimurium str. SL1344, whereas, the Δ*relA* Δ*spoT* mutant shows significant differences. Data are representative of two replicates.

Figure 2.S5



Chapter III – Genome-wide effects of (p)ppGpp signaling in invasive *Salmonella enterica* serovar Typhimurium

Chapter III – Co-authorship Statement

Chapter III consists of unpublished work:

Chau, N. Y E. and Coombes, B. K. Genome-wide effects of (p)ppGpp signaling in invasive *Salmonella enterica* serovar Typhimurium. (2021)

(1) Experiments were performed by N. Y E. C.

(2) Experimental design and writing done by N. Y E. C.

Genome-wide effects of (p)ppGpp signaling in invasive *Salmonella enterica* serovar Typhimurium

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Abstract

Salmonella enterica serovar Typhimurium ST313 is a newly emerging pathogenic strain in sub-Saharan Africa that causes a debilitating and often fatal systemic disease in humans. This is in contrast to *S.* Typhimurium ST19 that causes acute gastroenteritis and is most prevalent in developed countries. Genetic comparisons between ST313 and ST19 show that their core genomes differ by about 1,000 single nucleotide polymorphisms (SNPs), and some of these SNPs account for phenotypic differences suggestive of host adaptation by ST313. One mechanism that allows bacteria to sense and adapt to the host environment is the stringent response, which is coordinated by the nucleotide second messengers, ppGpp and pppGpp ((p)ppGpp). Here, we sought to examine the genome-wide effects of (p)ppGpp signaling in ST313 compared to ST19 using RNA-sequencing. Consistent with previous findings, ST313 and ST19 show similarities in (p)ppGpp mediated regulation of the virulence genes within *Salmonella* pathogenicity island-1 and -2. However, we also identified that while there are signatures of (p)ppGpp-dependent regulation of flagellar-based motility in ST19, this was significantly less pronounced in ST313. We propose that the differences in swimming motility regulated by (p)ppGpp is another example of a host-adapted trait in ST313 as it shifts towards a systemic lifestyle.

Introduction

Salmonella enterica is a pathogenic species of bacteria that poses a significant risk to human health globally. In developed countries, non-typhoidal strains of *Salmonella* such as serovar Typhimurium causes gastroenteritis that is usually self-limiting, and bloodstream infections are rare. This is in contrast to multi-drug resistant strains of *Salmonella* Typhimurium in sub-Saharan Africa that are associated with systemic disease and a high case fatality rate (3). Invasive *S*. Typhimurium disproportionally affects immunocompromised individuals such as those with human immunodeficiency virus (HIV), malaria, or malnutrition (3). To date, invasive *S*. Typhimurium has killed over 650,000 people worldwide (202).

S. Typhimurium associated with acute gastroenteritis belongs to the sequence type ST19, whereas, most of the *S*. Typhimurium isolates in Africa belong to lineage 2 of the sequence type ST313 (4). In comparison to *S*. Typhimurium ST19, ST313 has a distinct repertoire of plasmids and prophages, but their core genomes are well conserved and only differ by about 1,000 single nucleotide polymorphisms (SNPs) (23, 24). However, despite significant genome conservation, studies have shown that ST313 exhibits differences in certain virulence traits compared to ST19. For example, ST313 is more resistant to killing by the complement system and macrophages (63-65). Moreover, consistent with ST313 shifting towards a systemic lifestyle, genetic variations of the *macAB* efflux pump, or the loss of gene function in virulence factors such as *sseI* promotes its dissemination to the spleen and liver (62, 69).

The stringent response is a well-conserved mechanism that enables bacteria to adapt to the host environment. Activation of the stringent response by the RelA and SpoT enzymes results in the production of the nucleotide second messengers, ppGpp and pppGpp ((p)ppGpp herein), which alters bacterial physiology by coordinating changes in gene expression or regulating protein activity post-translationally (82). In *S.* Typhimurium, it has been well described that (p)ppGpp regulates genes associated with host cell invasion and intracellular survival (120, 134-136). However, the full complement of pathways regulated by (p)ppGpp in *Salmonella* is not fully understood. Moreover, given the phenotypic differences between ST19 and ST313 that have been identified thus far, we hypothesized that there may be differences in host adaptation mediated by (p)ppGpp. To this end, we sought to compare the stringent response between *S.* Typhimurium SL1344 of the ST19 sequence type and *S.* Typhimurium D23580 belonging to ST313 using transcriptomics profiling.

Results

Comparative RNA-sequencing reveals the global impact of (p)ppGpp regulation in *Salmonella* Typhimurium

RelA and SpoT modulate the levels of (p)ppGpp and are the main regulators of the stringent response in *Salmonella* (82). To investigate the effects of (p)ppGpp signaling in SL1344 and D23580, we performed RNA-seq of an SL1344 or D23580 Δ*relA* Δ*spoT* mutant unable to synthesize (p)ppGpp ((p)ppGpp^0) and compared each mutant to the respective wild type strain. RNA extracted from the mutant and wild type grown to early

stationary phase in LB medium to mimic stringent response conditions was sequenced using the Illumina MiSeq platform and analyzed using DESeq2 (**Fig. 3.1A**). Three biological replicates were sequenced to approximately 1.5 million single-end reads per sample. Based on our analysis, we identified 357 genes in SL1344, 364 genes in D23580, and 804 shared genes that were significantly up-regulated >2-fold or down-regulated <2-fold in the (p)ppGpp^0 mutant relative to wild type (adjusted *p*-value < 0.05) (**Fig. 3.1B, Fig. 3.S1**). In addition, each overall dataset was ranked by their adjusted *p*-value and the uncharacterized hits among the top 10 differentially regulated genes were highlighted in each overall dataset. In SL1344, we identified *pagK*, a putative xylanase/chitin deacetylase (*SL1344_3106*), a putative amidohydrolase (*SL1344_3107*), and *SL1344_1602* that were significantly repressed (**Fig. 3.1C**). In D23580, six genes including a putative type II anti-toxin from the ParD family (*STMMW_29181*), a putative amidohydrolase (*STMMW_30931*), a putative xylanase/chitin deacetylase (*STMMW_30921*), *STMMW_15131*, *STMMW_17231*, and *STMMW_17251* were down-regulated in the (p)ppGpp^0 mutant (**Fig. 3.1D**). We also examined the datasets for the expression profiles of genes significantly regulated in both strains, specifically those associated with SPI-1 and SPI-2 based on previous reports on the stringent response in *S.* Typhimurium. Indeed, the activation of genes encoded within or co-regulated with SPI-1 and SPI-2 were dependent on (p)ppGpp, demonstrating the validity of our approach (**Fig. 3.S1**). The remaining significantly regulated genes shared between SL1344 and D23580 were regulated in a similar manner by (p)ppGpp, thus, we only listed the differentially

regulated genes specific to either strain in **Table A2.1** and **Table A2.2**, which are potentially of interest for follow-up work.

(p)ppGpp differentially regulates flagellar-based motility in D23580 compared to SL1344

Our transcriptomic analysis revealed (p)ppGpp-dependent regulation of flagellar-based motility in SL1344 that was not apparent in D23580 (**Fig. 3.2A**). To confirm the differences we observed from the RNA-seq, we performed bacterial swimming assays on soft LB agar. Consistent with previous reports, wild type D23580 was considerably less motile than wild type SL1344 (24, 65, 66). We next tested the role of (p)ppGpp in the swimming assays and found that an SL1344 Δ*relA* mutant swam similarly to the wild type strain, whereas, motility was significantly impaired in the Δ*relA* Δ*spoT* double mutant and this could be complemented by the expression of SpoT *in trans* from its native promoter (**Fig. 3.2B, C**). In contrast, both the Δ*relA* and the Δ*relA* Δ*spoT* mutant in D23580 swam similarly to wild type (**Fig. 3.2B, 3.2C**). This phenotype was consistent with the activation of *flhDC* and *fliC*, which encode for the master transcriptional regulator of the flagellar cascade and flagellin subunit, respectively. The *flhDC* and *fliC* promoters were both repressed in the Δ*relA* Δ*spoT* mutant in SL1344 but remained largely insensitive to (p)ppGpp input in D23580 (**Fig. 3.2D**). We also measured FliC protein levels in the two strains of *S*. Typhimurium using western blotting and found that the production of flagellin was dependent on (p)ppGpp in SL1344 and not in D23580

(**Fig. 3.2E**). Together, these data indicate that *S*. Typhimurium D23580 has evolved away from the activation of flagellar genes by (p)ppGpp.

Divergence in flagellar motility is unlikely due to SNPs in D23580

Some of the phenotypic differences between *S*. Typhimurium D23580 and SL1344 are caused by certain SNPs that directly modulate virulence gene expression or protein function. Previous work identified a single nucleotide deletion and 11 SNP differences in the flagellar regulon of D23580 compared to the parent strain of SL1344, *S*. Typhimurium 4/74. These include mutations upstream of *motA*, *flhD*, and *mcpA*; synonymous mutations in *flgK*, *cheA*, and *fliP*; and non-synonymous mutations in *flhA*, *flhB*, *fliB*, and *mcpC* (24). To determine whether any of these mutations were responsible for the divergent regulation of flagellar genes between D23580 and SL1344, we reverted the SNPs in D23580 to the SL1344 variants using allelic exchange and performed swimming assays. Given that (p)ppGpp modulates transcription or post-translationally regulates protein activity, we focused on the mutations that are in intergenic regions or non-synonymous mutations found in protein coding sequences (**Table A1.3**). Interestingly, the SL1344 genetic variants of each of the flagellar genes made in the Δ*relA* Δ*spoT* D23580 mutant did not result in any changes in motility compared to the wild type strain (**Fig. 3.3**). These findings suggest that the SNPs in the flagellar regulon likely do not have an impact on (p)ppGpp mediated regulation or that it was not possible to evaluate each mutation using this approach due to other mutations present in the D23580 background.

Discussion

S. Typhimurium D23580 from the ST313 lineage isolated from Malawi in Africa is frequently associated with systemic disease compared to ST19 strains such as *S*. Typhimurium SL1344 that causes acute gastroenteritis (4). The stark contrast in pathogenicity of the two strains of *Salmonella* has been linked to various genetic changes in D23580 that are suggestive of adaptation to the host environment. In this study, we examined the signaling effects of the nucleotide second messenger, (p)ppGpp, in *S*. Typhimurium SL1344 and D23580 using RNA-sequencing. We found that there were similarities in the regulation of SPI-1 and SPI-2 associated genes by (p)ppGpp, but flagellar-based motility genes were differentially regulated between the two strains.

Our data is consistent with the stringent control of motility seen in SL1344. SL1344 lacking the DnaK suppressor A (DksA) protein that modulates RNA polymerase activity with (p)ppGpp is deficient in swimming motility and shows significant down-regulation of flagellar genes (203). Given that motility is required for *S*. Typhimurium to successfully colonize the intestine, these data are also concordant with the positive regulation of its invasion-associated genes during the stringent response. Pathogenic species such as *Vibrio cholerae* and *Legionella pneumophila* similarly require DksA for the activation of motility (204, 205). Interestingly, however, there have been conflicting reports on the role of DksA in the regulation of the flagella cascade, particularly in *E. coli*. One study found that both a Δ*relA* Δ*spoT* mutant and a Δ*dksA* mutant were less motile than the wild type strain (206). However, others have reported that an *E. coli*

Δ*dksA* mutant displayed increased flagellin production and was hypermotile both in stationary phase and following amino acid starvation (207, 208). The hyper motility phenotype was attributable to DksA mediated repression of the flagellar regulators, *flhDC* and *fliA*, and is thought to prevent the expenditure of energy and resources towards flagella biosynthesis during unfavorable growth conditions (208). The differences in the regulation of motility by (p)ppGpp likely reflects the lifestyle of each bacterial species.

Similar to previous findings, we observed that wild type D23580 was significantly less motile and produced a lower level of flagellin than wild type SL1344 (24, 65, 66). The decrease in swimming motility by D23580 is in part due to a non-synonymous mutation in *flhA* as engineering of the ST19 variant of *flhA* into the D23580 background resulted in a modest increase in motility compared to wild type D23580 (24). Moreover, these data are consistent with the increase in survival of ST313 strains in macrophages and the dampened activation of the NAIP/NLRC4 inflammasome by flagellin compared to ST19 (65). Interestingly, SL1344 evades inflammasome activation by SsrB-mediated repression of the flagellar gene cascade (13, 14, 75). SsrB is encoded within SPI-2 and is a master regulator of *Salmonella* intracellular virulence. The repression of motility genes is mediated by SsrB binding to the promoter region of *flhDC* in SL1344, but not in the SsrB-naïve species, *Salmonella bongori* (14). This indicates the evolution of *cis*-regulatory inputs following the acquisition of SsrB to fine-tune gene expression and optimize the virulence of *S.* Typhimurium SL1344. Although our study has focused on non-typhoidal servoars of *Salmonella*, *S.* Typhi that is restricted to infecting humans and

also causes a systemic disease similar to ST313 similarly represses flagellin intracellularly and prolongs macrophage survival (209).

Several virulence-associated traits such as invasion of gut epithelial cells and survival against complement proteins have been compared between ST313 and ST19, and differences in these traits have been ascribed to SNPs that have accumulated in ST313 (61, 63, 64). We tested whether some of the SNPs identified in the flagellar regulon in ST313 accounted for the changes in (p)ppGpp-mediated regulation of swimming motility (**Table A1.4**) (24). However, generation of the SL1344 genetic variants in *motA*, *flhA*, *flhB*, *flhD*, and *mcpC* in the D23580 Δ*relA* Δ*spoT* mutant background did not result in changes in motility compared to the wild type or mutant D23580 strains. These data remain inconclusive, as it is possible that other mutations present in ST313 are confounding our ability to associate a particular SNP with the motility phenotype. Similarly, this was also observed with a SNP in the diguanylate cyclase, STM1987, where generation of the ST19 variant in ST313 did not result in an observable phenotype, whereas, generating the ST313 SNP in the ST19 background resulted in an increase in bacterial survival within macrophages and enhanced *Salmonella* fitness in mice (68).

Our study highlights that there are differences in the stringent response between *S.* Typhimurium ST19 and ST313 despite significant conservation in their core genomes. To our knowledge, this is the first study to explore the effects of (p)ppGpp signaling in invasive *S.* Typhimurium and report differences in flagellar-based motility compared to

strains of *S.* Typhimurium that cause acute gastrointestinal disease. We hypothesize that the evolved regulatory control of flagellar genes during the stringent response helps to facilitate the transition of ST313 to a systemic lifestyle in human hosts. The focus of future work will be to determine the mechanism accounting for the motility phenotype, and to investigate the significance of evolving away from the positive regulation of motility by (p)ppGpp. We detail these aims in Chapter IV.

Experimental Procedures

Bacterial Strains and Growth Conditions

Salmonella enterica serovar Typhimurium (SL1344 or D23580) and isogenic derivatives were used in the study and listed in **Table 3.2**. Bacteria were grown in LB at 37°C with shaking and antibiotics were supplemented where appropriate (streptomycin, 50 μg/mL; ampicillin, 200 μg/mL; gentamicin, 15 μg/mL).

Cloning and Mutant Generation

Chromosomal allelic exchange was used to generate the D23580 Δ*relA*::FRT Δ*spoT*::FRT double mutant. Gene specific regions of homology flanking the Δ*relA*::FRT in SL1344 was amplified using Phire Hot Start II DNA Polymerase (Thermo Fisher), digested with SacI/KpnI (Thermo Fisher), and cloned into pRE112. *E. coli* SM10 λ pir transformed with pRE112-Δ*relA*::FRT^{SL1344} was then mated with wild type D23580 at a 3:1 ratio of donor to recipient on LB agar overnight at 37°C. The next day, conjugations were plated onto LB agar supplemented with 50 μg/mL streptomycin and 15 μg/mL

gentamicin to select for recipient strains that have chromosomally integrated the allelic exchange vector. Single colonies were then streaked onto LB agar without salt and containing 5% sucrose to counter-select against pRE112, and the mutation was verified by PCR and Sanger sequencing (GENEWIZ®). Δ*spoT*::FRT and flanking regions of homology from SL1344 was also cloned into pRE112 using SacI/SmaI (Thermo Fisher). The process was then repeated to generate Δ*spoT*::FRT in the Δ*relA*::FRT D23580 mutant background.

Similarly, SL1344 mutations in *motA*, *flhD*, *flhA*, *flhB*, and *mcpC* and ~500 bp regions of homology flanking each gene mutation were generated in the D23580 Δ*relA*::FRT Δ*spoT*::FRT double mutant using allelic exchange as described above.

Bioluminescence reporters were generated by PCR amplifying 910 bp upstream of *flhD* and 256 bp upstream of *fliC*, followed by digestion of the PCR products with SnaBI (Thermo Fisher), and cloning into pGEN-*luxCDABE*-Gm^R digested with SnaBI/PmeI (Thermo Fisher).

All plasmid constructs were sequence verified by Sanger sequencing (GENEWIZ®) then transformed by electroporation (BioRad) into the appropriate strain backgrounds for downstream experiments. Plasmids used in the study are listed in **Table 3.3** and primers (Sigma Aldrich) are listed in **Table 3.4**.

RNA Isolation for Sequencing

Bacterial strains grown in LB overnight were sub-cultured (1:50) into LB for 4 h until the cells reached early stationary phase (OD_{600} = 0.8-1.0). Following growth, the equivalent of 1 OD of bacteria was pelleted and flash frozen in liquid nitrogen and then stored at -80°C. RNA was extracted by lysing cells using Tissue and Cell Lysis Solution (MasterPure™) containing 0.175 µg/mL proteinase K (Lucigen) followed by protein precipitation with 175 µL of MPC Protein Precipitation Reagent (MasterPure™). Samples were then mixed briefly by vortexing and centrifuged for 15 min at 4°C. The resulting supernatant was taken to a new RNase-free microcentrifuge tube (Axygen) and the RNA was precipitated using 100% isopropanol, followed by washing with 75% ethanol, and then resuspension of the RNA pellet in DEPC water (Ambion). RNA was treated with DNase I (Thermo Fisher Scientific) for 1 h at 37°C and inactivated with EDTA (Thermo Fisher Scientific) for downstream applications.

RNA-seq Library Preparation and Read Processing

RNA-seq data are produced from three biological replicates per strain and approximately 1.5 million reads per sample. Prior to RNA-sequencing, rRNA was depleted and cDNA libraries were processed using the NEBNext® Ultra™ II RNA Library Prep Kit for Illumina®. cDNA was sequenced using the Illumina MiSeq platform using the V3 1x150 bp configuration.

The quality of each RNA-seq library was assessed using FastQC v0.11.9 (210). Reads

were then processed with Trimmomatic v0.39 to remove Illumina TruSeq adapter sequences, leading and trailing bases with a Phred quality score below 20, and trim reads with an average base quality score of 20 over a 4-bp sliding window. Reads less than 40 bp were also discarded using Trimmomatic v0.39 (211). The remaining reads of each library were aligned to the corresponding reference genome for *S*. Typhimurium SL1344 (accession: NC_016810) or *S*. Typhimurium D23580 (accession: NC_016854.1) using Bowtie2 v2.3.5.1 (212, 213). Reads were assigned to genome features and tallied using featureCounts as part of samtools v1.9 (214). Differential gene regulation and RNA-seq statistics comparing Δ*relA* Δ*spoT* of *S*. Typhimurium SL1344 or *S*. Typhimurium D23580 relative to the corresponding wild type strain were calculated using DESeq2 (215).

Motility Assays

Bacterial strains grown overnight in LB were normalized to an OD_{600} = 1.0 and 3 uL of each strain was spotted onto 0.25% LB agar. The plates were incubated for 5-6 h at 37°C and then imaged using ChemiDoc™ MP Imaging System (BioRad). Motility zones were measured using a ruler.

SDS-PAGE and Western Blot

Bacterial strains grown in LB overnight were sub-cultured (1:50) into LB for 4 h until the cells reached early stationary phase (OD_{600} = 0.8-1.0). An equivalent of 1 OD of cells was centrifuged at 16,000 *g* and resuspended in 1 X Laemmli buffer and boiled for 5 min at 95°C. Protein samples were separated by SDS-PAGE, transferred to a nitrocellulose

membrane, and blocked overnight at 4°C with rocking in 1 X phosphate buffered saline containing 0.1% Tween-20 (PBST) with 5% skim milk. Membranes were probed with mouse anti-FliC (BioLegend, 1:1000) or mouse anti-RNAP (BioLegend, 1:5000) in PBST with 1.5% skim milk for 1 h at room temperature with rocking. Following incubation with primary antibody, the membranes were washed 3 times with PBST for 5 min each and then incubated with goat anti-mouse-HRP conjugated secondary antibody (Jackson Immunoresearch, 1:10000) in PBST with 1.5% skim milk for 1 h. Membranes were washed again 3 times with PBST for 5 min each and blots were developed with Clarity Max Western (BioRad) ECL substrate then imaged with the ChemiDoc XRS+ (BioRad).

Statistical Analysis

Data were analyzed using GraphPad Prism 5.0a software (GraphPad Inc., San Diego, CA) using one-way ANOVA. *P* values of <0.05 were considered significant.

Acknowledgements

We thank Jay Hinton for providing the D23580 strain. This work was supported by a grant to B.K.C. from the Canadian Institutes of Health Research (CIHR). B.K.C. holds the Canada Research Chair in Infectious Disease Pathogenesis. N.Y E.C is a recipient of an Ontario Graduate Scholarship.

Conflict of Interest: The authors declare they have no conflicts of interest with the contents of this article.

Table 3.1. Flagellar gene mutations in D23580 compared to SL1344.

Gene	Mutation	Location	Protein Function
flhD	A→G	Intergenic	Master transcriptional regulator of flagellar genes with FlhC
flhA	A166T	CDS	Required for formation of flagellar rod structure
flhB	I73N	CDS	Required for formation of flagellar rod structure
fliB	A10T	CDS	Lysine-N-methylase; post-translational methylation of flagellin
motA	1-bp del	Intergenic	Forms stator with MotB required for flagellar motor rotation
mcpA	C→T	Intergenic	Methyl-accepting chemotaxis protein A
	G→T	Intergenic	
	G→A	Intergenic	
mcpC	I138S	CDS	Methyl-accepting chemotaxis protein C

Table 3.2. Strains used in the study.

Strain	**Source or reference**
Wild type *S.* Typhimurium str. SL1344	Coombes *et al*. 2003 (198)
Δ*relA S.* Typhimurium str. SL1344	Chau *et al.* 2021 (140)
Δ*relA* Δ*spoT S.* Typhimurium str. SL1344	Chau *et al.* 2021 (140)
Wild type *S.* Typhimurium str. D23580	Kingsley *et al.* 2009 (23)
Δ*relA S.* Typhimurium str. D23580	This study
Δ*relA* Δ*spoT S.* Typhimurium str. D23580	This study
DH5α λ pir *E. coli*	Lab stock
SM10 λ pir *E. coli*	Lab stock
TOP10 *E. coli*	Invitrogen

Table 3.3. Plasmids used in the study.

Plasmid	Description	Source or reference
pGEN-MCS	Low-copy number cloning vector	Lane *et al.* 2007 (192)
pGEN-*luxCDABE*	Lux transcriptional reporter plasmid	Lane *et al.* 2007 (192)
pGEN-MCS-*spoT*^{SL1344}	*spoT* with 291 bp upstream of coding sequence cloned into pGEN-MCS for complementation experiments	Chau *et al.* 2021 (140)
pGEN-MCS-*spoT*^{D23580}	*spoT* with 291 bp upstream of coding sequence cloned into pGEN-MCS for complementation experiments	This study
pGEN-*luxCDABE*-*flhDC*^{SL1344}	Lux transcriptional reporter for *flhDC*^{SL1344} promoter	This study
pGEN-*luxCDABE*-*flhDC*^{D23580}	Lux transcriptional reporter for *flhDC*^{D23580} promoter	This study
pGEN-*luxCDABE*-*fliC*^{SL1344}	Lux transcriptional reporter for *fliC*^{SL1344} promoter	This study
pGEN-*luxCDABE*-*fliC*^{D23580}	Lux transcriptional reporter for *fliC*^{D23580} promoter	This study
pRE112-*relA*::FRT^{SL1344}	Allelic exchange vector	This study
pRE112-*spoT*::FRT^{SL1344}	Allelic exchange vector	This study
pRE112-*flhD*^{SL1344}	Allelic exchange vector	This study
pRE112-*motA*^{SL1344}	Allelic exchange vector	This study
pRE112-*flhA*^{SL1344}	Allelic exchange vector	This study
pRE112-*flhB*^{SL1344}	Allelic exchange vector	This study
pRE112-*mcpC*^{SL1344}	Allelic exchange vector	This study

Table 3.4. Primers used in the study.

Primer	Gene	Direction	Destination	Sequence (5´-3´)
EC306	*relA*	F	pRE112	GCCAGAGCTCTGCGCGCCGAGGATCGTGTCCTGGA
EC306	*relA*	R	pRE112	ATGCGGTACCCGATCGGCCTGTATTTCGCCAAAAT
EC307	*spoT*	F	pRE112	GCCACCCGGGGCCATTACGCCGAATATGATTATCT
EC307	*spoT*	R	pRE112	ATGCGAGCTCGTCGCTGGCGCTGGGCTTCATAAAG
EC314	*fliC*	F	pGEN-*luxCDABE*	TATGCGGTACCGCCTTCTTCCTTTTTGATTGCAAA
EC314	*fliC*	R	pGEN-*luxCDABE*	GTCATATACGTAGATCTTTTCCTTATCAATTACAAC
EC316	*flhD*	F	pGEN-*luxCDABE*	TATGCGGTACCCGTTTAATATTTAAGCTACTGTTTACTA
EC316	*flhD*	R	pGEN-*luxCDABE*	AGTCTACGTACCAGAATAACCAACTTTATTTTTGT
EC318	*flhD*	F	pRE112	GCCAGAGCTCTCAGGATCGGAAGCGAGAGT
EC318	*flhD*	R	pRE112	ATGCGGTACCAGGAGTAAATATGACAAATTGATGT
EC330	*motA*	F	pRE112	GCCAGAGCTCAAAGAATTACGCGGTAGCCCGCC
EC330	*motA*	R	pRE112	ATGCGGTACCAATTTCGAACGTATTCATGTTGCCG
EC331	*flhA*	F	pRE112	GCCAGAGCTCATGGCTAATCTGGTCGCGATGCT
EC331	*flhA*	R	pRE112	ATGCGGTACCTCCGGCGCTTTTTCTTCACGCC
EC332	*flhB*	F	pRE112	GCCAGAGCTCAGGTGGATACCTCGAAAGCTGGC
EC332	*flhB*	R	pRE112	ATGCGGTACCTTTGCCCTTAACATGCGGATCGC
EC347	*mcpC*	F	pRE112	GCCAGAGCTCCTTACTAATAATGTTCCGAAATAAAAAGCG
EC347	*mcpC*	R	pRE112	ATGCGGTACCTGTTTTTAACCGTGGCGGTAATCTG

Main Figures

Figure 3.1. Comparative RNA-seq reveals the effects of (p)ppGpp signaling in *Salmonella* Typhimurium. (**A**) RNA from wild type *S.* Typhimurium SL1344 or D23580 and the respective Δ*relA* Δ*spoT* mutant grown to early stationary phase (OD=1.0) in LB medium were sequenced using the Illumina MiSeq platform. (**B**) Total counts of significantly differentially regulated genes between Δ*relA* Δ*spoT* and wild type in SL1344, D23580, or both strains based on a 2-fold cut-off and q-value < 0.05. Volcano plots of (**C**) SL1344 and (**D**) D23580 datasets depicting the $\log_2$ fold change (Δ*relA* Δ*spoT* / wild type) of *S.* Typhimurium genes against the corresponding q-value ($-\log_{10}$). Blue dots represent genes with a q-value < 0.05 and grey dots represent genes with a q-value > 0.05. Vertical dashed lines indicate 2-fold cut-offs. Labeled genes include the top 10 differentially regulated genes in either SL1344 or D23580 ranked based on their q-value.

Figure 3.1

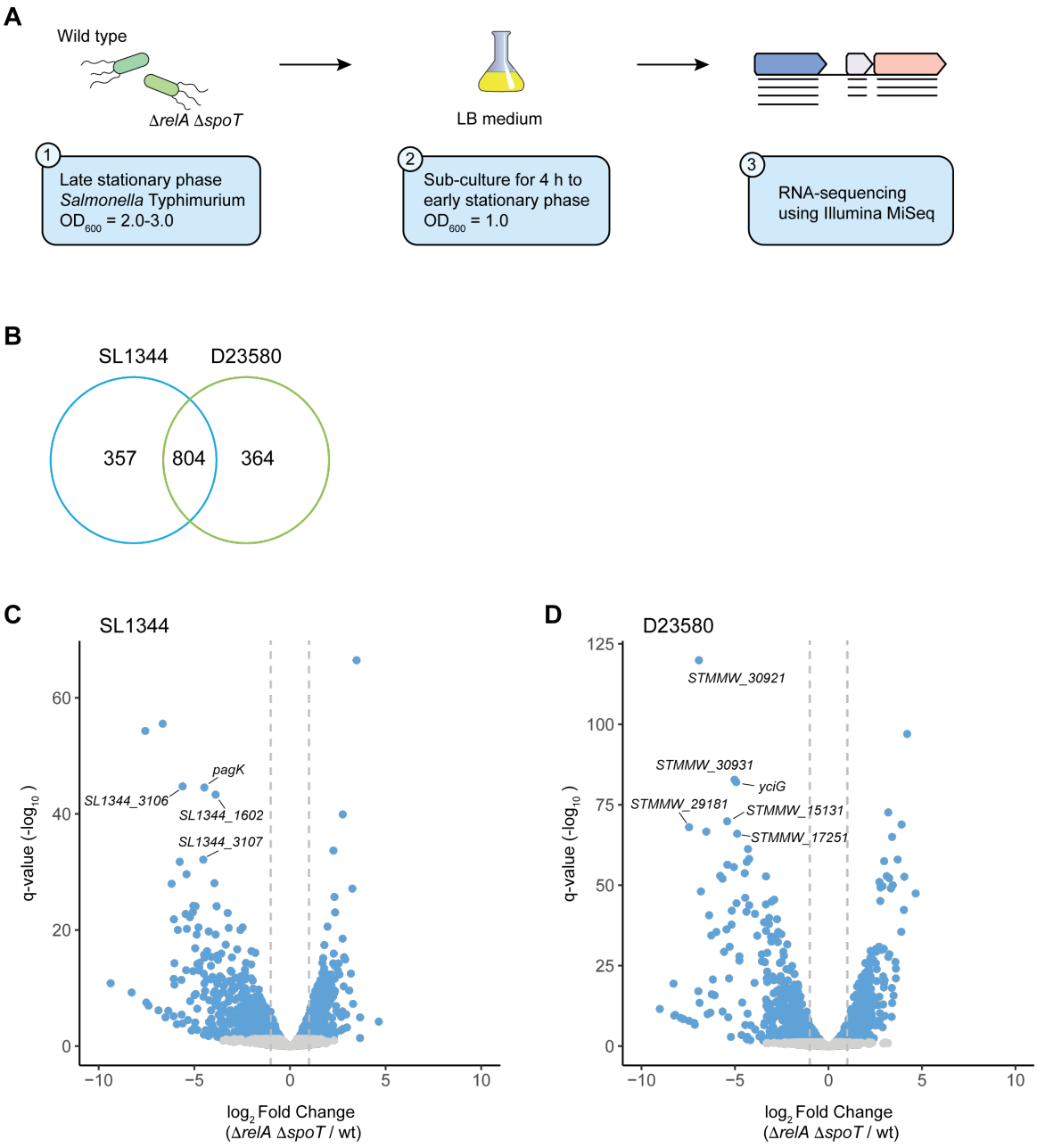

A
Wild type
ΔrelA ΔspoT
LB medium
1
Late stationary phase
Salmonella Typhimurium
OD600 = 2.0-3.0
2
Sub-culture for 4 h to
early stationary phase
OD600 = 1.0
3
RNA-sequencing
using Illumina MiSeq
B
SL1344
D23580
357
804
364
C
SL1344
pagK
SL1344_3106
SL1344_1602
SL1344_3107
q-value (-log10)
log2 Fold Change
(ΔrelA ΔspoT / wt)
D
D23580
STMMW_30921
STMMW_30931
yciG
STMMW_29181
STMMW_15131
STMMW_17251
q-value (-log10)
log2 Fold Change
(ΔrelA ΔspoT / wt)

Figure 3.2. Flagellar-based motility is differentially regulated by (p)ppGpp in *S.* Typhimurium. (**A**) Heat map depicting the $\log_2$ fold change in expression of flagellar-based motility genes (Δ*relA* Δ*spoT* / wild type) in SL1344 and D23580. (**B**) SL1344 Δ*relA* Δ*spoT* swims significantly less compared to the wild type SL1344 strain and this can be complemented by the expression of SpoT from its native promoter, whereas, the D23580 Δ*relA* Δ*spoT* mutant swims similarly to wild type D23580. Strains are carrying the empty pGEN-MCS vector unless otherwise specified. (**C**) Quantification of swimming zone diameters from the motility assays in (B). Data are the means ± SEM (error bars) of three independent experiments. (**D**) Transcriptional reporters of the full-length *flhDC* or *fliC* promoters in wild type and Δ*relA* Δ*spoT* of SL1344 or D23580. Data are the means ± SEM (error bars) of three independent experiments. (**E**) Western blot showing FliC protein levels are significantly less in the SL1344 Δ*relA* Δ*spoT* mutant relative to the wild type strain and this could be complemented by the expression of SpoT from its native promoter. The level of FliC protein in the D23580 Δ*relA* Δ*spoT* mutant is similar to its respective wild type strain. DnaK is used as a gel loading control. Data are representative of two independent experiments. Strains are carrying the empty pGEN-MCS vector unless otherwise specified. ns, not significant; ***, $P < 0.0001$.

Figure 3.2

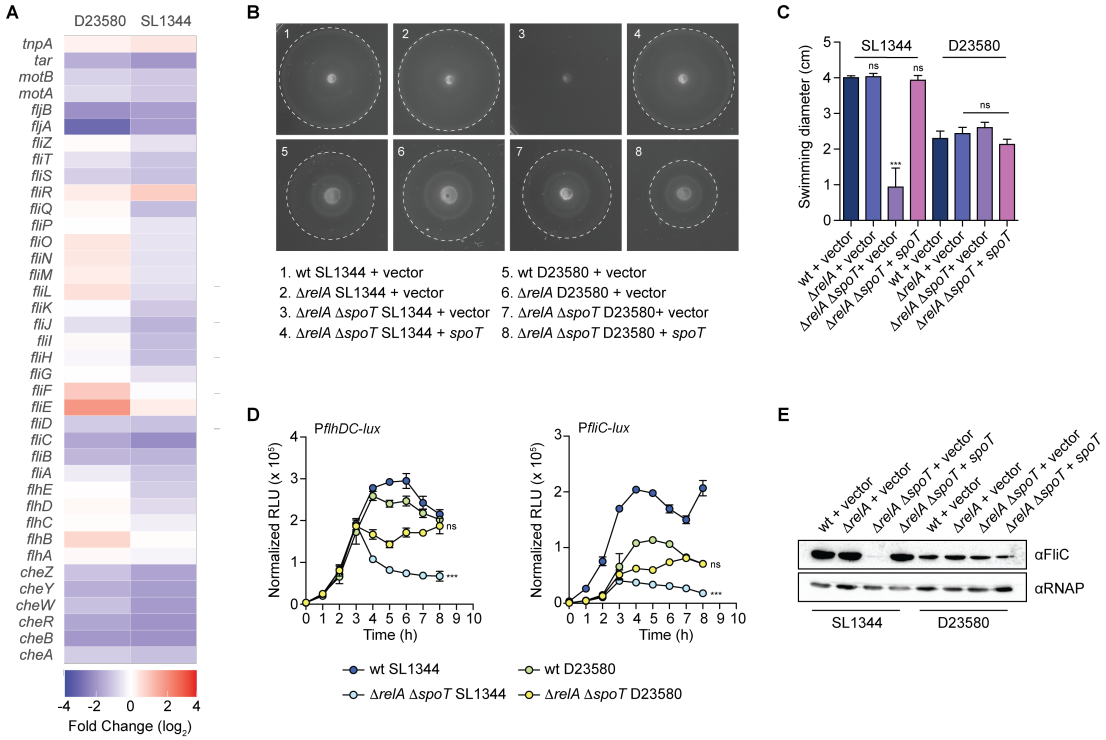
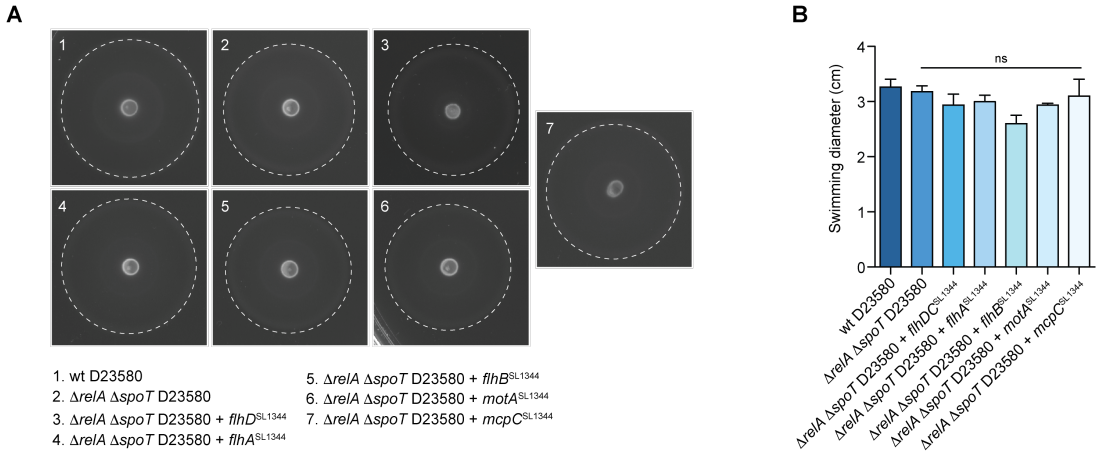

A
D23580
SL1344
tnpA
tar
motB
motA
flgB
flgA
fliZ
fliT
fliS
fliR
fliQ
fliP
fliO
fliN
fliM
fliL
fliK
fliJ
fliI
fliH
fliG
fliF
fliE
fliD
fliC
fliB
fliA
flhE
flhD
flhC
flhB
flhA
cheZ
cheY
cheW
cheR
cheB
cheA
-4 -2 0 2 4
Fold Change (log2)
B
1. wt SL1344 + vector
2. ΔrelA SL1344 + vector
3. ΔrelA ΔspoT SL1344 + vector
4. ΔrelA ΔspoT SL1344 + spoT
5. wt D23580 + vector
6. ΔrelA D23580 + vector
7. ΔrelA ΔspoT D23580+ vector
8. ΔrelA ΔspoT D23580 + spoT
C
SL1344
D23580
Swimming diameter (cm)
ns
***
wt + vector
ΔrelA + vector
ΔrelA ΔspoT+ vector
ΔrelA ΔspoT + spoT
D
PflhDC-lux
PfliC-lux
Normalized RLU (x 10^5)
Time (h)
wt SL1344
wt D23580
ΔrelA ΔspoT SL1344
ΔrelA ΔspoT D23580
E
αFliC
αRNAP
SL1344
D23580

Figure 3.3. Divergence in the regulation of flagellar genes by (p)ppGpp in D23580 is unlikely to be associated with SNPs in the flagellar cascade. (**A**) D23580 Δ*relA* Δ*spoT* containing the SL1344 genetic variant of various flagellar genes swam similarly to wild type and Δ*relA* Δ*spoT* D23580. (**B**) Quantification of swimming zone diameters from the motility assays in (A). Data are the means ± SEM (error bars) of three independent experiments. ns, not significant.

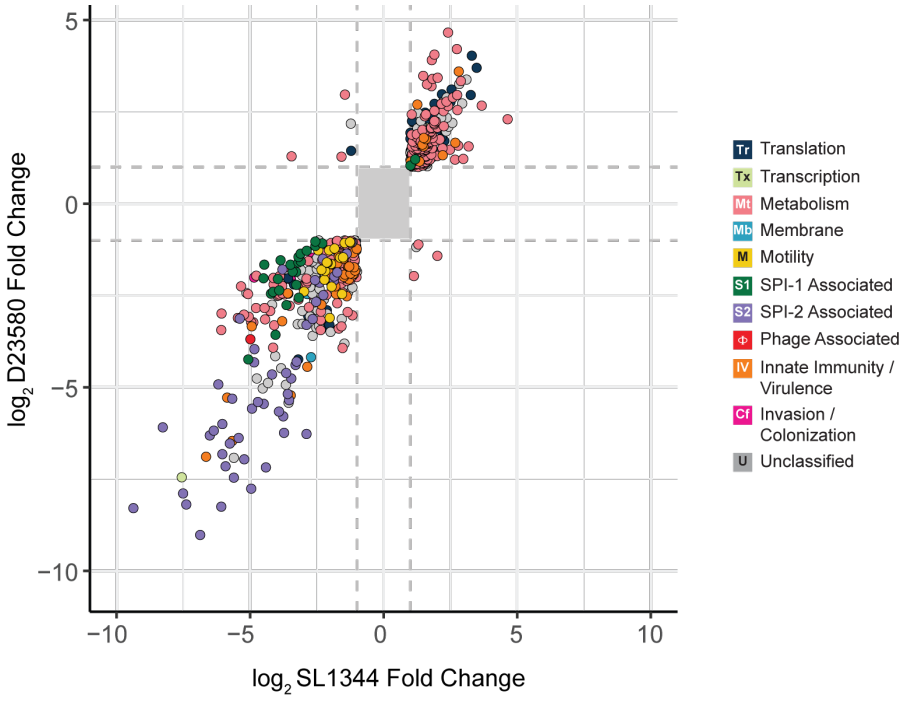
Figure 3.3


A
1
2
3
4
5
6
7
1. wt D23580
2. ΔrelA ΔspoT D23580
3. ΔrelA ΔspoT D23580 + flhD SL1344
4. ΔrelA ΔspoT D23580 + flhA SL1344
5. ΔrelA ΔspoT D23580 + flhB SL1344
6. ΔrelA ΔspoT D23580 + motA SL1344
7. ΔrelA ΔspoT D23580 + mcpC SL1344
B
Swimming diameter (cm)
ns
0
1
2
3
4
wt D23580
ΔrelA ΔspoT D23580
ΔrelA ΔspoT D23580 + flhDC SL1344
ΔrelA ΔspoT D23580 + flhA SL1344
ΔrelA ΔspoT D23580 + flhB SL1344
ΔrelA ΔspoT D23580 + motA SL1344
ΔrelA ΔspoT D23580 + mcpC SL1344

Supplementary Figures

Figure 3.S1. Distribution of genes differentially regulated by (p)ppGpp in *S.* Typhimurium SL1344 and D23580. Scatterplot shows 804 genes categorized by functional groups that were up- or down-regulated by at least 2-fold in the Δ*relA* Δ*spoT* mutant relative to wild type and have a q-value < 0.05 in both SL1344 and D23580.

Figure 3.S1


5
0
−5
−10
−10
−5
0
5
10
log2 D23580 Fold Change
log2 SL1344 Fold Change
Tr Translation
Tx Transcription
Mt Metabolism
Mb Membrane
M Motility
S1 SPI-1 Associated
S2 SPI-2 Associated
Φ Phage Associated
IV Innate Immunity / Virulence
Cf Invasion / Colonization
U Unclassified

Chapter IV – Discussion

Chapter IV – Discussion

The stringent response is a well-conserved signaling modality in bacteria that has primarily been associated with nutrient limitation, but many studies are beginning to unravel its impact on different aspects of cellular physiology including the regulation of host-bacterial interactions. This thesis describes the role of (p)ppGpp signaling in the pathogenesis of *Salmonella enterica* serovar Typhimurium. Here, I discuss the implications of our work and propose future areas of investigation that would enhance our understanding of the roles of (p)ppGpp at the host-pathogen interface.

Functional Roles of PpnN in Salmonella Typhimurium

The goal of Chapter II was to determine whether the stringent response is required for *Salmonella* evasion of the complement system, an important component of innate immunity (138). We established that SpoT-dependent production of (p)ppGpp is essential for bacterial survival in normal human serum, and identified that the regulation of the nucleotide hydrolase, PpnN, and biosynthesis of the LPS O-antigen, contributes to this phenotype (140). Based on previous work characterizing the function of PpnN in *Escherichia coli*, we confirmed that PpnN in *S.* Typhimurium hydrolyzes purine and pyrimidine nucleotide monophosphates to generate the corresponding nucleobases and ribose 5′-phosphate (139, 140, 163). In addition, deletion of *ppnN* or abrogation of its hydrolysis function through mutagenesis of the protein catalytic site resulted in a modest, but significant decrease in the survival of *S.* Typhimurium in human serum (140). These

data indicate that changes in metabolism are an important consideration upon the exposure of bacteria to infection-relevant conditions such as serum.

Our study would benefit from determining the mechanism of how the metabolic activity of PpnN contributes to complement resistance. Ribose 5´-phosphate is a common metabolite produced from the hydrolysis of purine and pyrimidine nucleotide monophosphates by PpnN. Previously, we considered that ribose 5´-phosphate could enter central carbon metabolism and trigger a downstream pathway that could confer resistance to complement killing. A systematic approach to investigate this would be to perform RNA-sequencing of wild type *S.* Typhimurium and a Δ*ppnN* mutant exposed to normal human serum and complement inactivated serum. An alternative strategy is to use untargeted metabolomics profiling, which may provide better resolution of the changes that may occur upon treatment of the bacteria with serum. These data may reveal metabolic pathways of interest that would warrant follow-up work.

Our data demonstrated that the deletion of *ppnN* in *S.* Typhimurium does not alter the synthesis of the LPS O-antigen or the protein composition of the outer membrane compared to the wild type strain (140). However, these experiments do not reveal whether PpnN contributes to outer membrane modifications that may protect the bacteria from complement recognition and ultimately, the formation of the membrane attack complex on the bacterial cell surface. LPS modifications, for example, are a well-established mechanism of complement evasion in pathogenic bacteria (216). Future work

could aim to use mass spectrometry to profile the outer membrane of a Δ*ppnN* mutant exposed to serum compared to wild type *S*. Typhimurium to determine whether PpnN contributes to modifying LPS.

The complement system is predominantly known as an extracellular host defense found in the intestinal lumen or in the blood suggesting that PpnN is important for *S*. Typhimurium virulence in either of these niches. However, (p)ppGpp is also required for the activation of the SsrA-SsrB two-component system that regulates genes associated with the intracellular lifestyle of *S*. Typhimurium (120, 136). Moreover, our RNA-sequencing data examining the regulatory landscape of SsrB showed that *ppnN* is up-regulated in an SsrB-dependent manner (14). Indeed, we confirmed using bioluminescence reporters that *ppnN* is significantly up-regulated in an Δ*ssrAB* mutant expressing a constitutively active variant of SsrB (D56E) relative to the Δ*ssrAB* mutant and wild type *S*. Typhimurium (**Fig. A1.1A**). We determined that SsrB-mediated regulation of *ppnN* occurs indirectly as gel shift assays demonstrated that the C-terminal DNA binding domain of SsrB (6HIS-SsrBc) did not bind to the promoter of *ppnN*, nor the negative control *phoH*, but it did bind to the promoter of *ssaG*, a SPI-2 encoded gene (**Fig. A1.1B**). Interestingly, *S*. Typhimurium lacking the SsrA-SsrB two component system was not more susceptible to killing in normal human serum (NHS) compared to the wild type strain (**Fig. A1.2**). These data suggest that SsrB-mediated regulation of *ppnN* likely does not have a role in protecting *S*. Typhimurium against complement in the intestinal lumen where there are basal levels of SsrB activity (217). Moreover, despite regulatory input from SsrB, a Δ*ppnN* mutant was

not attenuated in an acute mouse model of salmonellosis, nor did it display a replication defect in murine macrophage-like cells (**Fig. A1.3A, A1.3B**). As expected, however, an Δ*ssaR* mutant containing a lesion in the T3SS-2, showed reduced levels of intracellular replication. We hypothesized that the lack of an *in vivo* phenotype is likely due in part to the low levels of complement activity found in mice and showed that both wild type *S.* Typhimurium and the Δ*ppnN* mutant survived similarly in normal mouse serum (NMS) (**Fig. A1.3C**) (218). There is also a possibility that metabolic enzymes with similar functions to PpnN such as AMP nucleosidase (Amn) confounded our ability to assess its contributions to the intracellular lifestyle of *S.* Typhimurium and its effect on bacterial fitness within a host using a single deletion mutant (219). Thus, it may be worthwhile to perform similar experiments using a Δ*ppnN* mutant that overexpresses *ppnN*. These data may reveal another role for PpnN that allows *S.* Typhimurium to establish an intracellular niche in immune cells, in addition to mediating complement resistance.

Finally, it is also important to consider that PpnN contributes more prominently to another stringent response related phenotype such as antibiotic persistence. A Δ*ppnN* mutant in *E. coli* displays a significant fitness defect relative to wild type when the bacteria transition between nutrient rich and nutrient poor media (139). Slow growth is a characteristic feature of persister cell formation upon exposure to antibiotics (220). Consistent with these findings, an *E. coli* Δ*ppnN* mutant exposed to ofloxacin and ciprofloxacin produced significantly more persister cells than wild type and this could be restored to native levels by the expression of *ppnN in situ* (139). These data suggest that

the role of PpnN in *S*. Typhimurium persister biology should be investigated. Our collaboration with Dr. Yong E. Zhang at the University of Copenhagen will be essential in pursuing this work.

(p)ppGpp Signaling in Invasive Non-typhoidal Salmonella

In Chapter III, we compared the stringent response in invasive *S*. Typhimurium D23580 to *S*. Typhimurium SL1344 that causes acute gastrointestinal disease. Using RNA-sequencing, we identified 357 genes in SL1344, 364 genes in D23580, and 804 shared genes that are differentially regulated between the respective wild type strain and a (p)ppGpp-deficient mutant. Interestingly, our data revealed that the regulation of flagellar-based motility genes in D23580 was not dependent on (p)ppGpp compared to in SL1344. We validated these data using swimming motility assays and found the levels of flagellin protein to be consistent with our results. Although we began to investigate possible mechanisms accounting for the motility phenotype we observed in D23580, our findings are inconclusive and this remains the focus of follow up work. Our data highlights significant differences in the stringent response between the two strains of *S*. Typhimurium that warrant further examination.

One of the main focuses of future research will be to determine the mechanism accounting for the change in regulatory input of flagellar genes by (p)ppGpp in D23580. An important consideration to start with is whether the stringent response is regulated in a similar manner between SL1344 and D23580. Although our sequencing data for the

SL1344 and D23580 Δ*relA* Δ*spoT* mutant indicate that they are deficient in (p)ppGpp, a control to validate these strains is to measure the levels of (p)ppGpp. Previously, our collaborators measured (p)ppGpp levels in SL1344 under histidine starvation using thin layer chromatography (TLC) and showed that (p)ppGpp was undetectable in both the Δ*relA* and Δ*relA* Δ*spoT* mutant (**Fig. A2.4**). These data should be repeated with the strains grown to stationary phase in LB to mimic our experimental conditions, and by using high performance liquid chromatography (HPLC), which is a more robust technique for measuring metabolite abundance (221). A complementary set of experiments would be to test the activation of *relA* and *spoT* in SL1344 and D23580 under different growth conditions. Both the promoters and coding sequences of *relA* and *spoT* are conserved between the two strains of *S*. Typhimurium suggesting that they may be similarly regulated. However, it is possible that the regulation of the stringent response could be affected by differences in the core and accessory genomes between SL1344 and D23580, which would result in phenotypic differences. Our study also started to examine the SNPs in the flagellar cascade that were previously identified by genomic comparisons between ST19 and ST313 (**Table 3.1**) (24). Given that (p)ppGpp coordinates physiological changes in *S*. Typhimurium by binding to RNAP or effector proteins, we followed up on nine mutations in intergenic regions or non-synonymous mutations within gene coding sequences (82). I propose that each of the SNPs in D23580 should be engineered into the SL1344 Δ*relA* Δ*spoT* background and the motility of each strain should be tested in swimming assays. This approach will mitigate any confounding effects that other mutations in D23580 may have on bacterial motility. The SNPs should also be generated

in various combinations in the SL1344 Δ*relA* Δ*spoT* mutant to test for epistatic interactions between motility genes.

The goal of future work will also be to determine why ST313 has evolved away from (p)ppGpp-dependent activation of the flagellar cascade. The biosynthesis of flagella as well as fueling its function is energetically costly to bacteria. One hypothesis is that changes in the regulatory control of flagellar genes by (p)ppGpp contributes to conserving cellular resources to promote bacterial survival during stress. Competitive growth assays could be done to test the fitness of a D23580 Δ*relA* Δ*spoT* mutant compared to an SL1344 Δ*relA* Δ*spoT* mutant. Similar assays can also be performed to test the SNPs in the flagellar genes if one was identified to be responsible for the motility phenotype. Another possibility is that the level of flagellin expression in ST313 is sufficient for the bacteria to transit through the host environment and cause disease, and that additional regulatory input increases the likelihood of stimulating the immune system. Flagellin is a unique component of bacteria that can be recognized by host Toll-like receptor 5 (TLR5) and the NAIP/NLRC4 inflammasome during infection leading to the release of pro-inflammatory cytokines such as TNF-α and IL-1β (222, 223). To test whether the stringent response is involved in evading TLR5 or inflammasome activation in D23580, the levels of TNF-α and IL-1β can be measured using enzyme-linked immunosorbent assays (ELISAs) following infection of cultured epithelial cells and macrophages with wild type D23580 and the Δ*relA* Δ*spoT* mutant. In comparison to ST19, strains of ST313 exhibit a greater ability to evade host defenses, which is consistent with its systemic

lifestyle. Thus, these data may provide insight into the divergence in regulation of flagellar genes by (p)ppGpp between SL1344 and D23580.

Conclusions

Our understanding of the stringent response has greatly expanded since the discovery of (p)ppGpp 50 years ago. The enzymes that synthesize and degrade (p)ppGpp have been identified in nearly all species of bacteria, demonstrating their pervasive role in bacterial physiology. Although (p)ppGpp was initially characterized in mediating adaptation to nutrient stress, emerging research continues to highlight its role in regulating cellular homeostasis in response to diverse environmental stressors including infection relevant conditions. Our results support the role of (p)ppGpp as a major regulator of virulence gene expression in host-bacterial interactions. Moreover, the widespread regulatory functions of (p)ppGpp in bacteria have made it an attractive target for antimicrobial therapy. A more thorough investigation of (p)ppGpp signaling in the future may reveal new strategies to combat infections in the age of antibiotic resistance.

Appendix I

This appendix contains unpublished supplementary material pertaining to Chapter II.

Ethics Statement

Experimental Procedures

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Ethics Statement

Animal experiments were conducted according to guidelines set by the Canadian Council on Animal Care, using protocols approved by the Animal Review Ethics Board at McMaster University under Animal Use Protocol #17-03-10.

Experimental Procedures

Bacterial Strains and Growth Conditions

Salmonella enterica serovar Typhimurium (SL1344) and isogenic derivatives were used in the experiments in Appendix II and listed in **Table A1.1**. Plasmids are listed in **Table A1.2**. Bacteria were grown in LB or an acidic minimal medium containing low magnesium, low phosphate (LPM) (pH 5.8) for SPI-2 inducing conditions. Antibiotics were added where appropriate (streptomycin, 100 μg/mL; kanamycin, 50 μg/mL; ampicillin, 200 μg/mL) and strains were grown at 37°C with shaking.

Bioluminescence Reporter Assay

S. Typhimurium strains containing *lux*-transcriptional fusions were sub-cultured (1:50) and grown to mid-exponential phase in LB (OD_{600} = 0.4 to 0.5) then sub-cultured (1:50) again into LPM in black 96-well flat, clear bottom polystyrene plates (Corning). Plates were incubated at 37°C with shaking, and luminescence and A_{600} were measured every 1 h up to 5 h using the PerkinElmer® Plate Reader. Luminescence was normalized to A_{600}.

Protein Purification and Electrophoretic Mobility Shift Assays (EMSAs)

Purification of 6HIS-SsrBc using Ni^{2+}-NTA-agarose affinity chromatography, and EMSAs were performed as previously described (148). Briefly, ~100 ng of PCR amplified DNA fragments for *ppnN*, *ssaG*, and *phoH* was incubated with 6HIS-SsrBc for 20 min at room temperature in binding buffer containing 10 mM Tris pH 7.5, 50 mM KCl, 2.5% glycerol, 5 mM $MgCl_2$, and 0.05% Nonident P-40, and protein-DNA interactions were separated by electrophoresis on 6% non-denaturing acrylamide gels in 0.5 X Tris-borate-EDTA (TBE) buffer at room temperature. Gels were visualized with ethidium bromide on an Alpha-Imager UV transilluminator (Alpha Innotech Corp.).

Competitive Infection of Animals

Female C57BL/6 mice were infected by intraperitoneal injection with a mixed inoculum containing ~10^5 cfu of a chloramphenicol marked *S.* Typhimurium SL1344 strain (*ushA*::*cat*) and an unmarked mutant strain under investigation. The spleen and liver were harvested from mice at end-point (~72 h point-infection), which was defined as 15-20% body weight loss. Bacterial load was enumerated from each tissue by plating organ homogenates on LB-agar supplemented with the appropriate antibiotics. The competitive index (CI) was calculated on cfu as: (mutant /*ushA*::*cat*)$_{output}$/(mutant/ *ushA*::*cat*)$_{input}$.

Macrophage Infections

RAW264.7 cell infections were performed in triplicate for each strain of interest in 24-well plates. Twice the amount of wells was infected to measure bacterial cfus at time 2 h

(T_2) and 20 h (T_{20}) post-infection. Bacteria grown overnight in LB were harvested and normalized to an OD_{600} of 1.0 then opsonized in 20% normal human serum for 30 min at 37°C prior to infection. RAW cells were infected at an MOI of 50:1 and the plates were spun down for 2 min at 500 *g* to facilitate bacterial attachment to host cells then incubated for 30 min at 37°C to allow phagocytic uptake. Following incubation, cells were treated with DMEM containing 10% fetal bovine serum (FBS) and 100 μg/mL gentamicin for 1.5 h to kill extracellular bacteria, washed three times with 1 X phosphate buffered saline (PBS), then lysed with 1 X PBS containing 1% triton X-100 and 0.1% SDS. The remaining infected wells were treated with 12 μg/mL gentamicin and incubated at 37°C overnight. Cells were washed three times with 1 X PBS the next day and lysed with 1 X PBS containing 1% triton X-100 and 0.1% SDS. Cell lysates were serial diluted and spot plated onto LB agar supplemented with 100 μg/mL streptomycin. Percent replication was calculated as the cfu/mL at T_{20} relative to T_2 of each strain compared to wild type.

Serum Bactericidal Assays

Bacteria grown overnight in LB medium were harvested and normalized to an OD_{600} of 0.5. Cells were washed and resuspended in PBS and diluted (1:10) further for the assay. The equivalent OD_{600} of 0.005 of each strain was incubated in 90% pooled normal human serum (Innovative Research) or C57BL/6 mouse serum (Innovative Research) at 37°C. Serum treated with 5 units/mL of cobra venom factor (Quidel) for 30 min at 37°C was used as a negative control for each strain. The number of viable bacteria was determined by plating on LB agar supplemented with 200 μg/mL of ampicillin or 50 μg/mL

kanamycin to select for strains carrying the pGEN-MCS or pWSK129 vectors, respectively. Percent survival was calculated as the cfu/mL at 30 min relative to 0 min.

Measurement of (p)ppGpp by Autoradiography

The L-histidine auxotrophy of *S.* Typhimurium SL1344 was used to elicit the stringent response. Wild type *S.* Typhimurium, Δ*relA,* and Δ*relA ΔspoT* were grown to mid-logarithmic phase (OD_{600} = 0.4) in MOPS medium with 0.2 mM K_2HPO_4 and supplemented with 20 amino acids then diluted in fresh MOPS media to an OD_{600} of 0.05 for the assay (224). 100 μCi/mL $H_3{}^{32}PO_4$ (PerkinElmer®) was added to the cultures and then the cells were incubated at 37°C with mixing at 600 rpm for 35 min in a ThermoMixer® (Eppendorf). Following incubation, 40 μL of each culture was removed and mixed with 8 μl of ice-cold 2 N formic acid. These are the time zero unstressed samples. The remaining cultures were spun down and the cells were resuspended with the same volume of fresh pre-warmed MOPS medium, but without L-histidine to elicit starvation and (p)ppGpp production by *S.* Typhimurium. After incubation at 37°C in the ThermoMixer® for 5 min, 40 μL of each culture was taken and mixed with formic acid as above. The samples were stored at -20°C and spun down at 14000 rpm for 2 min upon thawing for downstream applications. 5 μL of supernatant from the samples were resolved by Thin Layer Chromatography (TLC, PEI Cellulose F, MERKE) with the use of 1.5 M K_2PO_4, pH 3.4 as the mobile phase (139). The TLC plate was visualized with a Typhoon FLA 7000 (GE).

Statistical Analysis

Data were analyzed where possible using GraphPad Prism 5.0a software (GraphPad Inc., San Diego, CA) using one-way ANOVA. *P* values of <0.05 were considered significant.

Table A1.1. Strains used in the study.

Strain	**Source or reference**
Wild type *S*. Typhimurium str. SL1344	Coombes *et al*. 2003 (198)
Δ*ssrAB S*. Typhimurium str. SL1344	Mulder *et al.* 2015 (59)
Δ*ppnN S*. Typhimurium str. SL1344	Chau *et al.* 2021 (140)
TOP10 *E. coli*	Invitrogen

Table A1.2. Plasmids used in the study.

Plasmid	Description	Source or reference
pGEN-MCS	Low-copy number cloning vector	Lane *et al.* 2007 (192)
pGEN-*luxCDABE*	Lux transcriptional reporter plasmid	Lane *et al.* 2007 (192)
pWSK129	Low-copy number cloning vector	Wang and Kushner, 1991 (200)
pWSK129-*ssrB* (D56E)	*ssrB* with the D56E mutation cloned into pWSK129 under its native promoter located upstream of *ssrA* for complementation experiments	Pérez-Morales *et al.* 2017 (148)
pGEN-MCS-*ppnN*	*ppnN* with 800 bp upstream of the coding sequence cloned into pGEN-MCS for complementation experiments	Chau *et al.* 2021 (140)
pGEN-*luxCDABE-ppnN*	Lux transcriptional reporter for *ppnN* promoter	Chau *et al.* 2021 (140)
pGEN-*luxCDABE-sseA*	Lux transcriptional reporter for *sseA* promoter	Osborne *et al.* 2011 (217)

Supplementary Figures

Figure A1.1. *ppnN* is indirectly regulated by SsrB. Transcriptional reporter of the full-length (**A**) *ppnN* and (**B**) *sseA* promoter, a canonical SPI-2 associated gene target, showed SsrB-dependent up-regulation. Strains are carrying the empty pWSK129 vector control unless otherwise specified. ns, not significant; ***, $P < 0.0001$. Electrophoretic mobility shift assay (EMSA) of SsrBc incubated with (**C**) P*ppnN* or (**D**) P*ssaG*. P*phoN* was included as a negative control on each gel. Protein-DNA complexes are indicated with an asterisk.

Figure A1.1

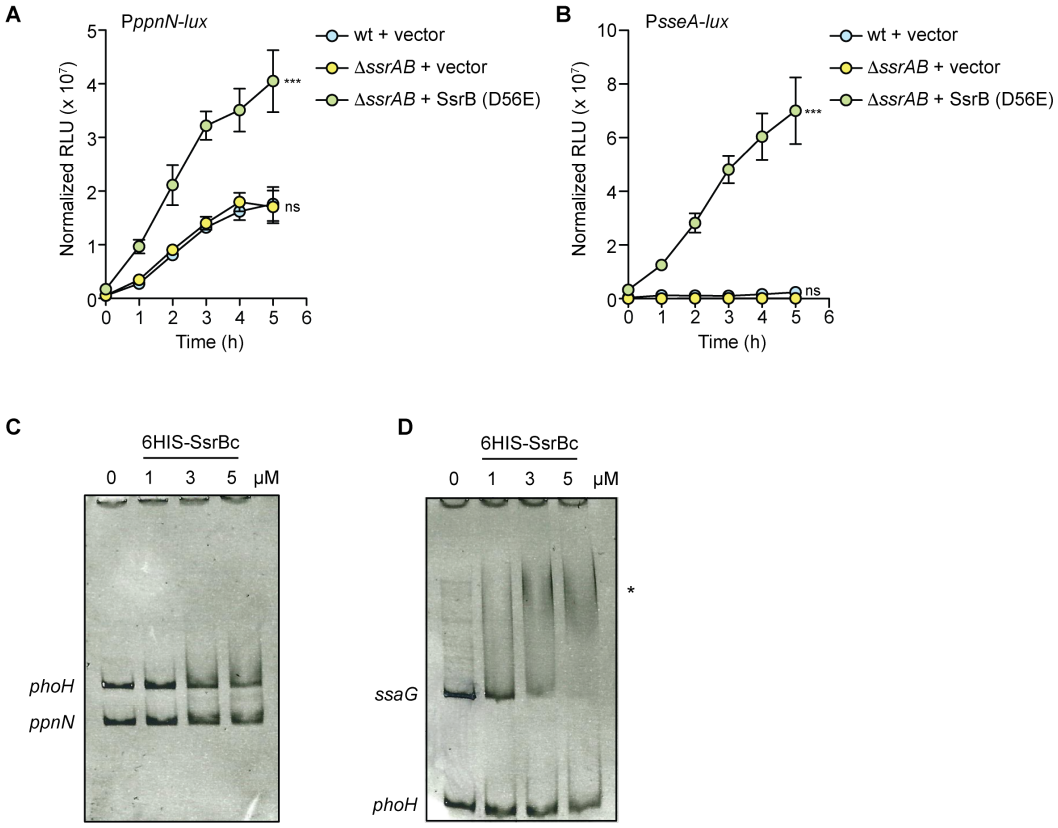

A
PppnN-lux
wt + vector
ΔssrAB + vector
ΔssrAB + SsrB (D56E)
Normalized RLU (x 10^7)
Time (h)
***
ns
B
PsseA-lux
wt + vector
ΔssrAB + vector
ΔssrAB + SsrB (D56E)
Normalized RLU (x 10^7)
Time (h)
***
ns
C
6HIS-SsrBc
0 1 3 5 μM
phoH
ppnN
D
6HIS-SsrBc
0 1 3 5 μM
*
ssaG
phoH

Figure A1.2. PpnN is dispensable for *S.* Typhimurium within-host fitness and survival in mouse serum. (**A**) C57BL/6 mice were infected with an equal mixture of *S.* Typhimurium marked with a Cm cassette (*ushA*::*cat*) and a Δ*ppnN* mutant. Competitive indices were determined for the mutant in the spleen and liver 3 days post-infection. Each data point represents one animal, and horizontal bars indicate geometric means. Data are from two independent experiments. (**B**) RAW264.7 cells were infected with wild type *S.* Typhimurium, an Δ*ssaR* mutant, or a Δ*ppnN* mutant. Percent replication was calculated as the cfu/mL at 2 h relative to 20 h post-infection of each strain relative to wild type. Data are the means ± SEM (error bars) of two independent experiments. (**C**) Δ*ppnN* is not susceptible to killing in normal mouse serum (NMS) isolated from C57BL/6 mice. Strains are carrying the empty pGEN-MCS vector control unless otherwise specified. Percent survival is depicted as the number of viable bacteria at 30 min relative to 0 min. Data are the means ± SEM (error bars) of two independent experiments.

Figure A1.2

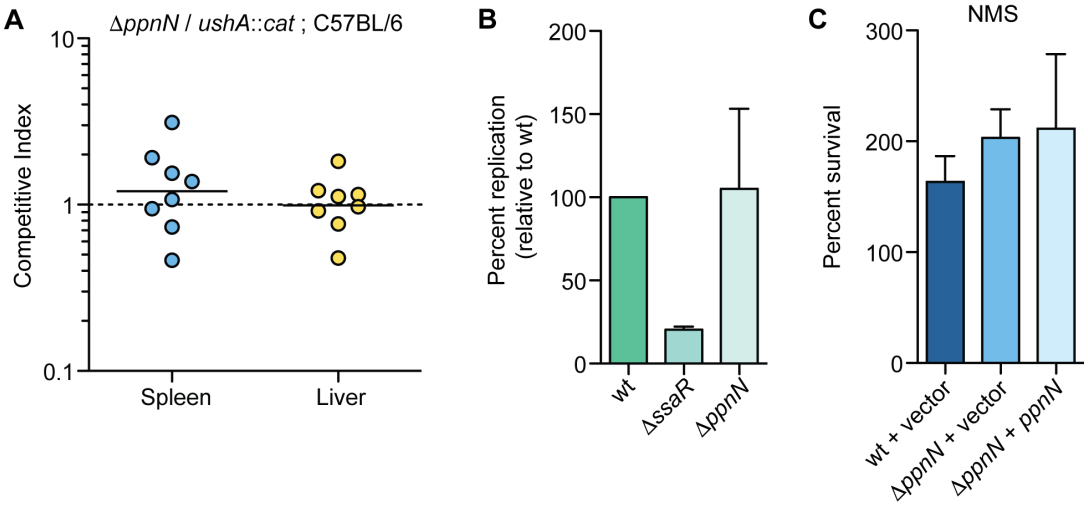

A
ΔppnN / ushA::cat ; C57BL/6
Competitive Index
10
1
0.1
Spleen
Liver
B
Percent replication (relative to wt)
200
150
100
50
0
wt
ΔssaR
ΔppnN
C
NMS
Percent survival
300
200
100
0
wt + vector
ΔppnN + vector
ΔppnN + ppnN

Figure A1.3. SsrB is not required for complement resistance in *S*. Typhimurium. Δ*ssrAB* is not susceptible to killing in normal human serum (NHS). Serum was pre-treated with cobra venom factor (CVF) as a control. Strains are carrying the empty pWSK129 vector control unless otherwise specified. Percent survival is depicted as the number of viable bacteria at 30 min relative to 0 min. Data are the means ± SEM (error bars) of three independent experiments. ns, not significant.

Figure A1.3

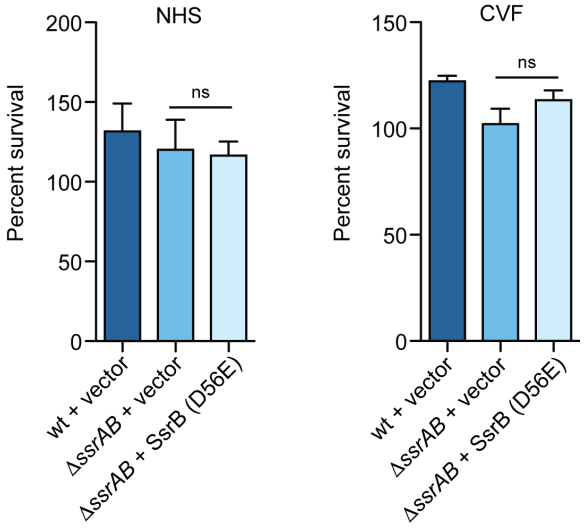
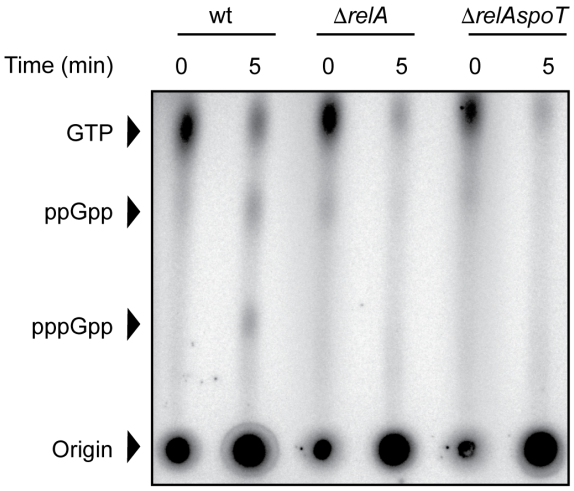

NHS
CVF
Percent survival
200
150
100
50
0
ns
wt + vector
ΔssrAB + vector
ΔssrAB + SsrB (D56E)

Figure A1.4. *S.* Typhimurium str. SL1344 Δ*relA* and Δ*relA* Δ*spoT* mutants starved for histidine are deficient in (p)ppGpp. Thin-layer chromatogram with ^{32}P-labelled nucleotides from wild type *S.* Typhimurium, Δ*relA*, and Δ*relA* Δ*spoT* starved for L-histidine in defined MOPS minimal medium.

Figure A1.4



Appendix II

This appendix contains unpublished supplementary material pertaining to Chapter III.

List of Tables

Table A2.1. *S.* Typhimurium str. SL1344 specific genes differentially regulated by (p)ppGpp.

Locus Tag	Gene Name	F.C.	q-value	Locus Tag	Gene Name	F.C.	q-value
SL1344_0994		-5.00	7.84E-04	**SL1344_2582**		-1.15	2.31E-02
SL1344_1794		-4.50	1.04E-02	**SL1344_0485**		-1.15	5.21E-03
SL1344_0698		-4.27	1.80E-02	**SL1344_1885**	*fliA*	-1.15	3.30E-03
SL1344_1795		-4.11	1.85E-05	**SL1344_2259**	*yfaV*	-1.14	9.25E-03
SL1344_0538	*fimC*	-3.89	1.09E-08	**SL1344_1404**	*ompN*	-1.13	2.45E-02
SL1344_1602		-3.89	4.82E-44	**SL1344_1729**	*cvrA/ycgO*	-1.13	8.13E-05
SL1344_4248		-3.74	8.67E-10	**SL1344_3307**	*yhcH*	-1.13	4.27E-03
SL1344_RS14910		-3.63	8.64E-06	**SL1344_1066**		-1.13	4.77E-02
SL1344_4521	*sthA*	-3.60	4.14E-02	**SL1344_1471**		-1.12	1.55E-02
SL1344_0974		-3.50	1.34E-02	**SL1344_1462**	*hyaF2*	-1.12	1.91E-03
SL1344_1793	*pagO*	-3.46	5.49E-10	**SL1344_3779**	*ccmH1*	-1.12	3.30E-02
SL1344_3099		-3.33	2.65E-03	**SL1344_2009**		-1.11	2.35E-02
SL1344_0539	*fimD*	-3.22	2.14E-08	**SL1344_2696**		-1.10	9.42E-03
SL1344_0540	*fimH*	-3.17	1.76E-06	**SL1344_0737**	*aroG*	-1.10	7.13E-06
SL1344_2674	*sopE*	-3.15	2.93E-05	**SL1344_1478**		-1.10	1.06E-02
SL1344_1597		-3.08	3.99E-02	**SL1344_3861**	*ilvG*	-1.10	3.57E-02
SL1344_2675		-3.05	2.34E-10	**SL1344_4244**		-1.10	4.02E-03
SL1344_0354		-3.03	3.61E-10	**SL1344_1313**	*orf48*	-1.10	4.99E-03
SL1344_4135		-2.93	1.39E-02	**SL1344_1903**	*fliK*	-1.09	2.47E-02
SL1344_1070	*nanT*	-2.91	3.56E-03	**SL1344_1857**	*motB*	-1.09	9.20E-03
SL1344_4395	*argF*	-2.73	5.03E-09	**SL1344_1231**	*ydjA*	-1.09	1.16E-04
SL1344_2576		-2.63	1.51E-03	**SL1344_3498**		-1.09	4.28E-02
SL1344_1873		-2.59	9.63E-21	**SL1344_2990**		-1.09	1.51E-03
SL1344_2568		-2.58	1.94E-05	**SL1344_3919**	*metE*	-1.09	3.65E-03
SL1344_2867	*spaR*	-2.57	2.44E-04	**SL1344_2553**		-1.08	4.91E-02
SL1344_2814	*srlB*	-2.50	2.15E-02	**SL1344_2309**		-1.08	4.24E-03
SL1344_0340		-2.39	3.25E-03	**SL1344_1858**	*motA*	-1.08	1.59E-02
SL1344_RS20070	*ilvL*	-2.36	2.18E-04	**SL1344_0128**	*murG*	-1.08	4.62E-05
SL1344_2874	*spaK*	-2.33	3.25E-04	**SL1344_1809**	*yobA*	-1.08	3.93E-04
SL1344_2872	*spaM*	-2.32	5.07E-04	**SL1344_3424**	*kefB*	-1.07	4.77E-03
SL1344_0991	*sseI*	-2.31	2.28E-03	**SL1344_1260**	*ghoS*	-1.07	4.19E-03
SL1344_1800	*mig-3*	-2.28	7.44E-06	**SL1344_0760**	*modC*	-1.07	1.61E-02
SL1344_1477		-2.25	1.17E-08	**SL1344_3494**		-1.06	7.17E-04
SL1344_2283	*cheV*	-2.23	2.45E-07	**SL1344_3921**	*dlhH/ysgA*	-1.06	2.55E-04
SL1344_2875	*invA*	-2.22	6.55E-04	**SL1344_1266**	*rfc/wzy*	-1.06	5.73E-03
SL1344_3130	*yqhC*	-2.21	9.32E-05	**SL1344_1740**	*ycgM*	-1.05	6.10E-04
SL1344_0963		-2.17	2.55E-02	**SL1344_0440**	*bolA*	-1.05	1.54E-02
SL1344_0975		-2.14	9.05E-03	**SL1344_2192**	*rtn*	-1.05	1.69E-03
SL1344_2851	*prgK*	-2.11	5.28E-03	**SL1344_2597**	*recEb*	-1.05	1.37E-02
SL1344_2581		-2.10	2.14E-02	**SL1344_1983**		-1.05	4.67E-02
SL1344_0490		-2.09	1.35E-02	**SL1344_0129**	*murC*	-1.04	1.30E-05
SL1344_0973		-2.06	2.56E-02	**SL1344_3723**	*misL*	-1.04	2.54E-02
SL1344_0638		-2.05	2.43E-02	**SL1344_1808**	*copD*	-1.04	2.74E-04
SL1344_4252		-2.05	9.89E-04	**SL1344_1546**		-1.04	2.73E-02
SL1344_2855	*hilD*	-2.04	7.05E-05	**SL1344_1085**	*ymdC*	-1.04	1.09E-03
SL1344_1802		-2.00	2.18E-02	**SL1344_3499**		-1.03	3.16E-02
SL1344_2850	*orgA*	-1.97	2.43E-03	**SL1344_0002**	*thrA*	-1.03	5.58E-03
SL1344_0541	*sfmF*	-1.97	7.19E-03	**SL1344_4161**	*yjbH*	-1.03	1.86E-02
SL1344_4158	*yjbE*	-1.95	1.49E-04	**SL1344_4265**	*yjeH*	-1.03	1.89E-02
SL1344_0966	*nucD*	-1.95	3.66E-02	**SL1344_3308**	*nanK*	-1.03	4.24E-02
SL1344_RS14085		-1.94	4.89E-09	**SL1344_1202**		-1.02	3.97E-03
SL1344_1264		-1.93	5.70E-03	**SL1344_3206**	*yhaH*	-1.02	3.29E-04
SL1344_2854	*prgH*	-1.91	6.42E-03	**SL1344_3750**		-1.02	3.94E-02
SL1344_4235	*melA*	-1.90	3.85E-04	**SL1344_1056**	*agp*	-1.01	1.10E-04
SL1344_2876	*invE*	-1.90	8.29E-04	**SL1344_1212**		-1.01	1.34E-02
SL1344_4180	*pspG*	-1.89	5.96E-03	**SL1344_0872**		-1.01	1.03E-02
SL1344_3956		-1.89	2.73E-02	**SL1344_3997**	*rhaS*	-1.00	6.97E-03
SL1344_2595		-1.89	2.62E-03	**SL1344_3356**	*prmA*	1.00	3.87E-02
SL1344_2510	*asrA*	-1.87	3.15E-04	**SL1344_0633**	*nadD*	1.00	5.25E-03
SL1344_4393		-1.85	4.09E-03	**SL1344_1910**	*fliR*	1.00	4.65E-02
SL1344_3624	*yiaB*	-1.81	7.50E-03	**SL1344_1568**		1.00	1.24E-02
SL1344_RS20565		-1.78	2.02E-06	**SL1344_4038**	*rraA*	1.00	8.83E-04
SL1344_4205	*yjcE*	-1.77	1.99E-04	**SL1344_1834**	*yebC*	1.00	6.75E-05
SL1344_0989		-1.77	4.84E-02	**SL1344_1366**	*rnt*	1.00	4.72E-04
SL1344_1731	*dadO*	-1.77	4.82E-04	**SL1344_0858**		1.00	1.77E-02
SL1344_2698		-1.76	3.81E-03	**SL1344_3616**	*cueP*	1.01	3.52E-02
SL1344_0738		-1.75	1.16E-03	**SL1344_3050**		1.01	4.59E-02
SL1344_2991	*lysA*	-1.73	2.57E-02	**SL1344_2132**	*metG*	1.02	4.67E-06
SL1344_2569		-1.72	4.32E-05	**SL1344_3668**	*grxC*	1.02	1.25E-04
SL1344_4247		-1.72	4.78E-03	**SL1344_1002**	*uup*	1.03	1.68E-03

SL1344_2530	*tadA*	-1.71	2.77E-04	**SL1344_2907**	*truD*	1.03	4.34E-05
SL1344_2586		-1.71	4.50E-02	**SL1344_3804**	*dnaN*	1.03	1.07E-03
SL1344_2710		-1.70	1.88E-03	**SL1344_3259**	*infB*	1.03	1.53E-05
SL1344_2592	*clla*	-1.69	4.03E-02	**SL1344_3053**	*yggG*	1.03	1.47E-03
SL1344_2815	*srlD*	-1.68	9.93E-03	**SL1344_3669**	*yibN*	1.03	1.51E-03
SL1344_4236	*melB*	-1.66	9.05E-05	**SL1344_3153**	*mdaB*	1.03	7.98E-03
SL1344_0739		-1.66	3.59E-04	**SL1344_0187**	*dksA*	1.04	8.52E-05
SL1344_4396	*arcC*	-1.66	6.80E-05	**SL1344_4281**	*epmA*	1.04	5.06E-03
SL1344_2078	*wcaK*	-1.66	4.60E-02	**SL1344_1207**	*yoaG*	1.04	2.98E-02
SL1344_2849	*orgB*	-1.66	7.45E-03	**SL1344_4406**	*holC*	1.05	8.35E-03
SL1344_2564		-1.64	1.34E-03	**SL1344_1153**	*mfd*	1.05	8.19E-07
SL1344_1482		-1.63	5.25E-03	**SL1344_3313**	*sspB*	1.05	1.44E-04
SL1344_0971		-1.63	1.97E-03	**SL1344_3256**	*rpsO*	1.05	3.18E-04
SL1344_3521	*ugpE*	-1.63	8.82E-03	**SL1344_1123**	*rluC*	1.05	6.19E-03
SL1344_3619	*yafP*	-1.61	1.44E-02	**SL1344_2931**	*eno*	1.06	3.39E-02
SL1344_2102	*yegD*	-1.58	4.02E-02	**SL1344_2075**	*galF*	1.06	8.53E-05
SL1344_3303	*gltD*	-1.58	2.29E-04	**SL1344_1697**	*ychN*	1.06	7.22E-04
SL1344_2406	*cysT*	-1.58	1.18E-02	**SL1344_3661**		1.06	1.70E-03
SL1344_RS23405		-1.57	2.17E-08	**SL1344_0717**	*sdhB*	1.06	4.33E-02
SL1344_1770		-1.57	2.95E-03	**SL1344_3271**	*greA*	1.07	2.88E-04
SL1344_2747		-1.57	7.22E-04	**SL1344_3260**	*nusA*	1.07	1.64E-04
SL1344_2845	*avrA*	-1.55	4.28E-02	**SL1344_0730**	*tolB*	1.07	2.45E-07
SL1344_1902	*fliJ*	-1.54	2.19E-03	**SL1344_2946**	*yqcC*	1.07	3.97E-02
SL1344_4394		-1.54	1.66E-05	**SL1344_0780**	*moaC*	1.08	1.87E-03
SL1344_0520	*allC*	-1.53	1.97E-03	**SL1344_2204**	*yejL*	1.08	1.91E-03
SL1344_4111	*zraP*	-1.53	2.47E-02	**SL1344_0530**	*cysS*	1.08	3.14E-06
SL1344_1658	*trpA*	-1.52	2.55E-04	**SL1344_1774**	*prc*	1.08	3.97E-03
SL1344_0197	*stfC*	-1.52	2.11E-02	**SL1344_0241**	*arfB*	1.09	7.38E-03
SL1344_1040		-1.51	2.79E-02	**SL1344_3824**	*pstS*	1.09	1.89E-03
SL1344_1861	*uspC*	-1.51	1.33E-03	**SL1344_1165**		1.10	1.44E-02
SL1344_2008		-1.50	1.19E-04	**SL1344_3452**	*damX*	1.10	2.02E-06
SL1344_1992	*cobT*	-1.50	4.05E-05	**SL1344_3955**	*typA*	1.10	2.36E-04
SL1344_3190	*aer*	-1.49	8.41E-03	**SL1344_1310**	*lppB*	1.11	1.24E-02
SL1344_1996	*cbiO*	-1.49	1.31E-08	**SL1344_1683**	*galU*	1.11	4.04E-04
SL1344_2881		-1.49	4.52E-02	**SL1344_3450**	*rpe*	1.11	4.22E-05
SL1344_2365	*pgtB*	-1.48	8.01E-06	**SL1344_0482**	*hemH*	1.12	4.05E-04
SL1344_4517	*creD*	-1.48	2.47E-03	**SL1344_0849**	*ybjC*	1.12	1.74E-02
SL1344_0748		-1.46	3.74E-02	**SL1344_3951**	*hemN*	1.13	3.88E-06
SL1344_2001	*cbiK*	-1.46	1.05E-05	**SL1344_3562**	*gorA*	1.13	1.77E-05
SL1344_3748		-1.46	3.00E-03	**SL1344_3683**	*rfaJ*	1.14	1.60E-03
SL1344_RS13645		-1.45	4.75E-02	**SL1344_0471**	*mscK*	1.14	2.38E-03
SL1344_2856	*hilA*	-1.45	1.72E-03	**SL1344_1869**	*yecH*	1.15	2.69E-02
SL1344_2407	*cysP*	-1.45	1.24E-02	**SL1344_3054**	*speB*	1.15	3.24E-04
SL1344_2624	*raiA/yfiA*	-1.45	2.77E-04	**SL1344_4023**	*lsrA*	1.16	3.88E-03
SL1344_0972		-1.45	1.09E-05	**SL1344_3682**	*rfaY*	1.16	1.67E-03
SL1344_1645	*cysB*	-1.44	2.84E-04	**SL1344_1763**	*mntP*	1.18	9.97E-03
SL1344_1083		-1.44	2.06E-02	**SL1344_0173**	*yadG*	1.19	2.50E-04
SL1344_RS09290		-1.43	1.88E-05	**SL1344_3681**	*waaZ*	1.19	2.00E-03
SL1344_2154		-1.42	1.12E-02	**SL1344_1160**	*potC*	1.20	4.31E-03
SL1344_3552	*yhiI*	-1.42	1.74E-03	**SL1344_0821**	*moeB*	1.21	5.74E-04
SL1344_1543		-1.41	3.87E-02	**SL1344_2236**	*apbE*	1.21	7.45E-03
SL1344_2165	*mglC*	-1.41	3.97E-03	**SL1344_0757**	*ybhT*	1.21	2.94E-04
SL1344_4345		-1.40	2.60E-02	**SL1344_0763**	*ybhC*	1.21	1.58E-06
SL1344_3959		-1.40	1.48E-02	**SL1344_2932**	*pyrG*	1.22	5.31E-05
SL1344_2567		-1.40	2.99E-04	**SL1344_2392**	*cysZ*	1.24	6.83E-04
SL1344_1251	*celF*	-1.39	2.37E-02	**SL1344_4232**	*adiY*	1.24	2.28E-04
SL1344_2857	*iagB*	-1.39	4.76E-03	**SL1344_3930**	*tatD*	1.25	1.12E-03
SL1344_2725		-1.39	7.31E-04	**SL1344_2394**	*ptsH*	1.25	2.43E-02
SL1344_2572		-1.39	1.74E-02	**SL1344_0729**	*tolA*	1.25	5.70E-04
SL1344_0980		-1.38	1.12E-03	**SL1344_3280**	*ibaG*	1.26	4.52E-03
SL1344_1458		-1.37	4.43E-02	**SL1344_3906**	*corA*	1.27	7.19E-04
SL1344_3551	*rbbA*	-1.37	1.01E-04	**SL1344_0775**	*uvrB*	1.27	2.49E-07
SL1344_0777	*yvcK*	-1.35	9.01E-06	**SL1344_0419**	*thiI*	1.27	1.37E-05
SL1344_2769	*tctD*	-1.34	2.95E-02	**SL1344_4166**	*malE*	1.29	4.77E-02
SL1344_3197	*yqjT*	-1.34	5.13E-03	**SL1344_1459**	*ompC*	1.29	4.05E-02
SL1344_1901	*fliI*	-1.33	1.19E-02	**SL1344_0243**	*proS*	1.30	3.70E-07
SL1344_2844	*sitD*	-1.33	2.09E-02	**SL1344_0901**	*serS*	1.30	7.38E-07
SL1344_2730		-1.32	7.15E-03	**SL1344_4487**	*yjjG*	1.31	2.65E-04
SL1344_2261	*yfaX*	-1.31	4.07E-02	**SL1344_3762**	*ilvB*	1.31	6.20E-03
SL1344_1900	*fliH*	-1.31	2.42E-02	**SL1344_3685**	*waaB*	1.31	4.05E-05
SL1344_1446	*ydeE*	-1.30	1.27E-02	**SL1344_3148**	*parC*	1.32	2.70E-06
SL1344_2768	*tctE*	-1.30	3.83E-03	**SL1344_2612**	*yfiF*	1.33	1.98E-06
SL1344_2993		-1.30	4.16E-04	**SL1344_1032**		1.34	1.38E-04
SL1344_0964		-1.29	2.82E-03	**SL1344_1099**	*dinI*	1.34	1.67E-02
SL1344_3747		-1.28	1.52E-02	**SL1344_2809**	*recA*	1.36	2.68E-06
SL1344_4344	*ytfP*	-1.28	6.50E-05	**SL1344_3017**	*prfB*	1.36	2.09E-07

SL1344_2713		-1.27	2.37E-02	**SL1344_3222**	*garL*	1.37	3.02E-03
SL1344_1464	*hypC*	-1.27	2.38E-02	**SL1344_3687**	*rfaP*	1.37	2.05E-05
SL1344_2140	*yehW*	-1.26	6.22E-04	**SL1344_1527**	*ydcX*	1.37	3.15E-04
SL1344_1469		-1.25	1.39E-04	**SL1344_1379**	*mliC*	1.38	2.28E-04
SL1344_2152		-1.25	1.25E-02	**SL1344_4168**	*lamB*	1.39	2.79E-02
SL1344_1890	*fliS*	-1.24	6.92E-03	**SL1344_3611**	*yiaD*	1.41	1.14E-07
SL1344_0969		-1.24	5.52E-03	**SL1344_3389**	*rpmD*	1.42	1.12E-04
SL1344_1463	*hyaE2*	-1.23	4.84E-02	**SL1344_2959**	*fucR*	1.43	3.06E-05
SL1344_0124	*murF*	-1.23	5.00E-05	**SL1344_4425**	*brxC*	1.44	1.55E-06
SL1344_2953	*fucO*	-1.23	1.23E-02	**SL1344_1053**	*scsB*	1.44	4.85E-02
SL1344_2447		-1.23	1.48E-02	**SL1344_1681**	*tdk*	1.45	1.25E-04
SL1344_4203		-1.23	4.75E-04	**SL1344_2058**	*gndA*	1.47	5.01E-08
SL1344_0593	*ybdN*	-1.23	3.62E-02	**SL1344_3051**		1.47	2.56E-02
SL1344_1572	*azoR*	-1.22	1.39E-02	**SL1344_2439**	*ypfG*	1.48	1.65E-03
SL1344_2772		-1.22	2.39E-03	**SL1344_4022**	*lsrR*	1.51	8.73E-07
SL1344_1084	*ymdB*	-1.22	5.61E-03	**SL1344_3016**	*lysS*	1.52	6.93E-10
SL1344_2597A		-1.22	3.27E-02	**SL1344_2070**	*rfbI*	1.54	1.17E-04
SL1344_2879	*invH*	-1.22	1.18E-02	**SL1344_0153**	*aceF*	1.55	1.79E-07
SL1344_RS13405		-1.21	1.52E-03	**SL1344_1031**	*orfX*	1.58	4.97E-08
SL1344_1879	*sdiA*	-1.21	1.08E-02	**SL1344_3704**		1.63	7.66E-05
SL1344_2899		-1.21	1.63E-02	**SL1344_0805**	*glnH*	1.63	7.04E-08
SL1344_1872		-1.20	9.92E-05	**SL1344_1159**	*potD*	1.65	3.32E-09
SL1344_2163		-1.20	7.99E-04	**SL1344_2480**	*yfgJ*	1.65	3.58E-02
SL1344_2767	*nixA*	-1.20	2.38E-02	**SL1344_2708**	*cI*	1.69	6.66E-06
SL1344_2531	*yfhb*	-1.19	3.11E-04	**SL1344_2810**	*pncC*	1.69	6.04E-08
SL1344_3427		-1.19	7.10E-04	**SL1344_1192**		1.72	3.25E-03
SL1344_2662		-1.18	1.53E-03	**SL1344_3728**	*mgtA*	1.76	8.13E-03
SL1344_2787		-1.18	5.55E-03	**SL1344_2785**	*ygaC*	1.78	4.02E-08
SL1344_0881	*macB*	-1.16	4.73E-05	**SL1344_3729**	*mgtC*	1.81	1.25E-02
SL1344_1891	*fliT*	-1.16	2.54E-03	**SL1344_1193**		1.94	2.24E-02
SL1344_RS19550	*tisB*	-1.16	9.48E-03	**SL1344_3413**	*fusA*	2.15	4.57E-13
SL1344_0109A	*sgrT*	-1.15	3.10E-03	**SL1344_3615**	*cspA*	2.26	2.83E-04
SL1344_3656	*sadA*	-1.15	3.06E-04				

[1]New locus tags SL1344_RS##### are used where an old locus tag is unavailable.
[2]F.C. is the $\log_2$ Fold Change (Δ*relA* Δ*spoT* / wild type).
[3]Blue represents down-regulated genes.
[4]Red represents up-regulated genes.

Table A2.2. *S.* Typhimurium str. D23580 specific genes differentially regulated by (p)ppGpp.

Locus Tag	Gene Name	F.C.	q-value	Locus Tag	Gene Name	F.C.	q-value
STMMW_RS24830		-5.66	1.84E-11	**STMMW_18421**	*pphA*	-1.15	3.51E-03
STMMW_16931	*steC*	-5.17	8.30E-43	**STMMW_43791**	*yjgA*	-1.15	2.60E-05
STMMW_27451		-5.06	2.25E-56	**STMMW_13121**	*astD*	-1.15	1.10E-02
STMMW_23821		-4.45	8.12E-47	**STMMW_15391**		-1.14	8.06E-08
STMMW_RS25575		-4.36	6.08E-58	**STMMW_14671**	*ydgA*	-1.13	2.94E-05
STMMW_31261		-4.36	7.24E-03	**STMMW_19751**	*cspB*	-1.13	2.41E-03
STMMW_03161		-4.35	1.27E-03	**STMMW_16461**	*nifJ*	-1.12	3.26E-08
STMMW_RS12475		-3.56	4.43E-27	**STMMW_44551**	*hilE*	-1.10	1.48E-03
STMMW_44851		-3.42	6.55E-08	**STMMW_35081**	*rtcA*	-1.10	6.42E-04
STMMW_13621	*ydiP*	-3.41	2.72E-02	**STMMW_09361**		-1.09	1.12E-02
STMMW_15741	*narW*	-3.31	1.05E-14	**STMMW_42861**	*sugE*	-1.09	1.62E-03
STMMW_23811		-3.27	1.18E-20	**STMMW_07711**	*pxpA*	-1.09	1.35E-03
STMMW_04291	*sssA*	-3.18	1.13E-38	**STMMW_09791**	*ycaD*	-1.09	1.66E-02
STMMW_27371	*fljA*	-3.11	4.96E-06	**STMMW_20081**	*rzpR*	-1.08	3.85E-02
STMMW_03871		-3.00	8.93E-04	**STMMW_31311**	*ygiK*	-1.08	1.25E-02
STMMW_19231		-2.96	6.13E-06	**STMMW_04591**	*yaiA*	-1.08	6.87E-06
STMMW_30761	*yqgA*	-2.94	4.08E-17	**STMMW_19761**	*umuC*	-1.08	3.22E-03
STMMW_21681	*sseK2*	-2.86	3.92E-05	**STMMW_08921**	*ybiU*	-1.07	6.56E-03
STMMW_44811		-2.69	3.72E-03	**STMMW_40321**		-1.07	2.67E-04
STMMW_03091	*safA*	-2.67	1.14E-02	**STMMW_08651**	*ybhP*	-1.07	5.72E-03
STMMW_11391	*nanM/yjhT*	-2.64	1.27E-02	**STMMW_35451**	*ugpA*	-1.07	1.35E-02
STMMW_RS25725		-2.60	1.82E-18	**STMMW_19871**		-1.07	1.26E-02
STMMW_10241	*C1*	-2.58	8.84E-03	**STMMW_19951**		-1.07	1.08E-04
STMMW_03731	*Rz_BTP1*	-2.58	3.98E-03	**STMMW_RS25690**	*mgtL*	-1.06	2.13E-03
STMMW_23801		-2.56	3.39E-16	**STMMW_20951**	*phsA*	-1.06	1.47E-03
STMMW_15721	*narZ*	-2.56	5.63E-21	**STMMW_32261**	*sstT*	-1.05	1.38E-05
STMMW_31291		-2.51	6.31E-12	**STMMW_06781**		-1.05	2.44E-03
STMMW_04381	*prpB*	-2.49	6.40E-06	**STMMW_36661**		-1.05	1.10E-02
STMMW_RS24675		-2.48	8.41E-04	**STMMW_39711**	*glnA*	-1.04	1.60E-04
STMMW_09341		-2.44	3.25E-02	**STMMW_42291**	*nrfB*	-1.04	1.72E-02
STMMW_22741		-2.43	7.98E-03	**STMMW_09771**	*dmsB*	-1.04	1.00E-05
STMMW_17951	*dadA*	-2.43	3.89E-16	**STMMW_20341**		-1.04	3.82E-03
STMMW_42631		-2.38	7.25E-06	**STMMW_19891**		-1.04	3.06E-02
STMMW_41341	*aceK*	-2.37	3.90E-12	**STMMW_22331**	*yeiE*	-1.04	6.14E-06
STMMW_15421		-2.37	4.97E-05	**STMMW_40291**	*cdh-a/ushB*	-1.03	9.18E-05
STMMW_RS15945		-2.36	3.80E-10	**STMMW_43731**	*iolC*	-1.03	2.01E-02
STMMW_03551	*cro_BTP1*	-2.33	1.66E-03	**STMMW_09801**	*ycaM*	-1.03	1.76E-03
STMMW_20181		-2.32	2.14E-02	**STMMW_00901**	*kefF*	-1.02	2.67E-02
STMMW_22061	*dusC*	-2.27	9.79E-08	**STMMW_16711**		-1.02	6.62E-03
STMMW_27381	*fljB*	-2.26	7.00E-09	**STMMW_14731**		-1.02	4.57E-02
STMMW_12501	*envF*	-2.24	4.71E-02	**STMMW_36751**	*mtlR*	-1.01	1.53E-04
STMMW_10171		-2.22	4.96E-02	**STMMW_42281**	*nrfA*	-1.01	2.71E-03
STMMW_05981	*ylbE*	-2.22	3.18E-02	**STMMW_27581**	*gabD*	-1.01	8.11E-03
STMMW_03661	*ninG_BTP1*	-2.22	3.71E-02	**STMMW_44581**	*iadA*	-1.00	9.42E-05
STMMW_RS23430		-2.20	2.54E-32	**STMMW_34871**	*hslR*	1.00	4.95E-02
STMMW_RS02155		-2.17	7.12E-05	**STMMW_RS14445**		1.00	1.77E-02
STMMW_RS20095	*ilvL*	-2.17	1.24E-06	**STMMW_06541**	*fepE*	1.00	4.80E-03
STMMW_30771	*lgl*	-2.16	3.33E-09	**STMMW_31221**	*yghB*	1.00	9.83E-05
STMMW_11141	*hpaF*	-2.16	2.88E-03	**STMMW_33101**	*mlaD*	1.00	3.38E-03
STMMW_03821		-2.14	1.85E-02	**STMMW_10291**		1.01	2.10E-03
STMMW_44831		-2.14	7.28E-04	**STMMW_15871**	*ydcY*	1.01	2.86E-02
STMMW_04391	*prpC*	-2.14	3.24E-06	**STMMW_29791**	*kduD*	1.01	1.23E-02
STMMW_07301	*gltI*	-2.13	8.44E-12	**STMMW_22531**	*rsuA*	1.01	1.37E-03
STMMW_14101	*sseG*	-2.12	6.01E-04	**STMMW_05061**	*yajR*	1.01	4.05E-04
STMMW_07201	*ybeR*	-2.12	3.26E-02	**STMMW_01571**	*pdhR*	1.01	5.81E-04
STMMW_29371	*fucA*	-2.07	4.28E-03	**STMMW_14951**	*dmsD*	1.01	9.58E-04
STMMW_15441		-2.06	3.52E-02	**STMMW_06021**	*purE*	1.02	9.56E-03
STMMW_24381	*xapR*	-2.06	4.68E-02	**STMMW_26661**	*grcA*	1.02	5.05E-05
STMMW_44821		-2.04	1.22E-02	**STMMW_39401**	*rmuC*	1.03	1.43E-03
STMMW_37871	*tisB*	-2.00	4.15E-10	**STMMW_25951**	*recO*	1.04	5.70E-03
STMMW_15281		-2.00	2.52E-04	**STMMW_03301**	*crl*	1.04	1.14E-05
STMMW_39661		-1.97	9.62E-17	**STMMW_32031**		1.04	1.14E-03
STMMW_38171		-1.97	7.58E-03	**STMMW_34561**	*yhfA*	1.04	1.61E-05
STMMW_05251	*fadM*	-1.97	1.97E-10	**STMMW_02521**	*metI*	1.05	2.27E-02
STMMW_03801		-1.96	1.20E-04	**STMMW_18151**	*yoaH*	1.05	1.94E-02
STMMW_39771		-1.95	9.47E-06	**STMMW_01731**	*yacC*	1.06	3.97E-03
STMMW_44861		-1.94	4.55E-06	**STMMW_33431**	*rpsI*	1.06	1.46E-05
STMMW_20201		-1.92	4.99E-02	**STMMW_10231**		1.06	3.18E-04
STMMW_11081	*hpaC*	-1.91	2.90E-02	**STMMW_09251**	*nfsA*	1.07	4.96E-04
STMMW_32011		-1.87	4.77E-04	**STMMW_36031**	*dctA*	1.07	1.62E-05
STMMW_40311		-1.87	2.98E-03	**STMMW_05031**	*yajL*	1.08	2.02E-04

STMMW_03811		-1.84	1.08E-06	**STMMW_24501**	*ptsH*	1.08	3.42E-09
STMMW_42101	*siiC*	-1.84	2.19E-05	**STMMW_19491**	*fliF*	1.08	2.69E-03
STMMW_11331	*putA*	-1.83	1.09E-07	**STMMW_18571**	*holE*	1.09	3.41E-02
STMMW_25002	*ypfM*	-1.82	2.59E-12	**STMMW_42981**	*orn*	1.10	1.24E-04
STMMW_30121	*yhcN*	-1.82	1.07E-02	**STMMW_32221**	*ygjP*	1.10	1.40E-03
STMMW_20231		-1.81	1.09E-12	**STMMW_14591**	*rsxD*	1.10	1.08E-02
STMMW_33601		-1.81	1.16E-10	**STMMW_26591**	*rseA*	1.11	1.06E-08
STMMW_28991	*iap*	-1.81	1.49E-02	**STMMW_06441**	*ybdF*	1.12	1.42E-02
STMMW_44841		-1.79	2.21E-03	**STMMW_11961**		1.12	3.74E-03
STMMW_19981		-1.79	1.80E-05	**STMMW_03751**	*pid_BTP1*	1.13	4.35E-05
STMMW_07471	*tcuC*	-1.78	9.42E-06	**STMMW_26791**	*bamD*	1.13	4.51E-06
STMMW_16241	*steB*	-1.78	3.75E-07	**STMMW_23671**		1.13	5.88E-05
STMMW_26181		-1.75	1.74E-02	**STMMW_RS14440**		1.13	3.20E-02
STMMW_13681	*ydiM*	-1.74	4.68E-03	**STMMW_44531**	*uxuR*	1.13	5.96E-07
STMMW_03571	*O_BTP1*	-1.71	3.57E-03	**STMMW_29661**	*mutH*	1.14	7.90E-04
STMMW_00201	*bcfA*	-1.71	1.23E-02	**STMMW_05311**	*mdlA*	1.14	3.74E-05
STMMW_03741	*rha_BTP1*	-1.70	5.19E-03	**STMMW_16971**	*yciW*	1.14	1.33E-02
STMMW_03101	*papD*	-1.70	4.22E-02	**STMMW_12121**	*ptsG*	1.14	4.99E-03
STMMW_RS07825	*mgtS*	-1.68	5.31E-08	**STMMW_08541**	*moaA*	1.14	4.57E-04
STMMW_19851		-1.67	4.36E-03	**STMMW_RS21305**		1.15	1.31E-02
STMMW_16641	*ydaL*	-1.67	3.25E-02	**STMMW_13731**	*ydiH*	1.15	5.73E-04
STMMW_19811		-1.67	5.46E-04	**STMMW_14311**	*ydhC*	1.15	3.40E-03
STMMW_11341	*putP*	-1.66	2.63E-07	**STMMW_21241**	*rbl*	1.15	3.02E-06
STMMW_27601	*gabP*	-1.66	2.38E-04	**STMMW_28981**	*cysD*	1.15	8.11E-06
STMMW_10341		-1.66	3.64E-02	**STMMW_14971**	*dmsB*	1.16	2.72E-02
STMMW_RS24565		-1.65	1.54E-02	**STMMW_40461**	*tpiA*	1.16	3.48E-09
STMMW_22101	*gtdA*	-1.61	8.77E-04	**STMMW_38361**	*adeP*	1.16	5.11E-05
STMMW_20251		-1.61	3.92E-12	**STMMW_45271**	*yjjX*	1.16	3.79E-03
STMMW_37611		-1.59	4.73E-02	**STMMW_07891**	*sdhC*	1.18	7.66E-09
STMMW_05091		-1.59	1.37E-02	**STMMW_40921**	*yijD*	1.20	3.43E-05
STMMW_28421		-1.58	8.53E-06	**STMMW_RS14455**		1.21	1.05E-02
STMMW_31521	*assT*	-1.58	2.15E-04	**STMMW_40971**	*birA*	1.23	3.31E-05
STMMW_15711	*narU*	-1.58	1.64E-04	**STMMW_44171**	*miaE*	1.25	7.02E-05
STMMW_41151	*thiF*	-1.57	3.43E-02	**STMMW_21171**	*rfbU*	1.25	1.67E-04
STMMW_RS24125	*mntS*	-1.57	2.10E-08	**STMMW_34461**		1.27	2.50E-04
STMMW_07281	*gltK*	-1.56	6.03E-05	**STMMW_38661**	*rbsA*	1.27	3.25E-02
STMMW_12681		-1.54	6.70E-09	**STMMW_01141**	*tbpA*	1.27	2.61E-03
STMMW_25851	*yfhb*	-1.54	2.17E-10	**STMMW_24691**	*amiA*	1.27	5.58E-05
STMMW_40001	*fdxH*	-1.54	1.61E-15	**STMMW_27011**	*yfjG*	1.29	2.67E-08
STMMW_27591	*gabT*	-1.53	1.11E-07	**STMMW_RS11630**		1.31	1.63E-05
STMMW_35091	*rtcB*	-1.52	8.03E-06	**STMMW_31011**		1.31	4.68E-03
STMMW_RS12945		-1.52	6.17E-05	**STMMW_37271**	*yigC*	1.32	5.14E-03
STMMW_30841		-1.51	2.17E-02	**STMMW_30311**	*epd*	1.32	1.16E-08
STMMW_12571		-1.50	9.16E-03	**STMMW_08861**	*mntR*	1.32	1.36E-03
STMMW_RS10400		-1.49	1.26E-07	**STMMW_11441**	*ycdW*	1.32	7.33E-08
STMMW_RS09545		-1.47	6.82E-07	**STMMW_01441**	*yacG*	1.32	5.71E-04
STMMW_14441		-1.47	9.12E-08	**STMMW_32431**	*tdcC*	1.33	2.83E-03
STMMW_13141	*astE*	-1.46	4.62E-03	**STMMW_45001**	*holD*	1.33	5.11E-03
STMMW_19831		-1.46	8.29E-05	**STMMW_RS04245**		1.34	5.44E-05
STMMW_27691	*ygaM*	-1.46	8.05E-10	**STMMW_32401**	*tdcG*	1.36	1.35E-02
STMMW_07291	*gltJ*	-1.46	9.59E-05	**STMMW_44471**		1.36	1.29E-03
STMMW_19971		-1.46	2.81E-03	**STMMW_15881**	*srfA*	1.38	2.55E-03
STMMW_RS25810		-1.44	6.18E-03	**STMMW_45321**	*creC*	1.39	3.04E-04
STMMW_31161		-1.44	1.84E-05	**STMMW_36241**		1.40	9.09E-04
STMMW_27311		-1.44	2.79E-04	**STMMW_11831**	*flgC*	1.41	1.87E-04
STMMW_19921		-1.42	1.13E-07	**STMMW_23181**	*yfaZ*	1.42	5.85E-05
STMMW_36121	*bcsF*	-1.41	1.26E-02	**STMMW_20431**		1.43	1.47E-03
STMMW_17002	*osmB*	-1.41	9.34E-14	**STMMW_42761**	*fxsA*	1.43	4.73E-08
STMMW_20221		-1.41	8.51E-09	**STMMW_22231**	*galS*	1.43	1.39E-04
STMMW_06841	*citG*	-1.41	1.38E-02	**STMMW_14411**	*ydhL*	1.44	1.22E-03
STMMW_20001		-1.39	8.93E-07	**STMMW_25241**		1.46	4.34E-04
STMMW_07461	*ybfN*	-1.39	9.30E-03	**STMMW_33831**	*fis*	1.46	8.27E-05
STMMW_44201	*yjgN*	-1.37	3.60E-02	**STMMW_45021**	*yjjG*	1.47	6.22E-04
STMMW_19861		-1.37	1.71E-02	**STMMW_19471**		1.50	9.68E-03
STMMW_15691	*smvA*	-1.37	4.59E-06	**STMMW_32121**		1.52	6.77E-04
STMMW_22071		-1.36	4.22E-05	**STMMW_24371**	*gltX*	1.52	2.18E-11
STMMW_11131	*hpaD*	-1.36	1.65E-03	**STMMW_45301**	*creA*	1.54	5.16E-07
STMMW_44541	*trpS*	-1.35	2.24E-02	**STMMW_13261**	*cedA*	1.55	8.94E-05
STMMW_22081	*maiA*	-1.34	1.66E-02	**STMMW_27681**	*ygaC*	1.55	6.24E-08
STMMW_07271	*gltL*	-1.34	1.27E-04	**STMMW_34411**	*tusB*	1.56	4.52E-05
STMMW_16611		-1.34	3.85E-03	**STMMW_06011**	*purK*	1.56	3.85E-09
STMMW_13131	*astB*	-1.34	1.02E-03	**STMMW_29481**	*csdE*	1.56	5.99E-04
STMMW_01971	*fhuA*	-1.34	1.51E-05	**STMMW_RS24490**		1.60	4.91E-07
STMMW_03581	*P_BTP1*	-1.33	1.01E-03	**STMMW_38611**	*asnA*	1.62	4.26E-04
STMMW_40031		-1.32	2.12E-02	**STMMW_05741**	*cnoX*	1.63	4.49E-13

STMMW_42301	*nrfC*	-1.32	2.19E-03	**STMMW_37501**	*slsA*	1.66	9.12E-10
STMMW_RS14315		-1.31	2.63E-06	**STMMW_43981**	*treC*	1.66	5.83E-05
STMMW_30871		-1.30	1.99E-02	**STMMW_34951**	*feoA*	1.67	6.53E-06
STMMW_37821	*rbsK*	-1.30	8.82E-05	**STMMW_09661**		1.68	1.44E-02
STMMW_22091		-1.29	1.04E-02	**STMMW_12581**		1.69	7.12E-11
STMMW_20111		-1.29	1.54E-02	**STMMW_32421**	*tdcD*	1.71	3.35E-03
STMMW_12231	*ycfR*	-1.29	2.04E-03	**STMMW_22841**	*napA*	1.73	4.43E-13
STMMW_31791		-1.28	1.53E-02	**STMMW_44021**		1.77	7.10E-10
STMMW_20021		-1.28	5.11E-04	**STMMW_34381**	*fusA*	1.81	5.09E-10
STMMW_RS05270		-1.28	1.05E-02	**STMMW_28701**	*pphB*	1.84	4.65E-02
STMMW_24861	*eutT*	-1.27	4.69E-02	**STMMW_27921**	*pncC*	1.84	2.03E-07
STMMW_31821		-1.26	7.32E-05	**STMMW_37991**	*ibpA*	1.87	8.19E-09
STMMW_32311	*yqjK*	-1.26	4.74E-08	**STMMW_37361**	*xanP*	1.90	6.51E-06
STMMW_20191		-1.25	4.73E-02	**STMMW_42531**		1.94	2.01E-03
STMMW_36091	*bcsQ*	-1.23	3.24E-05	**STMMW_06241**	*gtrBa/yfdH*	2.03	4.62E-03
STMMW_06501	*fepA*	-1.22	5.80E-03	**STMMW_19481**	*fliE*	2.11	4.72E-09
STMMW_19911		-1.22	3.42E-04	**STMMW_33321**	*codB*	2.12	2.72E-11
STMMW_31971		-1.22	1.81E-02	**STMMW_32441**	*tdcB*	2.13	1.25E-02
STMMW_10901	*yccT*	-1.21	4.62E-03	**STMMW_40281**	*sbp*	2.15	9.63E-07
STMMW_39991	*fdoI*	-1.21	5.97E-09	**STMMW_02531**	*metN*	2.17	1.20E-07
STMMW_19121	*otsB*	-1.21	2.45E-07	**STMMW_13271**	*tcyP*	2.25	2.04E-22
STMMW_20261		-1.19	3.53E-06	**STMMW_28961**	*cysC*	2.27	1.54E-18
STMMW_40301		-1.19	2.66E-02	**STMMW_37791**	*uhpA*	2.42	3.40E-03
STMMW_06531	*entF*	-1.19	1.41E-02	**STMMW_37151**	*mutM*	2.45	6.12E-15
STMMW_RS23760		-1.19	1.94E-02	**STMMW_16811**	*pspE*	2.53	1.83E-26
STMMW_21561	*alkA*	-1.19	2.73E-02	**STMMW_24611**	*cysW*	2.64	1.59E-08
STMMW_19961		-1.18	3.53E-03	**STMMW_RS25180**		2.90	1.40E-06
STMMW_36081	*bcsA*	-1.18	3.75E-07	**STMMW_37981**	*ibpB*	2.92	2.32E-22
STMMW_19932		-1.18	4.54E-03	**STMMW_07571**		2.93	1.12E-13
STMMW_42261	*acs*	-1.18	1.74E-05	**STMMW_29091**	*cysH*	3.00	3.47E-21
STMMW_16681		-1.18	1.72E-03	**STMMW_29101**	*cysI*	3.20	1.92E-28
STMMW_09531	*ybjX*	-1.17	1.07E-07	**STMMW_28971**	*cysN*	3.39	8.33E-19
STMMW_19941		-1.17	2.61E-03	**STMMW_44051**	*pyrB*	3.41	4.06E-14
STMMW_21961	*yehY*	-1.17	2.00E-06	**STMMW_07581**	*potE*	3.58	7.02E-27
STMMW_43651	*iolA*	-1.16	2.36E-02	**STMMW_24601**	*cysA*	3.61	8.43E-25
STMMW_15541	*treY*	-1.16	2.94E-08	**STMMW_RS25485**		3.90	2.98E-36

[1]New locus tags STMMW_RS##### are used where an old locus tag is unavailable.
[2]F.C. is the $\log_2$ Fold Change (Δ*relA* Δ*spoT* / wild type).
[3]Blue represents down-regulated genes.
[4]Red represents up-regulated genes.

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