NICHE SPECIFIC GENE REGULATION IN *SALMONELLA ENTERICA*

IDENTIFYING NOVEL REGULATORY INPUTS GOVERNING *SALMONELLA ENTERICA* NICHE-SPECIFIC GENE EXPRESSION

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy

McMaster University DOCTOR OF PHILOSOPHY (2019) Hamilton, Ontario
(Biochemistry and Biomedical Sciences)

TITLE: Identifying the regulatory inputs governing *Salmonella enterica* niche-specific gene expression

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NUMBER OF PAGES: 173, xiii

LAY ABSTRACT

Salmonella enterica (*S. enterica*) is a species of bacteria that can cause food poisoning in various animals, including humans, through consumption of contaminated food and water. During an infection, host cells activate numerous defense mechanisms to prevent disease. *S. enterica* has evolved to turn specific genes on or off in response, resulting in modifications to bacterial and host cell behaviour that promote infection. The timing of these genetic changes is controlled by proteins that can sense specific environmental signals and adjust gene expression accordingly. The specific signals sensed by *S. enterica* that allow for adaptive gene expression within the host, and the types of genes that are regulated to promote survival, have not been fully identified. Here, we show that *S. enterica* evolved to repress genes involved in flagellar motility to hide from the host immune response. We further demonstrate that *S. enterica* can sense anti-bacterial molecules produced by the host, called reactive oxygen species, to trigger specific changes in gene expression. Together, this work reveals novel aspects for the molecular basis of *Salmonella enterica* pathogenesis.

ABSTRACT

Salmonella enterica is an enteric pathogen with a broad host tropism that can cause disease ranging from self-limited gastroenteritis to enteric fever. The evolution of *S. enterica* as a pathogen is driven by the horizontal acquisition of genes that promote virulence and survival within host immune cells, as well as the coordinated regulation of these and ancestral genes by two-component systems (TCS). TCS integrate environmental cues with the transcriptional reprogramming of bacteria, and in the case of *Salmonella*, result in niche-specific gene expression in response to anti-bacterial cues produced by the host. The TCS SsrA-SsrB in *S. enterica* is considered the master regulator for intracellular virulence, where SsrA is a sensor kinase that triggers the activation of the DNA binding protein SsrB. The full suite of genes regulated by SsrB in *S. enterica*, as well as the cues that activate this TCS, have not been fully characterized. Here, we demonstrated that horizontally acquired and ancestral genes in the *S. enterica* genome have evolved to be regulated by SsrB, and the repression of a set of ancestral genes involved in flagellar motility promotes evasion of the host immune system. Additionally, we identified the production of reactive oxygen species (ROS) by host immune cells as a signal that can activate a cluster of genes regulated by the SsrA-SsrB TCS, likely mediated by SsrA sensing of these ROS. Together, these results expand our understanding of the complex interplay between the pathogen *S. enterica* and the host that results in bacterial infections.

ACKNOWLEDGEMENTS

This thesis which would not have been possible without the support and guidance of my supervisor Dr. Brian Coombes. Brian, thank you for giving me this amazing opportunity to dive into a PhD and for having faith in me throughout. You have made me a better thinker, researcher, and communicator, and I am honored to have been a part of your lab. Secondly, thank you to my committee, Drs. Dawn Bowdish and Karen Mossman. You have both been stalwart pillars of support and encouragement, and my go-to source for understanding immunology. Thank you all for constantly asking more of me.

I have been fortunate enough to have wonderful mentors that have fueled my passion for science from a young age. To my high school biology and chemistry teachers Mr. Pinto and Ms. Tabbara, you taught me how to understand science and how to share that understanding with passion. I hope one day to be as great a proponent of science as either of you. Dr. Will Navarre, I fell in love with bacteria the day I attended your first MGY377 lecture. Thank you for opening my eyes to how awesome bacteria are, and for giving me the chance to work in your lab. You inspired me to strive for more.

I have also had the amazing fortune of being surrounded by wonderful people throughout graduate school. Drs. Joe McPhee and David Mulder, you are my favorite science nerds. Thank you both for being sounding boards for my many rants and for lending me perspective, usually accompanied by good food and drinks. Drs. Dustin Little

and Wael Elhenawy, your scientific rigor and wisdom remind me of how much I still need to learn. Thank you both for your encouragement and guidance. My fellow grad students, Caressa, Alex, Liz, Brian –you alone know the uniqueness of this journey, and I am so grateful that I have been able to share it with you. Alex, thank you for all the adventures that allowed me to grow as a person. Caressa, thank you for being the person I can talk to about literally anything, and for renewing my scientific curiosity. Liz, thank you for reminding me to be a better scientist and person. Brian, thanks for the early years, I will always look back on them fondly. To the rest of the Coombes lab and my extended lab family in the Burrows and Whitney labs, thank you for your companionship and support. Each of you has helped me get to this point in your own way, and I thank you all. Lisa Kush, thank you for always keeping your office open to me when I needed to figure out my next steps or summon up the courage to take them. Grad school would have been a much murkier place without your shining light guiding me through. Dr. Felicia Vulcu, you taught me how to teach. I am grateful for five years of TAship under your tutelage.

To my family - mum, dad, Ayman. You have made sacrifices for me and my decisions, even when you didn't necessarily understand or agree with them. Thank you for being constant cheerleaders, I could not have done it without you standing by me. Finally, thank you Kaizad. You have held my hand through it all, even when I made that hard for you to do. I am so honored to have embarked on this journey with you, thank you for waiting for me by the finish line. I could not have made it without you to share the load and I am excited to embark on many more of life's adventures with you. I love you.

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LIST OF ABBREVIATIONS AND SYMBOLS

AMP	Antimicrobial peptide
CP	Crossing point
DAO	D-amino acid oxidase
DTT	Dithiothreitol
E. coli	*Escherichia coli*
H_2O_2	Hydrogen peroxide
HGT	Horizontal gene transfer
H-NS	Histone like nucleoid structuring protein
HPLC	High performance liquid chromatography
HTS	High-throughput screen
ICAT	Isotopically labelled tags with cleavable linkers
IL-1β	Interleukin-1β
iNTS	Invasive non-typhoidal *Salmonella* disease
LPM	Low phosphate and magnesium
LPS	Lipopolysaccharide
M cell	Microfold cell
MPO	Myeloperoxidase
m/z	Mass/charge
NAIP	Neuronal apoptosis inhibitory protein
NK	Natural killer
NLR	Nod-like receptor
NRAMP	Natural resistance associated macrophage protein
PITS	pore-induced intracellular traps
PRR	Pattern recognition receptor
RR	Response receiver

ROS	Reactive oxygen species
RT-qPCR	Reverse transcriptase-quantitative polymerase chain reaction
SBG	*Salmonella bongori*
S. bongori	*Salmonella bongori*
SCV	*Salmonella* containing vacuole
SDM	Site-directed mutagenesis
SK	Sensor kinase
SNP	Single nucleotide polymorphism
SPI	*Salmonella p*athogenicity island
ST	Sequence type
STM	*Salmonella enterica* serovar Typhimurium
S. Typhimurium	*Salmonella enterica* serovar Typhimurium
T3SS	Type 3 secretion system
T6SS	Type 6 secretion system
TCS	Two component system
TLR	Toll-like receptor
V-ATPase	vacuolar H^+-ATPase

DECLARATION OF ACADEMIC ACHIEVEMENT

The work presented in this thesis is my own. Any experimental contributions by other researchers have been outlined at the beginning of each chapter. The design, experimentation, analyses, and writing of the chapters herein are my own work.

CHAPTER 1: INTRODUCTION

Portions of this chapter have been adapted from:

Ilyas, B., Tsai, C.N., and Coombes, B.K. (2017). Evolution of *Salmonella*-host cell interactions through a dynamic bacterial genome. Front. Cell. Infect. Microbiol. *7*.

Salmonella is an enteric pathogen with a broad host range and disease manifestation

Salmonella is a genus of pathogenic bacteria in the Enterobacteriaceae family that diverged from its closest evolutionary relative *Escherichia coli* (*E. coli*) ~100 million years ago (Cotter and DiRita, 2000). The genus *Salmonella* is comprised of only two species, *S. bongori* and *S. enterica*, but contains over 2500 species variants called serovars that are differentiated based on surface antigenicity (Issenhuth-Jeanjean et al., 2014). *S. enterica* has six subspecies (subsp.), of which subsp. II-VI, as well as all known strains of *S. bongori*, infect cold-blooded animals. Only strains of *S. enterica* subsp. I (enterica) are associated with disease in mammals and humans (Brenner et al., 2000; Grimont and Weill, 2007; Tindall et al., 2005). *S. enterica* subsp. enterica is comprised of over 1500 serovars; these serovars vary from those that can infect a variety of mammals, sometimes referred to as 'host-generalists', to host-adapted species that infect a single host or closely related hosts (Branchu et al., 2018). In addition to this wide range in host tropism, the *Salmonella* genus is also diverse in its disease manifestations, ranging from self-limiting gastroenteritis to bacteremia. *S. enterica* subsp. enterica is the best studied serovar due to its clinical and agricultural significance, since it can be transmitted to humans from livestock and can cause a range of diseases in humans (Majowicz et al., 2010).

The *Salmonella enterica* subsp. enterica serovars Typhi and Paratyphi are human restricted pathogens that cause enteric fever and associated bacteremia (Dougan and

Baker, 2014). *S. enterica* subsp. enterica serovars Enteritidis and Typhimurium (hereafter referred to as *Salmonella* Typhimurium or *S.* Typhimurium) are major causative agents of acute gastroenteritis, resulting in abdominal pain, fever, vomiting, and diarrhea in humans (Chai et al., 2012). This is typically self-limiting; however risk factors such as age, immunosuppression, and microbial dysbiosis can cause severe complications including systemic spread leading to septicemia (Hohmann, 2001). Further, the increasing incidence of antibiotic resistant *S.* Typhimurium and the emergence of a highly virulent and multidrug resistant variant of *S.* Typhimurium that can cause invasive disease and death (termed invasive non-typhoidal *Salmonella* disease or iNTS) has increased the relevance of studying this pathogen (Feasey et al., 2012; Glynn et al., 1998). The other *Salmonella* species, *S. bongori*, predominantly causes gastroenteritis in cold-blooded animals like reptiles, although it has on occasion been isolated from humans (Giammanco et al., 2002; Nastasi et al., 1988). However, this species is primarily studied comparatively to understand the evolution of the mammalian pathogen *S. enterica* (Fookes et al., 2011).

The evolution of pathogenesis of *Salmonella* is driven by a dynamic bacterial genome

The recent rise in comparative genomics methods has facilitated genetic comparisons between *S. enterica* and *S. bongori* and has cast a light on the molecular basis of pathogenesis. The study of the two *Salmonella* species has revealed that *Salmonella* pathogenesis is a result of evolution by "quantum leaps" through the acquisition of genes by horizontal gene transfer (HGT) (Bäumler, 1997; Bäumler et al.,

1998). Horizontal gene transfer, which is the acquisition of genes by bacterial conjugation, transduction via bacteriophage, or uptake of extracellular DNA by transformation, can confer new phenotypes to the recipient bacteria and is often the source of adaptive changes that maximize fitness in a given niche (Ochman et al., 2000; Soucy et al., 2015). In *Salmonella*, horizontally acquired multi-gene loci that are linked to infection are called *Salmonella* pathogenicity islands (SPIs), and have been described as the "molecular toolbox" for *Salmonella* pathogenesis (Gal-Mor and Finlay, 2006).

The *Salmonella* pathogenicity island-1 (SPI-1) is a major pathogenic marker of *Salmonella*. The acquisition of SPI-1 differentiates the *Salmonella* genus from *E. coli*, which is comprised of both commensal and pathogenic organisms (Leimbach et al., 2013). The SPI-1 genomic island encodes a type 3 secretion system (T3SS) – a molecular machine that injects host-modulating effectors into host cells, as well as the regulators and a few of the effectors for this T3SS. The SPI-1 T3SS (T3SS-1) effectors mainly mediate invasion of *Salmonella* into gut epithelial cells (Lostroh and Lee, 2001; McGhie et al., 2009; Que et al., 2013). Thus, acquisition of SPI-1 allowed *Salmonella* to exploit a new niche within intestinal epithelial cells, and SPI-1 deletion strains are typically avirulent in oral infections in a *Salmonella* mouse model.

The second major advancement in the evolution of *Salmonella* as a pathogen was the acquisition of the SPI-2 genomic island, which is present in *S. enterica* but not *S. bongori.* SPI-2, which encodes a second T3SS (T3SS-2), allowed *S. enterica* to modify

the intracellular niche and to survive within host immune cells like macrophages (Hensel et al., 1998; Ochman et al., 1996; Shea et al., 1996). This trait is necessary for transit through the bloodstream to systemic sites (Hensel et al., 1997; Ochman et al., 1996).

Although SPI-1 and SPI-2 are the major molecular markers that distinguish *Salmonella* from other Enterobacteriaceae and the two *Salmonella* species from each other, the pathogenesis of *Salmonella* is a product of the activity of these and numerous other horizontally acquired islands and host factors. Of over 21 SPIs that have been identified, only a subset are present in all *Salmonella* serovars and different SPIs are relevant for pathogenesis depending on the animal infection model or host (Hensel, 2004; McClelland et al., 2001; Morgan et al., 2004). This genetic flux within the *Salmonella* species and subspecies defines the variety in host range and disease phenotypes (Bäumler, 1997; Fookes et al., 2011). Perhaps the best-studied serovar of *Salmonella* is *S.* Typhimurium. *S.* Typhimurium is amenable to molecular manipulation, can infect numerous cell types, and robust animal models have been developed to model both the self-limiting gastroenteritis and systemic infection that *Salmonella* infections can induce in humans (Finlay and Brumell, 2000; Tsolis et al., 2011). Together, these tools have laid the foundation for understanding the genetic basis for *Salmonella* virulence and have helped researchers understand the host response to infection. Data from this serovar form the basis of the rest of this introduction.

Niche-specific virulence factors drive different stages of *Salmonella* infection

Genetically susceptible mice (*Nramp/SLC11A1*$^{-/-}$) orally infected with *S.* Typhimurium develop systemic disease characterized by high bacterial burdens in the spleen and liver, gross intestinal pathology, and death from systemic bacteremia (Cuellar-Mata et al., 2002; Santos et al., 2001). Streptomycin pre-treatment in this model lowers intrinsic resistance to host colonization and intensifies the bacterial-driven intestinal inflammation, a finding that has been extensively leveraged to understand how *S.* Typhimurium competes metabolically in the inflamed intestine (Barthel et al., 2003; Winter et al., 2010a, 2010b). This mouse model of infection is used to understand the complex interplay of virulence factors and the host during an *S.* Typhimurium infection.

Following transit through the stomach, *S.* Typhimurium colonizes the lumen of the small intestine, invades epithelial cells or is taken up by M cells, and invades or is phagocytosed by immune cells such as macrophages and neutrophils. In this permissive niche for replication, systemic dissemination proceeds through the lymphatic system and bloodstream to shuttle bacteria to sites such as the spleen and liver. At each step in this process, specific virulence factors are activated that interact with the host to make the environment more conducive to bacterial survival and replication (Erhardt and Dersch, 2015).

Luminal colonization is driven by a combination of metabolic genes, swimming motility, and horizontally acquired effectors to modify the lumen environment. An

uncontrived gut microbiota limits nutrient availability and provides colonization resistance towards *S.* Typhimurium attempting to colonize the intestinal lumen (Lawley and Walker, 2013; Patel and McCormick, 2014). *S.* Typhimurium can directly target commensal microbes through the expression of a type 6 secretion system (T6SS), a nano-machine used primarily for inter-bacterial antagonism that is encoded on SPI-6 (Mulder et al., 2012; Sana et al., 2016). *S.* Typhimurium can also overcome nutrient limitation by promoting luminal inflammation, which in turn generates metabolic by-products that can be used as alternate electron acceptors for anaerobic respiration. Inflammation-derived metabolites such as nitrate, which is produced from reactive oxygen and nitrogen species within the lumen, and tetrathionate produced from reduced thiosulfate, are alternative energy sources for *S.* Typhimurium through anaerobic respiration (Lopez et al., 2012; Winter et al., 2010a). *Salmonella*-specific genes such as the tetrathionate utilization operon (*ttr)* allow the bacteria to use these alternate metabolites to outcompete commensal microbes (Hensel et al., 1999; Winter et al., 2010a). Inflammation is driven by the activation of pro-inflammatory pathways by SPI-1 effector proteins (Santos et al., 2009). These effectors, injected into epithelial cells, activate cytokine and chemokine production via the NF-κB and Nod-like receptor (NLR) pathways (Müller et al., 2009; Zhang et al., 2003). This signaling is amplified by T cells, natural killer (NK) cells, and dendritic cells, producing a cytokine storm that promotes neutrophil recruitment and trans-migration through into the intestinal lumen, resulting in acute intestinal inflammation (Godinez et al., 2008; Mumy and McCormick, 2009, 2009; Tükel et al., 2006).

S. Typhimurium can also stimulate inflammation by up-regulating flagella production on the bacterial surface. These molecules can be detected by host pattern recognition receptors (PRR) on the basolateral surface of epithelial cells, inducing a pro-inflammatory cytokine burst. Flagellar expression and up-regulation of chemotaxis genes in the lumen promotes bacterial motility towards these nutrients, allowing for efficient colonization of the host lumen (Rivera-Chávez et al., 2013; Stecher et al., 2004, 2007). Expression of flagella and chemotaxis genes also allows for flagellar-based swimming motility that allows *S.* Typhimurium to swim towards the host epithelial cells and initiate attachment to epithelial cells, promoting host cell invasion (Horstmann et al., 2017). Together, these genes allow *S.* Typhimurium to successfully overtake the intestinal lumen environment.

The attachment and invasion of *S.* Typhimurium to host epithelial cells provides an access route to deeper tissues and the bloodstream. As *S.* Typhimurium approaches the epithelial barrier within the intestine, it can reach the lamina propria via endocytosis by microfold (M) cells, specialized intestinal epithelial cells that sample the lumen for foreign microbes (Martinez-Argudo and Jepson, 2008; Ohno, 2016). Entry into the lamina propria allows for toll-like receptor (TLR) activation by flagellin on the basolateral surface, uptake by macrophages and neutrophils, and activation of B and T cells (Johansson et al., 2006). In addition to luminal sampling by M cells, *S.* Typhimurium can mediate uptake by host epithelial cells through the cooperative interaction of several virulence factors encoded on SPI-1, SPI-3, and SPI-4, as well as adhesins encoded on the

core *Salmonella* genome (Dorsey et al., 2005; Gerlach et al., 2007; Kolachala et al., 2007). The T3SS-1-mediated invasion of *S.* Typhimurium exerts its main function at this stage as well, injecting effectors into the host cell that allow for bacterial uptake. The net result of T3SS-1 effector translocation upon host cell contact is the induction of major cytoskeletal changes in the epithelial cell, leading to membrane ruffling and bacterial internalization. Once within epithelial cells, *S.* Typhimurium establishes a specialized compartment called the *Salmonella* containing vacuole (SCV) (Steele-Mortimer, 2008). SPI-1 effectors can recruit host factors to the vacuolar membrane to redirect the vacuole in the endosomal trafficking pathway, thus limiting lysosomal fusion and promoting bacterial survival. SPI-1 associated genes are also implicated in escape from the SCV in non-phagocytic cells, allowing for *S.* Typhimurium survival and replication in the cytosolic compartment (Knodler et al., 2010, 2014). Cytosolic *S.* Typhimurium can hyper-replicate, induce pyroptosis, and escape back into the lumen to infect other epithelial cells or be taken up by macrophages (Knodler, 2015). SCV-contained *S.* Typhimurium are eventually released into the lamina propria through cell death or extrusion (Flieger et al., 2018; Hallstrom and McCormick, 2011).

Whether through bacterial mediated pyroptosis, trafficking of the SCV to the basolateral surface of epithelial cells, or by direct sampling of luminal bacteria by dendritic cells, *S.* Typhimurium eventually reaches the lamina propria where it is taken up by phagocytic cells through a combination of bacterial-mediated endocytosis and immune cell driven phagocytosis (Haraga et al., 2008). The systemic spread of *S.* Typhimurium to

the liver and spleen is contingent upon survival within the macrophage environment; mutants in *S.* Typhimurium that cannot survive within this environment are avirulent in a systemic mouse model of infection. *Salmonella*-containing macrophages enter the mesenteric lymph node, where they are shuttled to the liver and spleen (Watson and Holden, 2010). Replication within macrophages is also essential for colonization at the liver and spleen, as analyses of cell types within these tissues containing *S.* Typhimurium identify macrophages, and to a degree neutrophils, as the primary cells at infection foci (Geddes et al., 2007; Thöne et al., 2007).

The intracellular niche reprograms *S.* Typhimurium gene expression

Understanding the adaptations of *S.* Typhimurium to the intracellular niche requires a parallel exploration of the intrinsic antibacterial mechanisms that the host generates. Following uptake by professional phagocytes like neutrophils and macrophages, the phagosome typically fuses with other intracellular vesicles like early endosomes, late endosomes, and lysosomes (or granules in the case of neutrophils), allowing for maturation and modification of the vesicular membrane and contents (Aderem and Underhill, 1999). Phagosome maturation is associated with numerous antimicrobial defenses such as the activation of the oxidative burst by NADPH oxidase, acidification of the phagosome, metal and ion sequestration through the activity of metal transporters, and fusion with vesicles or granules containing proteases and antimicrobial peptides such as defensins (Flannagan et al., 2009; Gogoi et al., 2018). During this process, cytosolic

immune sensing mechanisms like TLRs and the inflammasome are also active in performing surveillance for intracellular pathogens to stimulate a pro-inflammatory anti-bacterial response. Survival and replication of *S.* Typhimurium within this intracellular niche is dependent on numerous bacterial genes, both horizontally acquired and ancestral, that are activated within this environment (Srikumar et al., 2015). Indeed, infection dynamics modeling demonstrates that the host anti-bacterial response initially limits *S.* Typhimurium burdens, but adaptive gene expression gradually allows for resistance and replication within this environment, thereby promoting dissemination (Mastroeni and Grant, 2011).

The phagocytosis of *S.* Typhimurium does not result in a small, restricted phagosome, but rather a modified, spacious vacuole called the SCV (Alpuche-Aranda et al., 1994). The modification of the phagosome to form the SCV, as well as SCV localization, are largely mediated by effector proteins secreted by the T3SS-2. The T3SS-2 effectors SifA and SseJ modify the SCV membrane to allow for subsequent control of SCV localization by the effector proteins SseF and SseG, which interact with the microtubule network within cells (Beuzón et al., 2000). These and other SPI-2 effectors also control protein assembly on the SCV membrane by targeting proteins for ubiquitination, and *S.* Typhimurium encodes genes that prevent fusion of the SCV with the lysosome, thereby limiting the extent of acidification of the SCV (Brumell and Grinstein, 2004; Holden, 2002; LaRock et al., 2015). Thus, T3SS-2 effectors are critical for evading a large part of the anti-bacterial response within the host. Indeed, mutants of

the T3SS-2 or of many of these effectors render *S.* Typhimurium avirulent in macrophages and in systemic mouse models of infection (Jennings et al., 2017).

Phagocytosis is coupled to the assembly of host NADPH oxidase on the phagosome membrane, generating reactive oxygen species (ROS) that can cause protein and DNA damage (Minakami and Sumimotoa, 2006). NADPH oxidase consists of the catalytic subunit, $gp91^{phox}$, and numerous regulatory subunits. These regulatory subunits maintain an "off" state for NADPH oxidase by spatial separation until phagocytosis is initiated through direct binding. $gp91^{phox}$ and one of the regulatory subunits $p22^{phox}$ are present on the phagocyte membrane while the other components are in the cytosol. Once phagocytosis is initiated, the membrane components are incorporated on the phagosome membrane and can associate with the cytosolic components (p40, p47, and p67) to activate the enzyme. Activated NADPH oxidase on the phagosome membrane converts molecular oxygen to superoxide ($O_2\bullet^-$). Superoxide has a short half-life and can react with protons to form hydrogen peroxide (H_2O_2). Together, superoxide and hydrogen peroxide can form hydroxyl radicals, and in neutrophils, the enzyme myeloperoxidase (MPO) can convert hydrogen peroxide to hypochlorous acid (Nauseef, 2008; Winterbourn and Kettle, 2013). Neutrophils also have a second source of H_2O_2, produced as a by-product during the selective oxidation of D-amino acids by the enzyme D-amino acid oxidase (DAO), which has also been shown to be a relevant source of antimicrobial ROS during bacterial infections (Nakamura et al., 2012; Sacchi, 2013; Tuinema et al., 2014) Superoxide, hydrogen peroxide, and hypochlorous acid are all potent antimicrobials as they can target

numerous bacterial molecules such as proteins, DNA, and iron-sulfur clusters, resulting in metabolic and functional defects (Craig and Slauch, 2009; Fang, 2011; Slauch, 2011; Vazquez-Torres et al., 2000a). The murine NADPH oxidase is important for the restriction of *S.* Typhimurium loads early on in an infection (Grant et al., 2008). *S.* Typhimurium encodes several redundant detoxifying enzymes including catalases, reductases, and dismutases (*ahpC, katEGN, sodCI, II,* and *III*) to protect against the oxidative burst that are vital to allow for subsequent expansion of *S.* Typhimurium following this initial restriction (De Groote et al., 1997; Fang et al., 1999; Hébrard et al., 2009; Uzzau et al., 2002). Although there are differences in the contribution of all these genes towards *S.* Typhimurium virulence, the redundant roles of these genes and the increased susceptibility of NADPH oxidase deficient mice to *S.* Typhimurium infections speak to the importance of the respiratory burst as an anti-bacterial strategy (Hébrard et al., 2009). SPI-2 has also been implicated in blocking this NADPH oxidase assembly on the SCV, although the effector responsible for this has not been identified and the relevance of this process during a *S.* Typhimurium infection has been debated (Aussel et al., 2011; Fang, 2011; Vazquez-Torres and Fang, 2001; Vazquez-Torres et al., 2000b).

As the phagosome matures, there is a gradual acidification through the assembly of the vacuolar H^+-ATPase (V-ATPase) on the phagosome membrane. The V-ATPase is a multimeric proton pump that hydrolyses ATP by the V_1 cytoplasmic domain to translocate H^+ through the phagosome membrane by the V_0 subunit (Marshansky and Futai, 2008; Maxson and Grinstein, 2014). The accumulation of H^+ in the phagosome

reduces the pH of the phagosome compartment. The inhibition of phagosome-lysosome fusion prevents the pH of the phagosome from dropping low enough to induce acid shock (pH <4), but *S.* Typhimurium benefits from a moderately acidified SCV as neutralization of the phagosome has been shown to decrease *S.* Typhimurium virulence (Rathman et al., 1996). *S.* Typhimurium has numerous acid tolerance response (ATR) genes that are activated in response to the low pH of the phagosome, which function to maintain intracellular pH homeostasis by pumping out the protons or by using H^+ in catalytic reactions by lysine or arginine decarboxylases (Álvarez-Ordóñez et al., 2010, 2012; Foster and Spector, 1995; Wilmes-Riesenberg et al., 1996).

Phagocytosis is also associated with a pro-inflammatory response via TLR and NOD-like receptors (NLR) signaling and activation of NF-κB. TLR2, 4, and 5 on the macrophage membrane detect bacterial ligands such as lipopolysaccharide (LPS) and other lipoproteins, and flagellin before *S.* Typhimurium is phagocytosed (Li and Cherayil, 2003). Although TLR activation induces the production of pro-inflammatory cytokines, it also promotes acidification of the phagosome. Moderate phagosome acidification promotes virulence gene expression so intracellular *S.* Typhimurium benefit from this TLR signalling (Arpaia et al., 2011). However, once *S.* Typhimurium is within the phagosome, the neuronal apoptosis inhibitory protein (NAIP)/NLR inflammasome complex in the macrophage cytosol can detect *S.* Typhimurium flagellin monomers and T3SS-1 components to produce the pro-inflammatory cytokines IL-1β and IL-18 (Broz and Dixit, 2016; Miao and Rajan, 2011; Vance, 2015). Activation of the inflammasome

during *S.* Typhimurium infection leads to macrophage cell death by pyroptosis, neutrophil recruitment, and bacterial clearance by efferocytosis (Jorgensen et al., 2016a, 2016b). The T3SS-2 effectors involved in maintaining an intact SCV limit *S.* Typhimurium detection by the inflammasome. However, flagellin monomers can also be secreted through the T3SS-1 and -2 due to the similarity between these secretion systems and the flagellar secretion system (Lyons et al., 2004). Genetic analysis has shown that flagellin and SPI-1 gene expression are suppressed in the intracellular niche to evade this inflammasome response (Behnsen et al., 2015; Miao et al., 2010; Srikumar et al., 2015).

Within the phagosome environment, both sequestration of essential metals as well the fusion of granules containing antimicrobial peptides (AMPs) with the SCV act together to restrict bacterial replication (Hood and Skaar, 2012). Granule fusion is especially relevant in neutrophils, with primary granules containing MPO contributing to the increased ROS burst observed in neutrophil phagocytes (Borregaard et al., 1995). These granules also contain serine proteases and membrane damaging peptides like α-defensins that form pores in bacterial outer membranes (Faurschou and Borregaard, 2003). Specific granules, which are MPO negative, also contain anti-microbial components, such as lysozyme, which degrades bacterial cell membranes; cationic AMPs that can form pores and penetrate cells to lyse bacteria or cause DNA damage; and metal binding agents like the natural resistance associated macrophage protein (Nramp), lactoferrin, and ferritin, which sequester zinc, manganese, and iron away from bacteria within the phagosome (Gudmundsson and Agerberth, 1999; Le et al., 2017; Oram and

Reiter, 1968; Rest et al., 1978). The *S.* Typhimurium defense against AMPs and other membrane-targeting systems is driven largely by outer-membrane remodeling genes. SPI-11 encodes several genes that are critical for pathogenesis and survival at this stage of infection, including genes that encode the envelope proteins *pagC, envE,* and *envF*, although numerous other genes throughout the genome are important for these modifications (Groisman et al., 1992; Gunn et al., 1995; Miller et al., 1989). Efflux and proteolytic degradation of antimicrobial peptides are also relevant mechanisms for *S.* Typhimurium protection (Nizet, 2006). The sequestration of metal ions by Nramp poses a significant barrier to systemic *S.* Typhimurium infection. While *S.* Typhimurium can colonize the intestines of *Nramp1*$^{+/+}$ mice in a chronic infection model of colitis, infection in these mice does not induce the systemic bacteremia or high bacterial loads observed in *Nramp*$^{-/-}$ mice (Monack et al., 2004; Stecher et al., 2006). *S.* Typhimurium expresses two types of siderophores that can bind and transport iron; enterochelin, which is shared with *E. coli*, and the *S. enterica* specific salmochelin. While enterochelin can be detected and restrained by the host factor lipocalin-2, salmochelin cannot be detected by lipocalin-2 and can sequester iron that is taken up by *S.* Typhimurium through salmochelin specific receptors on the bacterial surface, thereby promoting bacterial survival in response to iron restriction (Crouch et al., 2008; Fischbach et al., 2006; Nairz et al., 2010; Raffatellu et al., 2009; Umler et al., 1998).

The intracellular environment of immune cells is rife with anti-microbial systems to restrict intracellular pathogens. *S.* Typhimurium has numerous mechanisms in place to

react to these antibacterial pathways through the precise modulation of gene expression, the outcome of which is greater resistance to host defense systems.

Adaptive gene expression is driven by environmental sensing

As *S.* Typhimurium transits through a host, it encounters a variety of environments that the bacteria can sense and respond to by reprogramming gene transcription. This adaptive gene expression is a result of regulatory proteins that control gene expression in response to environmental cues. In bacteria, the translation of these environmental cues to changes in gene expression is mediated by two-component systems (TCS) (Soncini and Groisman, 1996; Stock et al., 2000). As the name suggests, TCS are made up of two modules – a protein kinase that acts as a sensor and a response regulator (RR) that acts as an effector protein. Sensor kinase (SK) proteins are usually membrane bound, with a sensing domain that is periplasmic or extracellular and a transmembrane domain that is involved in signal transduction from the sensing domain to the catalytic kinase domain. The sensor domain of the SK can bind ligands or detect stimuli like light, osmolarity, or specific nutrients. Rotation or a piston-type movement of the transmembrane domain amplifies the sensory signal to induce conformational changes in the catalytic domain, allowing for exposure of a conserved histidine phosphorylation residue, dimerization of the kinase domain, and binding of the catalytic domain to ATP within the bacterial cytosol (Cheung and Hendrickson, 2010; Igo et al., 1989; Marina et al., 2005; Zhang and Hendrickson, 2010). This conformational change allows for

phosphotransfer from ATP to the histidine, thereby activating the sensor kinase. The cytosolic domain of the SK binds the RR, allowing for transfer of the phosphoryl group to the receiver domain of the RR. Phosphorylation of an aspartate residue on this receiver domain results in activation of the effector domain on the response regulator, usually by a conformational change in the response regulator that exposes the effector domain for interaction with its target or for dimerization. The effector domain is typically a DNA binding domain, but can also be enzymatic or RNA/protein interacting domains that exert their effects post-transcriptionally or post-translationally (Jacob-Dubuisson et al., 2018; Wang, 2012). Together, the interaction of the sensor kinase and response regulator result in transmission of an environmental stimulus to a change in bacterial behaviour. While the cytoplasmic histidine and catalytic domains of the sensor kinase and regulator domain of the response regulator are well conserved, the sensing and effector domains of SKs and RRs respectively vary widely, resulting in differences in the signals sensed and the resultant effect on the cell (Parkinson and Kofoid, 1992).

While TCS are important for physiological bacterial responses such as swimming and carbon metabolism, several of the *S.* Typhimurium TCS are associated with pathogenesis because mutants of these TCS are less fit in animal infection models (Beier and Gross, 2006). Further characterization of these phenotypes revealed that this is due to modification of the genetic programs in these mutants, indicating that these TCS regulate genes required for pathogenesis (Beier and Gross, 2006; Bijlsma and Groisman, 2003; Cirillo et al., 1998; Feng et al., 2003; Groisman, 2001). Each of the genetic programs

activated in the different niches that *S.* Typhimurium colonizes within a host, such as motility, invasion, and intracellular survival are regulated by specific TCS and by cross-talk between different TCS. Of particular interest are the TCS involved in gene regulation in the intracellular environment because they regulate genes required to adapt to the anti-microbial barrage induced within macrophages. Intriguingly, *S.* Typhimurium has evolved to integrate these immune responses as signals for the activation of these TCS, thereby linking the production of anti-microbial molecules to the adaptive gene expression that, ultimately, evades these very host defenses (Arpaia et al., 2011; Deiwick et al., 1999; Wong et al., 2009).

One system that detects host immunity is the PhoQ-PhoP regulatory system, which is highly conserved across bacterial species and governs several aspects of the *Salmonella* virulence program (Miller et al., 1989). PhoQ-PhoP regulates approximately 5% of genes and is necessary for virulence in mice and humans (Fields et al., 1989; Harari et al., 2010; Zwir et al., 2005). Consisting of a sensor kinase in the inner membrane (PhoQ), and a response regulator in the cytoplasm (PhoP), this system senses low Mg^{2+} *in vitro* (García Véscovi et al., 1996), and low pH (Bearson et al., 1997) and cationic antimicrobial peptides when *S.* Typhimurium is within macrophages (Bader et al., 2005) to mediate rapid adaptation of *Salmonella* to the host environment. PhoQ is a small sensor kinase protein within the bacterial inner membrane, with a periplasmic sensing domain that is conformationally modified following binding of AMPs. The periplasmic domain of PhoQ typically forms close contacts with the phospholipids on the bacterial inner membrane

through divalent cation bridges. Binding of AMPs disrupts coordinating magnesium ions, causing a conformational change in the periplasmic domain that leads to PhoQ activation (Hancock and McPhee, 2005). Low cytosolic pH can independently activate PhoQ through sensing by the C-terminal domain of PhoQ or can boost the AMP-dependent activation (Choi and Groisman, 2016, 2017; Gunn and Richards, 2007; Prost et al., 2007). The net result of PhoQ autophosphorylation by these signals is an overall increase in levels of phosphorylated PhoP, a response regulator with a DNA-binding domain (Shin and Groisman, 2005; Shin et al., 2006). The detection of these environmental cues within the macrophage environment results in the positive regulation of PhoP-activated (*pag*) gene expression through direct binding of phosphorylated PhoP to promoter elements of these genes, and negative regulation of PhoP-repressed (*prg*) gene expression (Groisman, 2001). The genetic program activated by PhoQ-PhoP modifies the *S.* Typhimurium outer membrane to promote resistance to cell lysis by AMPs, increases magnesium uptake and acid tolerance, and up-regulates both the PmrA-PmrB and SsrA-SsrB two-component systems, which are both important for intracellular survival (Bearson et al., 1998; Deiwick et al., 1999; Gunn and Miller, 1996). The loss of virulence in pH-neutralized SCVs is thought to be correlated to a reduction in acid-promoted PhoQ activity, indicating the importance of this TCS to survival within macrophages (Arpaia et al., 2011).

The OmpR-EnvZ TCS is also shared between *E. coli* and *S.* Typhimurium and was initially characterized as the master regulator of outer membrane porin genes in response

to changes in osmolarity (Matsubara et al., 2000; Mizuno and Mizushima, 1990). EnvZ is a sensor kinase with transmembrane and periplasmic domains, but it is thought to sense pH and osmolarity in the cytoplasmic histidine phosphotransfer domain *in vitro* and *in vivo* (Chakraborty et al., 2015; Foo et al., 2015; Wang et al., 2012). A 17-amino acid peptide around the reactive histidine residue has been implicated in detecting increased osmolarity through conformational changes induced by increased hydrogen bonding in this helical domain. This hydrogen bonding can be promoted by a low cytosolic pH, a condition that is thought to be established through the repression of lysine decarboxylase genes by OmpR (Chakraborty et al., 2015). While the specific *in vivo* dynamics of these cues and their role in EnvZ activation are still being characterized, autophosphorylation of EnvZ allows for phosphotransfer to OmpR, which activates the DNA-binding domain to promote transcription of OmpR regulated genes (Mattison and Kenney, 2002). The canonical genes activated by OmpR are *ompF* and *ompC,* both of which encode outer membrane porins that are important for *S.* Typhimurium virulence, and OmpR has also been shown to activate some acid tolerance response genes (Bang et al., 2000; Chatfield, 1991). OmpR promotes transcription of *ssrA* and *ssrB*, genes encoded on the SPI-2 genomic island leading to transcription of the T3SS-2 (Feng et al., 2003; Garmendia et al., 2003; Lee et al., 2000). Together, the intracellular sensing of pH and osmolarity promote survival within macrophages and *in vivo*, making EnvZ-OmpR an important TCS for *S.* Typhimurium virulence (Dorman et al., 1989).

The PhoQ-PhoP and EnvZ-OmpR TCS are important for *S.* Typhimurium virulence and regulate genes that are important in several environments. The osmolarity sensing and porin response, as well as AMP detection and outer membrane remodeling promote survival within the stomach, small intestine, and the gall bladder (Crawford et al., 2010; Richards et al., 2012; van Velkinburgh and Gunn, 1999). Additionally, neither of these TCS are sufficient for activation of the SPI-2 locus, which is required for survival within the SCV (Garmendia et al., 2003; Miao et al., 2002). Co-inherited with the T3SS-2 genes on SPI-2 was a two-gene operon that encodes the regulators for T3SS-2 gene transcription. These genes, *ssrA* and *ssrB*, were found to encode the SsrA-SsrB TCS (Cirillo et al., 1998). SsrA, a putative inner membrane sensor kinase, phosphorylates SsrB, which can bind to the promoters of genes within SPI-2 to activate their transcription. Transcription of *ssrA* is promoted by the activity of phosphorylated OmpR at this promoter and post-transcriptionally by phosphorylated PhoP, allowing for integration of the PhoQ and EnvZ signals to feed into SPI-2 gene expression (Bijlsma and Groisman, 2003; Feng et al., 2003; Garmendia et al., 2003). However, as a sensor kinase, SsrA is thought to respond to low osmolarity, low pH, low concentrations of cations (magnesium and calcium), and phosphate restriction *in vitro* to directly activate SPI-2 genes, independent of OmpR and PhoP, although the relative contributions of these signals has been contested (Coombes et al., 2004; Garmendia et al., 2003; Löber et al., 2006; Miao et al., 2002). Multiple histidine residues in the periplasmic domain of SsrA are thought to be protonated in the low pH environment within macrophages, however SsrA is still able to function in the absence of this signal, suggesting that pH promotes SsrA activity. Although there is no structural

information on SsrA, mutagenesis and sequence homology models suggest the presence of a *Salmonella*-specific ligand binding loop but the ligand responsible for SsrA activity has not been identified (Mulder et al., 2015). While the exact cues or ligands that activate SsrA are unknown, phosphorylated SsrA can phosphorylate the response regulator SsrB, exposing the dimerization and DNA-binding domains on SsrB (Carroll et al., 2009; Feng et al., 2004). SsrB can directly bind to genes within SPI-2, and outside of this island, and activate or repress their transcription (Walthers et al., 2007; Worley et al., 2000). This activity is mediated by a flexible 18 base pair palindromic sequence upstream of SsrB-regulated genes, allowing SsrB to regulate ~5% of the *Salmonella* genome (Tomljenovic-Berube et al., 2010). SsrB-activated genes, like the genes in SPI-2, are required for survival within macrophages, leading to SsrB being considered a master regulator of intracellular virulence (Xu and Hensel, 2010). Additionally, SsrB mediates a key regulatory cross-talk between SPI-1 and SPI-2. The SPI-2-encoded SsrB downregulates SPI-1 genes by repressing HilA and HilD (Pérez-Morales et al., 2017), and reciprocally, SPI-1-encoded HilD upregulates SPI-2 gene expression by directly binding the *ssrAB* operon (Bustamante et al., 2008). This demonstrates the role SsrB plays in mediating the cross-talk between pathogenicity islands to regulate the different lifestyles of *S.* Typhimurium during infection.

The EnvZ-OmpR, PhoQ-PhoP, and SsrA-SsrB TCS together regulate the majority of genes important for survival within macrophages in response to cues generated by the host. However, non-TCS also play roles in sensing the environment and activating adaptive gene expression. Perhaps the best-studied example of a cytoplasmic protein that

can sense a host-derived cue to modulate gene expression is the transcription factor OxyR. OxyR regulates gene expression of the catalases, peroxyreductases, and dismutases encoded in *E. coli* and *S.* Typhimurium in response to the oxidative burst by NADPH oxidase (Tartaglia et al., 1989). This was found to be mediated by cysteine residues on the protein that are oxidised, resulting in disulfide bond formation and a conformational change that promotes DNA binding at specific promoters in the genome (Aslund et al., 1999; Jo et al., 2015; Zheng et al., 1998). The genes regulated by OxyR are vital for the *S.* Typhimurium defense against host-derived ROS within macrophages. SlyA is another transcription factor that promotes intra-macrophage survival, although the cue for SlyA activation has not been identified (Buchmeier et al., 1997). Transcription of *slyA* can be promoted by PhoP, integrating SlyA into the regulatory network that controls genes required for intracellular survival. Analysis of the genes regulated by SlyA found direct transcriptional activation of numerous genes that modify the outer membrane to promote resistance to antimicrobial peptides, however SlyA has also been shown to be important for protection against ROS and the regulation of genes involved in this response (Cabezas et al., 2018; Ellison and Miller, 2006; Navarre et al., 2005; Shi et al., 2004). Interestingly, SlyA can directly promote the transcription of *ssrA* and *ssrB*, suggesting it plays a role in promoting T3SS-2 activity as well (Linehan et al., 2005). The function of SsrB can also be modified by host-generated reactive nitrogen species (RNS) independently of signaling through SsrA (Husain et al., 2010). RNS generated in the intestinal environment during the pro-inflammatory phase of an *S.* Typhimurium infection can be detected through a cysteine residue on the dimerization domain of SsrB, C203,

which hinders the ability of SsrB to bind to DNA and activate SPI-2 gene expression. This mechanism is thought to be one way in which *S.* Typhimurium controls the lifestyle switch between intracellular survival, where SPI-2 genes are required, and a more chronic lifestyle during a long-term colonization model of infection.

The host anti-bacterial response is well developed to restrict survival and replication of intracellular pathogens. However, a combination of horizontally acquired and core genes in the *S.* Typhimurium genome, along with a finely tuned, integrated regulatory network that can directly sense these cues to mount a defense response, allow *S.* Typhimurium to thrive in this niche and render the macrophages and neutrophil environment amenable for bacterial replication and dissemination (Mastroeni and Sheppard, 2004).

Regulatory evolution links horizontally acquired genes and regulation by TCS

Although the acquisition of genes through horizontal transfer has been essential to the evolution of *Salmonella* pathogenesis, the majority of foreign DNA is detrimental to bacteria (Buckling and Rainey, 2002; Navarre et al., 2007). Insertion within coding sequences, overexpression of energetically taxing genes, and activation of unfavourable gene products are all examples of the drawbacks to horizontal gene transfer. The integration of these genes into the core regulatory circuitry of TCS or cytosolic transcription factors is critical for appropriate temporal gene expression and to ensure that these genes do not antagonize existing cellular functions. Until such time as this

integration occurs, transcriptional silencing of horizontally acquired genes protects the bacteria from these laterally acquired genes (Singh et al., 2016).

Seminal work over the past decade has identified the histone like nucleoid structuring protein (H-NS), a DNA binding protein conserved across Gram-negative bacteria, as a broad repressor of horizontally acquired genes (Lucchini et al., 2006; Navarre et al., 2006). The mechanism of this repression relies on the intrinsic curvature of DNA rich in adenine and thymine (AT-rich) (Navarre et al., 2006), which allows for H-NS binding in the minor groove, followed by nucleation and bridging along the DNA that ultimately blocks RNA polymerase (Ali et al., 2012). The existence of H-NS as a global sentinel of horizontally acquired genes has been speculated to contribute to the preferential retention of AT-rich horizontally acquired genes, as H-NS can mitigate the immediate harmful effects of lateral gene transfer (Dorman, 2007; Higashi et al., 2016). De-repression of H-NS by environmental stimuli is one way through which horizontally acquired genes can be activated in the right environments. Changes to DNA curvature induced by osmolarity or temperature shifts can modify the bridging activity of H-NS at specific promoters, allowing for transcription to proceed (Hinton et al., 1992). However, directed activation of virulence genes predominantly occurs through regulatory rewiring of H-NS binding elements to put these genes under the control of virulence regulators, or to allow for transcription factor binding that displaces H-NS bridges from the DNA, through evolution of the regulatory circuitry in *S.* Typhimurium.

Perhaps the best studied example of H-NS counter-silencing in *S.* Typhimurium is by the transcription factor PhoP. The majority of genes identified to be regulated by PhoQ-PhoP have been acquired by horizontal gene transfer (Groisman, 2001) and integrated into the PhoP regulon over evolutionary time. This suggests that horizontally acquired genes co-evolve with the *S.* Typhimurium genome to become assimilated into existing core regulatory architecture, such that the spatiotemporal expression of virulence determinants is tightly controlled. Interestingly, PhoP appears to act differentially to regulate promoters acquired by horizontal gene transfer relative to those predicted to be ancestral (Will et al., 2014). A comparison of the promoter architectures between foreign and ancestral genes suggests that those that were horizontally acquired bind PhoP flexibly with high variability at a number of positions, whereas those that are part of the core genome interact with PhoP in a conserved manner at one binding site (Zwir et al., 2005, 2012). Furthermore, PhoP is capable of activating ancestral promoters directly via RNA polymerase holoenzyme interaction (Will et al., 2014), but horizontally acquired promoters only by counter-silencing of H-NS (Will et al., 2015). The importance of PhoQ-PhoP in regulating horizontally acquired genes is also evident from the divergence in PhoP targets despite the conservation of this regulatory system across diverse taxa. For example, *Yersinia pestis* (Grabenstein et al., 2006)*, Shigella flexneri* (Moss et al., 2000)*, Erwinia carotovora* (Flego et al., 2000)*, Klebsiella pneumoniae* (Cheng et al., 2010)*,* and *Sodalis glossinidius* (Toh et al., 2006) all contain PhoQ-PhoP and are severely attenuated for fitness in its absence, but have strikingly different lifestyles and highly distinct PhoP regulons (Groisman, 2001). These findings indicate that PhoQ-PhoP is a broadly

conserved regulatory system that can flexibly integrate ancestral and acquired genes to accommodate bacterial lifestyles ranging from endosymbiosis to parasitism.

Unlike PhoQ-PhoP, the SsrA-SsrB two-component regulatory system was horizontally acquired on SPI-2 along with the T3SS-2 genes (Fass and Groisman, 2009). Interestingly, in the absence of H-NS, SsrB has a reduced effect on SPI-2 gene expression, and relatively recent work has demonstrated that SsrB can directly displace H-NS polymers along DNA (Walthers et al., 2011). The classical definition of *cis*-regulatory evolution rests upon the accumulation of mutations in noncoding DNA that drift in the nearly neutral range (Stone and Wray, 2001). Those mutations that generate a fitness-increasing quantitative output to alter gene expression may sweep to fixation, creating novel regulatory nodes that result in the flexible expansion of complex genetic networks (Wray, 2007). Adaptation within *cis*-regulatory elements is proposed to contribute to genetic tunability in response to environmental cues, a critical component of host colonization. However, up until recently, empirical evidence for this in the context of bacterial pathogenesis was largely lacking. Recent work has shown that mutations in noncoding DNA are targets for polymorphism-fixing selection to assimilate genes into the SsrB regulon, and that divergence in the regulatory patterns between *S. enterica* and *S. bongori* (which lacks SsrB) confers pathoadaptive fitness differences. For example, following acquisition of SsrA-SsrB by *S. enterica,* promoters that regulate ancestral genes evolved responsiveness to SsrB to fine-tune fitness in the host (Osborne et al., 2009). This involved rewiring the *cis*-regulatory element controlling the ancestral gene that generated

phenotypic diversity among the bacterial population that is selective in the host setting. Work by other groups has since verified and extended these findings, showing that regulatory evolution drives diverse bacterial traits including immune evasion (Tuinema et al., 2014), antibiotic resistance (Horii et al., 1999), and virulence (Li et al., 2009).

The rewiring of gene expression following horizontal gene transfer has allowed *S.* Typhimurium to co-express genes across the genome to promote survival, linking genes that have diverse effects on the bacteria together due to their phenotypic contributions.

Purpose and goals of the present study

While much is known about the genes involved in promoting *S.* Typhimurium survival during an infection, the intracellular environment poses unique barriers to *S.* Typhimurium infection due to the various antibacterial pathways that act to restrict bacterial survival and replication. Given the importance of transcriptional changes to adapt to different environments for bacteria to cause successful infection, my hypothesis was that **certain regulatory modifications in *S.* Typhimurium gene expression evolved to selectively adapt to this intracellular environment**. The aims of this study were to 1) characterize the evolution of transcriptional regulation by SsrB in *S.* Typhimurium and its contribution to intracellular survival; and to 2) identify host cues specific to the intracellular niche that promote adaptive gene expression.

Specific goals highlighted in the following chapters

1. Regulatory evolution drives inflammasome evasion by *Salmonella* Typhimurium

- Horizontal acquisition of SsrB rewired the *Salmonella* Typhimurium genome
- SsrB divergently regulates motility gene expression in *S.* Typhimurium and *S. bongori*
- An evolved SsrB binding region upstream of *S.* Typhimurium *flhDC* drives repression of motility in this species
- SsrB repression of motility in *S.* Typhimurium promotes evasion of the host inflammasome during infection

2. *Salmonella* Typhimurium senses host-derived reactive oxygen species to launch an intracellular virulence program

- Reactive oxygen species induce virulence gene expression in *S.* Typhimurium
- Host NADPH oxidase activity activates an intracellular virulence program
- The regulator SsrA may be involved in sensing reactive oxygen species to drive the activation of *S.* Typhimurium virulence genes

CHAPTER 2: REGULATORY EVOLUTION DRIVES INFLAMMASOME EVASION BY *SALMONELLA* TYPHIMURIUM

Chapter 2: Co-authorship statement

Chapter 2 is a manuscript that has been published in:

Ilyas, B., Mulder D.T., Little D.J., Elhenawy W., Banda M.M., Pérez-Morales D. Tsai, C.N., Chau N. Y E., Bustamante V.H., and Coombes, B.K. (2018). Regulatory evolution drives evasion of host inflammasomes by *Salmonella* Typhimurium. Cell Reports. 25, 825-832.

The manuscript was written by BI and BKC.

The following experiments were performed by collaborators other than myself:

(1) RNA-seq data analysis was performed by DTM and CNT (Figure 2.1, 2.S1, 2.S2)

(2) Western blot analysis was performed by DJL (Figure 2.2D, E, 2.4A, B)

(3) Electron micrograph images were obtained by WE (Figure 2.2B)

(4) Electro-mobility shift assays were performed by DP-M and MMB (Figure 2.3C-E, 2.S3 B,C)

Regulatory evolution drives inflammasome evasion by *Salmonella* Typhimurium

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Abstract

Bacterial two-component regulatory systems (TCS) couple the detection of niche-specific cues with adaptive gene expression to optimize fitness. In Salmonella Typhimurium (STM), the SsrA-SsrB TCS regulates virulence genes needed for survival within host cells, yet the impact of this TCS on regulatory evolution in this pathogen remains incompletely understood. Here, we show that SsrB alters a transcriptional network controlling bacterial motility to limit inflammasome activation during host cell infection. Using comparative RNA sequencing between STM and S. bongori (SBG) engineered to express SsrB, we show that SsrB represses flagellar gene expression in STM but activates this pathway in SBG, which has evolved in the absence of SsrB. Motility repression in STM is driven by an SsrB-binding region upstream of flhDC that appears to have evolved in STM following divergence from SBG. These data reveal a divergent regulatory circuit in non-coding DNA that reduces flagellar gene expression to evade host defenses.

Introduction

The evolution of *Salmonella* as a pathogen has been shaped prominently by genomic evolution via horizontal gene transfer. Horizontally acquired loci, called *Salmonella* Pathogenicity Islands (SPIs), are differentially distributed in different strains and serovars, and contribute to strain-specific traits. For example, the acquisition of SPI-2 following divergence of *S. enterica* from *S. bongori*, conferred on *S. enterica* the ability to survive and replicate within immune cells (Jennings et al., 2017). Thus, the acquisition of SPI-2 allowed *S. enterica* serotypes to exploit new intracellular host niches, thereby expanding their pathogenic potential.

Co-inherited with SPI-2 was SsrA-SsrB, a two-component regulatory system (TCS) that controls expression of SPI-2 genes following intracellular invasion. Under acidic conditions, the SsrA sensor kinase phosphorylates the SsrB response regulator, which then binds to the regulatory region of target genes to regulate their expression directly, or through counter-silencing of H-NS (Mulder et al., 2015; Tomljenovic-Berube et al., 2010; Walthers et al., 2007, 2011). We and others have shown that genes outside SPI-2 have also been captured into the SsrB regulon through mutations in regulatory DNA (Osborne et al., 2009; Pérez-Morales et al., 2017; Tomljenovic-Berube et al., 2010; Worley et al., 2000). The optimized transcriptional output of these genes, in addition to that of the classical SPI-2 virulence genes, contributes to bacterial fitness within the host.

Many of the genes outside of SPI-2 that are controlled by SsrB have orthologs in *S. bongori* (SBG), which is interesting because *S. bongori* lacks SPI-2 and has evolved in the absence of SsrA-SsrB. Here, we used comparative transcriptomics between *S.* Typhimurium (STM) and SBG, engineered to express SsrB, to probe the extent to which SsrB has shaped the regulatory landscape in STM. We identified 396 orthologous *Salmonella* genes from 11 functional classes that were divergently regulated by SsrB in both species, including over 100 genes of unknown function. One of these functional classes, bacterial motility, was systematically repressed by SsrB in STM but was activated in SBG. We demonstrate that the SsrB-mediated repression of flagellar-based motility is required to limit inflammasome activation during intracellular infection of macrophages.

Results

Comparative RNA-sequencing reveals divergent gene regulation by SsrB

The SsrB response regulator is essential for *Salmonella* virulence because it controls expression of genes required for intracellular survival. To investigate the extent of regulatory rewiring in STM, we compared gene expression in the SsrB-naïve species, SBG, to that of an SsrB-adapted species, STM. We engineered a constitutively active SsrB variant (SsrB D56E) that does not require input from SsrA, and expressed it in SBG and in STM Δ*ssrAB*. RNA extracted from SsrB expressing strains ($SsrB^+$) and from vector-controlled strains ($SsrB^-$) was sequenced and analyzed by Rockhopper (McClure et

al., 2013; Tjaden, 2015). Sequencing reached an average of ~10 million reads from three biological replicates per strain with ~150× genome coverage. We identified 331 genes (~6% of the genome) that were up-regulated >2-fold and 224 genes down-regulated >2-fold in $SsrB^+$ STM relative to $SsrB^-$ (Fig. 2.S1A). Among the genes with altered regulation in STM, 62% of the up-regulated genes and 87% of the down-regulated genes had orthologs in SBG as identified by OrtholugeDB (Whiteside et al., 2013). We were particularly interested in orthologous genes for which SsrB-dependent regulation was discordant between STM and SBG as these likely represent the outcome of regulatory evolution in STM (Fig. 2.1A). This included 20 genes up-regulated in STM but down-regulated in SBG, and 14 genes down-regulated in STM but up-regulated in SBG.

To understand the types of genes influenced by SsrB, up- and down-regulated genes were assigned to functional classes and COG categories (Tatusov et al., 2000) (Fig. 2.1B and S2.1C). The 156 genes unique to STM that were affected by SsrB were mainly in genomic islands, such as SPI-2 and T3SS-2 effector cargo (Fig. 2.S1B-D). Among the orthologs present in the two organisms, this analysis revealed three striking regulatory patterns in STM; (*i*) SsrB-mediated up-regulation of genes involved in innate immunity defense, (*ii*) SsrB-mediated down-regulation of cellular invasion machinery, and (*iii*) SsrB-mediated down-regulation of motility (Fig. 2.1B, C, and 2.S2). In SBG, motility genes appeared to follow a regulatory pattern of up-regulation in response to SsrB.

SsrB divergently regulates flagellar motility in STM and SBG

Our transcriptomic analysis revealed an SsrB-dependent program that systematically repressed genes involved in flagellar-based motility in STM, while having a disordered effect in SBG (Figure 2.S2). This discordant effect of SsrB on motility was confirmed in bacterial swimming assays where active SsrB significantly decreased swimming in STM and increased it in SBG (Fig. 2.2A). This phenotype was consistent with a significant decrease in the number of peritrichous flagella per cell in $SsrB^+$ STM and a significant increase in surface flagella in $SsrB^+$ SBG measured by electron microscopy, with concomitant differences in total FliC protein levels in the two species (Fig. 2.2B-D). The level of SsrB expressed in STM and SBG did not account for these motility phenotypes, as SsrB protein levels were similar in STM and SBG (Fig. 2.2E). Together, these data indicated that STM contains a regulatory mechanism to repress flagellar-based motility by SsrB, a transcription factor involved in intracellular survival.

SsrB binds to the *flhDC* promoter in STM

Previous studies showed that flagellar expression is repressed in STM under SPI-2-inducing conditions and during macrophage infections through unknown mechanisms (Brown et al., 2014; Kröger et al., 2013; Srikumar et al., 2015). Flagellar gene expression is organized into a hierarchy of three promoter classes (Kalir et al., 2001), where the class I promoter activates the master transcriptional regulator, FlhDC, followed by the apparatus and chemotaxis genes through the class II and III promoters. We confirmed our

RNA-seq data by showing SsrB-dependent repression of all levels of the motility hierarchy in STM by RT-qPCR (Fig. 2.3A). The *flhDC* promoter is a hotspot for transcriptional input into the motility regulatory cascade by activators (Erhardt and Dersch, 2015) and repressors (Mouslim and Hughes, 2014). For example, SlyA is a repressor of *flhDC* that is activated by SsrB; however, SsrB was able to repress motility in a Δ*slyA* mutant equivalently to wild type, thus ruling out contributions by SlyA (Fig. 2.S3A). HilD is a transcriptional regulator shared by STM and SBG that is known to activate *flhDC* (Singer et al., 2014). Although we found that purified HilD bound equally well to the STM and SBG *flhDC* promoters (Fig. 2.S3B), this interaction did not impact the binding of SsrBc to the STM *flhDC* promoter or vice versa (Fig. 2.S3C), thus ruling out a competitive inhibition by SsrB. We also confirmed that the evolved intergenic region in front of *flhDC* in STM conferred this repression because when this sequence was replaced with the intergenic region upstream of *flhDC* from SBG and expressed in STM, repression of *flhD* was lost (Fig. 2.3B).

To probe the interaction of SsrB with the motility program more deeply, we incubated purified SsrBc with DNA from intergenic regions upstream of *flhDC, fliA,* and *fliC* from STM and upstream of *flhDC* from SBG. SsrBc shifted the full-length 910 bp *flhDC* promoter fragment from STM, but not the corresponding fragment from SBG or the full-length promoter fragments of *fliA* and *fliC* from STM (Fig. 2.3C and D). These data are consistent with SsrB-mediated repression occurring at the top of the motility regulatory hierarchy. Transcriptional fusions of the full-length (-827+83) and truncated (-

328+83, -197+83) *flhDC* promoters were made to report luciferase activity in response to SsrB. Consistent with the previous binding experiments, the -827+83 fragment showed SsrB-dependent repression of *flhDC* (Fig. 2.S3D). We further narrowed this down to a -328+83 fragment upstream of the P1 transcriptional start site that also retained repression and was shifted by SsrBc (Fig. 2.3E and 2.S3E). However, a -197+83 fragment (downstream of P1) was no longer bound by SsrBc and did not confer SsrB-dependent repression (Fig. 2.3E and 2.S3F). Since deletion of P1 completely eliminates motility (Mouslim and Hughes, 2014), we conclude from these data that the SsrB binding site must be within the 131 bp region between -328 and -197 around the P1 transcriptional start site.

SsrB-dependent repression of flagellar gene expression limits inflammasome activation

During intracellular infection of macrophages, cytoplasmic flagellin induces NAIP/NLRC4 inflammasome-dependent activation of caspase-1 leading to the release of IL-1β and eventual pyroptosis, which can lead to bacterial clearance (Broz and Dixit, 2016; Jorgensen et al., 2016a, 2016b; Vance, 2015). Therefore, we speculated that the selective driver for SsrB-mediated repression of motility was to limit caspase-1 activation and IL-1β release. We first confirmed that SsrB repressed flagellin *in vivo* by measuring luciferase activity from the *fliC* promoter. Mice infected with either $SsrB^-$ or $SsrB^+$ STM that expressed a *fliC-lux* transcriptional fusion showed that $SsrB^+$ STM had significantly reduced luciferase activity compared to $SsrB^-$ STM (Fig. 2.S4). We additionally

demonstrated that, similar to our *in vitro* results, FliC was more abundant in macrophages infected with $SsrB^-$ STM and was undetectable in cells infected with $SsrB^+$ STM (Fig. 2.4A). Next, the effect of these FliC levels on inflammasome activation was tested in bone marrow-derived macrophages (BMM) using secretion of caspase-1 and the pro-inflammatory cytokine IL-1β as readouts following infection with STM and SBG (Mariathasan et al., 2004; Miao et al., 2006). Infection with $SsrB^-$ STM triggered caspase-1 processing, enriching for the active 20 kDa subunit, and significantly increased secretion of IL-1β (Fig. 2.4B and C). This activity was dependent on both FliC and SsrB because deletion of *fliC* in $SsrB^-$ STM, or expression of SsrB in *fliC*-positive STM, prevented caspase-1 processing and significantly inhibited IL-1β release from infected BMM (Fig. 2.4B and C). In contrast, SBG was unable to mediate repression of flagellin through SsrB. $SsrB^+$ SBG significantly induced caspase-1 activation and IL-1β secretion from infected BMM (Fig. 2.4B and C). Treating BMM with the caspase-1 inhibitor Z-YVAD-FMK fully prevented caspase-1 processing and IL-1β release from infected cells (Fig. 2.4B and C). Together, these data identified a regulatory circuit that represses flagellin in STM by using a horizontally-acquired TCS that becomes highly active in the intracellular setting. This evolved regulatory circuit limits inflammasome activation and dampens the host response from infected cells.

Discussion

The SPI-2 genomic island in STM is a key phylogenetic discriminator between STM and the closely related species, SBG. The absence of SPI-2 in SBG has provided a means to study how regulatory evolution influences pathogenic adaptation in *Salmonella* through appropriation of shared homologous genes (Osborne et al., 2009). Several studies show that SsrB regulates a large number of genes in STM involved in virulence traits (Brown et al., 2014; Colgan et al., 2016; Tomljenovic-Berube et al., 2010; Yoon et al., 2009) but few studies have determined whether SsrB-dependent regulation of homologous genes provides a fitness advantage to the bacteria during infection. Thus, whether or not these regulatory connections represent evolved and selective *cis*-regulatory inputs or reflect spurious gene expression has not been clear. We addressed this by comparing genome-wide SsrB-dependent regulatory patterns between STM, whose genome has evolved in the presence of SsrB, and SBG, whose genome is naïve to SsrB. In doing so we identified SsrB as a negative regulator of flagellar gene expression during the intracellular stages of STM infection in macrophages. The repression of flagellin limits NAIP/NLRC4 inflammasome-dependent caspase-1 activation by intracellular STM and prevents IL-1β release, an immune evasion event that is required for *Salmonella* virulence (Miao and Rajan, 2011). Thus, our comparative RNA-seq approach has allowed us to investigate the co-evolution of a pathogen with a virulence associated transcription factor to identify biologically relevant adaptations in pathogenic *Salmonella*.

Given the large number of SBG genes affected by SsrB, it is likely that the early SsrB regulon in STM also included genes whose intracellular expression was detrimental to bacterial survival. Our data is consistent with motility being the target of regulatory evolution, where there appears to have been selection for SsrB-dependent repression of this pathway to limit intracellular detection. Accounting for this phenotype was a differential interaction between active SsrB and the promoter region upstream of the flagellar master regulator, *flhDC,* where SsrB bound this DNA region in STM but not in SBG. This illuminates a regulatory solution to the problem of antagonistic pleiotropy created by the acquisition of a new transcription factor. The type 3 secretion system-1 (T3SS-1) rod protein, PrgJ, can also be detected by cytosolic NAIPs (Kofoed and Vance, 2011; Miao et al., 2010) and it is likely that control of this virulence machinery has also been selected by regulatory evolution. For example, SsrB has been shown to directly repress the *hilD* and *hilA* regulatory genes that activate SPI-1 gene expression involved in assembling the T3SS-1 (Pérez-Morales et al., 2017).

Although our study has focused on *S.* Typhimurium, there is evidence that control of intracellular flagellin might also play an important role in *S.* Typhi, the *Salmonella* serovar associated with typhoid fever in humans (Winter et al., 2015). An emerging clade of invasive non-typhoidal *Salmonella*, ST313, that causes life-threatening bloodstream infections (Feasey et al., 2012) also seem to express less flagellin compared to the phylogenetically related ST19 lineage commonly associated with gastrointestinal disease worldwide (Carden et al., 2015; Ramachandran et al., 2015). These data imply that the

ST313 lineage might also exploit regulatory evolution of homologous genes to modify its virulence properties (Hammarlöf et al., 2018).

An important case study for the evolution of regulatory networks leading to bacterial phenotypic diversity is the transcription factor PhoP (Perez and Groisman, 2009a, 2009b). In this system, functional changes in the PhoP protein and in *cis*-regulatory elements within the genomes of PhoP-containing bacteria enable the customization of the PhoP regulon. Similarly, STM and the human-restricted serovar *S.* Typhi both share the transcription factor, OmpR, and have maintained a set of core OmpR-regulated genes while diversifying themselves with a serotype-specific regulon that plays a role in host colonization (Perkins et al., 2013). By comparing the differential regulation of orthologous genes by SsrB, the RNA-seq dataset generated in our study provides a means to uncover additional sources of immune evasion by intracellular *Salmonella*, in addition to other infection traits. For example, investigation of the manually curated functional classes identified divergent regulation of genes involved in succinate metabolism between the two species. The *sucBCD* and *sdhABD* genes are repressed in STM but unchanged or up-regulated in SBG (Figure S2). Interestingly, these genes are also repressed during STM infection in macrophages but have been shown to be important for luminal colonization during *Salmonella* infection of the mouse gut (Spiga et al., 2017; Srikumar et al., 2015). Together, these data could indicate that SsrB has influenced regulatory evolution to optimize metabolic fitness in different host niches.

In summary, our data highlight that the mammalian immune system imposes a selective pressure on intracellular bacteria to repress the expression of immune-activating proteins, ultimately compromising host defenses. With antibiotic resistance on the rise and bacteria increasingly being linked to acute and chronic diseases, a thorough accounting of bacterial immune evasion mechanisms may ultimately guide new paradigms in antimicrobial treatments.

Acknowledgements

We thank Brandon Aubie for assistance with generating the RNA-seq figures. We thank the McMaster Genome Facility for RNA-sequencing. This work was supported by grants from the Canadian Institutes of Health Research (MOP-82704, 376674, and 388472 to B.K.C.), Consejo Nacional de Ciencia y Tecnología (CONACYT)/México (254531), and Dirección General de Asuntos del Personal Académico de la UNAM/México (IN203415) (to V.H.B.). D.J.L. was supported by a Michael G. DeGroote postdoctoral fellowship and a CIHR postdoctoral fellowship in partnership with the Canadian Association of Gastroenterology (CAG), M.M.B. was supported by a pre-doctoral fellowship from CONACYT/México (403748). B.K.C. is the Canada Research Chair in Infectious Disease Pathogenesis.

Materials and methods

Contact for Reagent and Resource Sharing

Further information and requests for reagents may be directed to and fulfilled by the corresponding author Brian K Coombes (coombes@mcmaster.ca).

Experimental model and subject details

Bacterial strains and culture conditions

A detailed list of strains and plasmids used in this study is provided in the key resources table.

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) strain SL1344 and *Salmonella bongori* 66:z41 were used in this study (Coombes et al., 2003; Osborne et al., 2009). Routine propagation of bacteria was in LB media supplemented with appropriate antibiotics. For SPI-2 inducing conditions, overnight cultures were grown in LB, sub-cultured 1:50 to mid log in LB, then sub-cultured into minimal media with low magnesium, low phosphate (LPM) at pH 5.8 with shaking for 5 h (mid-log) before downstream analysis (Coombes et al., 2004). Antibiotic concentrations used were chloramphenicol (34 μg/ml), kanamycin (50 μg/ml), and ampicillin (100 μg/ml) unless otherwise mentioned.

Mice

All animal experiments were performed according to the Canadian Council on Animal Care guidelines using protocols approved by the Animal Review Ethics Board at McMaster University under animal use protocol #17-03-20. Female, six-to-eight-week old C57BL/6J mice were purchased from Charles River Laboratories and used for all murine infections or for isolation of murine bone-marrow derived macrophages. Mice were stored in Biosafety Level 2 SPF barrier facility at the McMaster Central Animal Facility. Mice were fed a regular chow diet ad libitum.

Cell culture

Cell lines were maintained in a humidified incubator at 37°C and 5% CO_2. RAW264.7 macrophages were grown in DMEM, 10% FBS (Gibco) and seeded in 10 cm tissue culture treated dishes the day before infection. Bone marrow-derived macrophages were differentiated from the marrow isolated from the hind legs of 6-10-week old female C57BL/6 mice, grown in RPMI, 10% FBS, 100 U penicillin-streptomycin and 10% L929 cell conditioned media at 37°C and 5% CO_2. Cells were differentiated for 7 days in 150 mm petri dishes, then lifted with ice-cold PBS for seeding in tissue culture treated plates 16 hours prior to infection.

L929 conditioned media was collected from the supernatants of L929 fibroblasts grown in DMEM + 10% FBS for ten days.

Method details

Cloning and mutant generation

Primers used for cloning and mutant generation are listed in table S2.1. PCR's were performed with Phusion, Phire II, or Taq DNA polymerases (Thermo).

S. Typhimurium Δ*ssrAB* was generated in Mulder et al., 2015. pWSK129-P*ssrA-ssrB* - D56E was generated in Pérez-Morales et al., 2017 and transformed into *S.* Typhimurium Δ*ssrAB* and *S. bongori* to generate $SsrB^+$ strains. $SsrB^-$ strains were transformed with the empty pWSK129 vector.

pWSK129-P*ssrA-ssrB*-FLAG D56E was generated by amplification of P*ssrA-ssrB* D56E from pWSK129-P*ssrA-ssrB* D56E with primers that introduced a C-terminal FLAG-tag.

pACYC184-P$flhD_{SBG}$-$flhD_{STM}$ used for complementing the *S. bongori* promoter was generated by splicing by overlap extension (SOE) PCR of the SBG *flhDC* intergenic region and the STM *flhD* gene, followed by ligation into pACYC184. pACYC184-P$flhD_{STM}$-$flhD_{STM}$ was generated by amplifying the full STM *flhDC* intergenic region and *flhD* gene. Sequences were verified and plasmids transformed into *S.* Typhimurium.

Transcriptional reporters were generated by amplifying the intergenic regions upstream of the translational start site for *flhD* or *fliC* in STM, which were ligated into pGEN-*luxCDABE* (Lane et al., 2007). Sequence-verified plasmids were transformed into STM.

An in-frame mutant of *S.* Typhimurium *flhD* was generated by Lambda-Red recombination using pKD46 expressed in a Δ*ssrAB* background and a PCR product amplified from pKD3, following the protocol of Datsenko and Wanner, 2000. Red recombinase enzymes were induced with 100 mM freshly prepared L-arabinose (BioShop). Transformants were selected on LB-chloramphenicol (10 μg/ml) and verified by PCR. Flp-recombinase-expressing pFlp2 was used to flip out the antibiotic cassette (Choi and Schweizer, 2005). pACYC184-P*flhDC*$_{SBG}$-*flhD*$_{STM}$ or pACYC184-P*flhDC*$_{STM}$-*flhD*$_{STM}$ were transformed into Δ*ssrAB* Δ*flhD*, and pWSK129 or pWSK129-P*ssrA-ssrB* D56E were transformed into each of the strains.

In-frame, marked mutants of *S.* Typhimurium and *S. bongori fliC* were generated by Lambda-Red recombination using pKD46 and transformed linear PCR products amplified from pKD3. Transformants were selected on LB containing chloramphenicol (10 μg/ml) and knockouts were verified by PCR. The *S.* Typhimurium *fliC::cat* mutant was transduced into Δ*ssrAB* pWSK129 or pWSK129-P*ssrA-ssrB* D56E using P22 HT phage.

RNA isolation for sequencing and RT-qPCR

For all RNA isolation, bacterial strains were grown under SPI-2 inducing conditions. Following growth to OD_{500} 0.4-0.5, 10 ml of each strain was pelleted by centrifugation and resuspended in 2 ml Trizol (Invitrogen) for cell lysis. RNA was extracted by chloroform (BioShop) separation following the manufacturer's protocol, precipitated with 100% isopropanol (BioShop) and washed with 75% ethanol (Sigma), and treated with Dnase I (Invitrogen). RNA was then re-precipitated in isopropanol following Dnase I inactivation (Dnase inactivation beads, Ambion), washed with ethanol, and resuspended in DEPC water (Invitrogen). RNA concentrations were quantified by nanodrop prior to downstream analysis.

RNA-seq library preparation and gene expression analysis

All RNA-seq data are from three biological replicates per strain. Prior to RNA-sequencing, purified RNA was treated with Ribo-Zero for rRNA depletion and cDNA was barcoded for each sample. cDNA was sequenced on an Illumina Hi-Seq platform. Sequencing data was aligned against the reference genomes for *Salmonella* Typhimurium (NC_016810) and *Salmonella bongori* (NC_015761), and analyzed using Rockhopper.

Average gene expression values were calculated with normalization by the upper quartile of gene expression with Rockhopper (McClure et al., 2013). Differential expression for each species was calculated from expression values for the $SsrB^+$ and $SsrB^-$ strains ($SsrB^+/SsrB^-$). Genes were prioritized based on two conditions: an absolute read count of at least 100 in one condition ($SsrB^+$ or $SsrB^-$) in either species, and a differential

expression level of at least two-fold between conditions. Orthologous genes between the two species were identified using OrtholugeDB (Dataset S1). Total numbers of *S.* Typhimurium specific and orthologous genes that were differentially expressed were compiled in UpSet (Lex et al., 2014). Priority genes were manually classified into ten functional categories based on function reported in the literature. Genes with no reported function were classified as unknown. Genes were also classified based on COG categories (NCBI). All genes with over 100 transcript reads were mapped by genome location using Circos (Krzywinski et al., 2009). Heat maps were generated in Excel with a maximum cut off fold change of $\log_2$ 5 or -5. Scatter plots were generated using a custom Python script.

Motility assays

The relevant bacterial strains were grown to mid-log phase in LB, normalized to OD_{600} of 0.1, and 2 µl of each strain was spotted on LPM pH 5.8 media with 0.25% agar. Following stationary incubation for 5 h at 37°C, plates were imaged on a Canon scanner. Motility zones were measured using ImageJ, averaging three independent measurements per motility zone. Data are representative of three independent biological replicates.

Electron microscopy and flagellin levels *in vitro*

Strains were grown under SPI-2 inducing conditions without shaking at 37°C for 6 h. Following this, 10 µl of each bacterial culture was spotted onto Formvar-coated 200

mesh Cu/Pd grids for 10 min. Excess liquid was removed using filter paper and the samples were stained with 1% (w/v) uranyl acetate (Sigma-Aldrich) for 1 minute. Grids were washed for 2 min with water and dried and examined using a JEOL JEM 1200 EX TEMSCAN transmission electron microscope at 80 kV. Images were acquired with an AMT 4-megapixel digital camera (Advanced Microscopy Techniques). The number of flagella per cell were determined by manually counting attached flagella for fifty bacterial cells per strain. In parallel, bacteria grown in LPM pH 5.8 without shaking for 6 h were pelleted and lysed in 1× Laemmli buffer for western blot analysis of flagellin levels.

SsrB-FLAG analysis

Strains carrying pWSK-P*ssrA-ssrB*-FLAG D56E were grown under SPI-2 inducing conditions for 5 h, normalized to an OD_{600} of 0.5 and 1 ml was centrifuged at 10,000 *g* and resuspended in 1× Laemmli for western blot analysis.

RT-qPCR

cDNA was synthesized from purified RNA following growth of bacteria in SPI-2 inducing conditions using qScript cDNA Supermix (Quantabio), diluted 1:10 and used in quantitative PCR reactions for various motility genes and the housekeeping gene *rsmC* in a LightCycler 480 (Roche) with Quanta Perfecta SYBR Green Supermix (Quantabio). Quantification was determined based on a standard curve for each gene and normalized

ratios ($SsrB^+/SsrB^-$) were calculated relative to *rsmC*. Data are representative of three to six independent biological replicates.

In vivo bioluminescent imaging

Eight to ten-week-old female mice were orally gavaged with 20 mg streptomycin, and their abdomens were shaved and depilated with Nair hair removal cream in preparation for imaging one day prior to infection, as described previously (Tuinema et al., 2014). Mice were orally gavaged with 10^8 cfu of STM (pGEN-P*fliC*$_{STM}$-*lux*) in 0.9% NaCl and 100 mM HEPES and imaged for luminescence quantification with an IVIS Spectrum (Perkin Elmer) *In vivo* Imaging System (IVIS). Mice were anesthetised and luminescence was imaged every hour for 6 h. Flux/h was determined from defined regions of interest (ROI) for each mouse at each time point using Living Image software (PerkinElmer). Data are representative of four mice per strain and of two independent experiments.

Macrophage infections

Bacterial strains were grown in LB to $OD_{600} \sim 0.9$, pelleted, and resuspended in cell culture media for macrophage infections at a multiplicity of infection (MOI) of 40:1.

RAW cell infections were performed in 10 mm petri dishes. Bacteria were opsonized in 20% human serum for 30 min at 37°C prior to infection. Infected plates were spun for 2

min at 500 *g* to synchronize host cell attachment. Following 1 h of infection at 37°C and 5% CO_2, cell supernatants were decanted and cells were washed 3× with PBS, scraped from the plate and harvested by centrifugation at 10,000 g. Cell pellets were lysed in PBS containing 1% (v/v) Triton-X100, 0.1% (w/v) SDS and boiled in SDS loading dye for downstream analysis.

Differentiated bone-marrow derived macrophages were seeded in 96-well plates in RPMI+10% FBS for IL-1β quantification or in 12-well plates in RPMI only for caspase-1 quantification, 16 hours prior to infection. Three hours prior to infection, bone-marrow derived macrophages were stimulated with 5 μg/ml *Salmonella minnesota* LPS R595 (EMD Millipore) and treated with 200 μM Z-YVAD-FMK (BioVision) or an equal volume of DMSO (BioShop) as a vehicle control for caspase-1 inhibition. Lysis control wells were treated with 10X lysis buffer from the LDH cytotoxicity kit (Pierce) at the time of bacterial infection. Following 1 h of infection in BMMs, plates were centrifuged again and supernatant transferred to a storage plate frozen at -20°C for downstream IL-1β measurement. Total IL-1β was measured using a mouse IL-1β ELISA kit (Thermo Fisher Scientific). Cell cytotoxicity was measured by LDH production (Pierce LDH cytotoxicity kit) and mature IL-1β was calculated by adjusting total IL-1β levels to % cytotoxicity as described previously (Miao et al., 2006).

Supernatants from 12-well plates were precipitated with ice-cold 10% TCA overnight. followed by an acetone wash for secreted protein analysis by western blot for caspase-1.

SDS-PAGE and western blot analysis

Protein samples from bacterial cell pellets were boiled for 10 min and separated by SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane, blocked with Tris-buffered saline with 0.1% (w/v) Tween-20 (TBST) containing 5% skimmed milk and probed with mouse anti-DnaK (Enzo Life Sciences, 1:5000), mouse anti-FliC (BioLegend, 1:1000), or mouse anti-FLAG (SigmaAldrich, 1:5000) in TBST with 1% (w/v) skim milk. Secondary antibodies were goat anti-mouse-HRP conjugated antibodies (Jackson Immunoresearch, 1:5000) in TBST containing 1% (w/v) skim milk. Blots were developed with Clarity Max Western (BioRad) ECL substrate and imaged with a ChemiDoc XRS+ (BioRad). Protein fractions from macrophage infections were processed as described above and probed with mouse anti-DnaK (Enzo Life Sciences, 1:5000), mouse anti-FliC (BioLegend, 1:1000), or goat anti-GAPDH (R&D Systems Inc., 1:5000) in TBST with 1% (w/v) skim milk. Blots were developed as described previously using goat α-mouse-HRP conjugated (Jackson Immunoresearch, 1:5000) and donkey anti-goat-HRP conjugated (Santa Cruz Biotechnology, 1:5000) as secondary antibodies in TBST containing 1% (w/v) skim milk. For caspase-1 detection, protein fractions were separated by SDS-PAGE, transferred to PVDF, and blocked in TBST containing 3% (w/v) bovine

serum albumin (BSA). The membrane was probed with mouse anti-caspase-1 (Adipogen Life Sciences) using a 1:4000 dilution in TBST containing 3% (w/v) BSA. Caspase-1 was then detected using goat anti-mouse-HRP conjugated antibody as secondary (Jackson Immunoresearch) using a 1:5000 dilution in TBST containing 3% (w/v) BSA.

Protein purification and electrophoretic mobility shift assays

A His-tagged fusion protein 6H-SsrBc was expressed in *E. coli* BL21(DE3) and purified using Ni^{2+}-NTA-agarose affinity chromatography as described previously (Pérez-Morales et al., 2017). Maltose binding protein (MBP)-HilD was expressed in *E. coli* BL21(DE3) and purified using an amylose affinity column as described previously (Bustamante et al., 2008). 6H-SsrBc EMSAs were performed in binding buffer containing 10 mM Tris pH 7.5, 50 mM KCl, 2.5% glycerol, 5 mM $MgCl_2$ and 0.05% Nonidet P-40 at concentrations ranging from 0 to 2 μM. MBP-HilD EMSAs were performed in binding buffer containing 10 mM Tris-HCl, pH 8, 50 mM KCl, 1 mM DTT, 0.5 mM EDTA, 5% glycerol and 10 μg/ml BSA at concentrations ranging from 0 to 1 μM. PCR-amplified regulatory regions of *flhDC, fliA, fliC,* and *ppK* from *S.* Typhimurium and *flhDC* from *S. bongori* were purified with the QIAquick PCR purification kit (Qiagen). 100 ng of each PCR product was incubated with the relevant proteins for 20 min at room temperature. Protein-DNA reactions were separated by electrophoresis on 6% non-denaturing acrylamide gels in 0.5× Tris-borate-EDTA buffer at room temperature. DNA was

visualized with ethidium bromide on an Alpha-Imager UV transilluminator (Alpha Innotech Corp.).

For competitive EMSAs between 6H-SsrBc and MBP-HilD, the PCR-amplified *flhDC* regulatory region was either first mixed and incubated with 2 μM 6H-SsrBc or 1 μM MBP-HilD for 20 min and then mixed and incubated with 1 μM MBP-HilD or 2 μM 6H-SsrBc, respectively, for additional 20 min, or mixed and incubated at the same time with 2 μM 6H-SsrBc and 1 μM MBP-HilD for 20 min. Binding reactions were performed in the buffer described above for EMSAs with 6H-SsrBc. Protein-DNA complexes were analyzed as described above.

Quantification and Statistical Analysis

Statistical parameters such as number of biological replicates and exact P values of significant data are indicated in the figure legends. Data were analyzed using GraphPad Prism 6.0 software using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test unless otherwise mentioned. P values of <0.05 were considered significant.

Data and Software Availability

Raw RNA-seq data have been deposited in the NCBI Gene Expression Omnibus (Edgar et al., 2002) under accession number GSE120043.

Figures and Tables

Figure 2.1. SsrB changes global gene expression in STM and SBG.

(A) Comparative analysis of gene regulation in STM and SBG. Each bar represents the number of genes within an expression group (filled circle). Vertical bars represent the sum of all genes within the expression category. (B) Functional group and COG categories for orthologous genes in each set identified in (A). All differentially expressed genes were assigned to a COG category (outer ring) and by functional group through manual curation (inner ring). (C) Distribution of orthologous genes that were differentially regulated by SsrB in STM versus SBG. Dashed lines indicate cutoffs for up or down regulation in STM and SBG. See also Fig. 2.S1 and 2.S2.

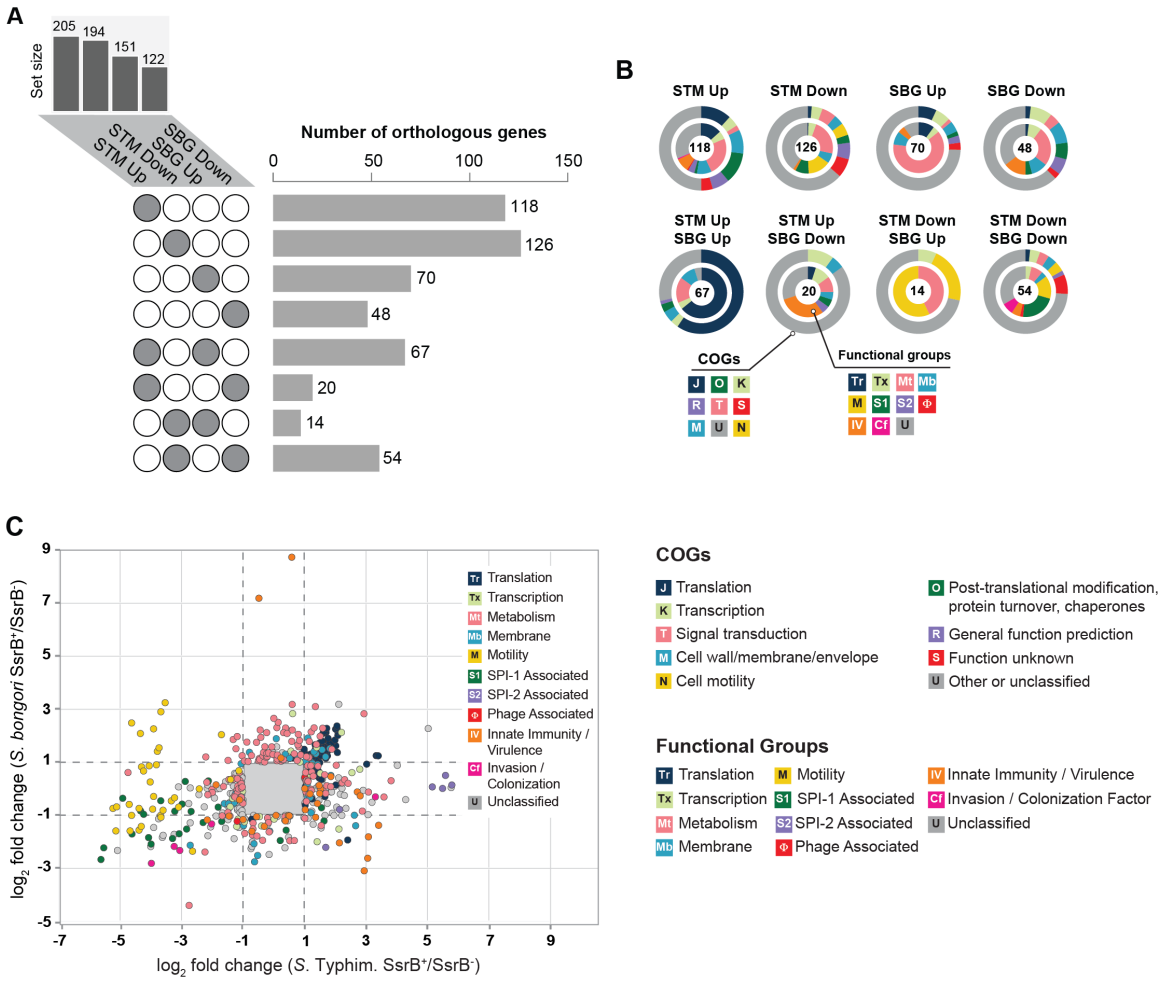
A
Set size
205
194
151
122
STM Up
STM Down
SBG Up
SBG Down
Number of orthologous genes
0
50
100
150
118
126
70
48
67
20
14
54
B
STM Up
STM Down
SBG Up
SBG Down
118
126
70
48
STM Up SBG Up
STM Up SBG Down
STM Down SBG Up
STM Down SBG Down
67
20
14
54
COGs
Functional groups
C
log2 fold change (S. bongori SsrB+/SsrB-)
log2 fold change (S. Typhim. SsrB+/SsrB-)
Translation
Transcription
Metabolism
Membrane
Motility
SPI-1 Associated
SPI-2 Associated
Phage Associated
Innate Immunity / Virulence
Invasion / Colonization
Unclassified
COGs
Translation
Transcription
Signal transduction
Cell wall/membrane/envelope
Cell motility
Post-translational modification, protein turnover, chaperones
General function prediction
Function unknown
Other or unclassified
Functional Groups
Translation
Transcription
Metabolism
Membrane
Motility
SPI-1 Associated
SPI-2 Associated
Phage Associated
Innate Immunity / Virulence
Invasion / Colonization Factor
Unclassified

Figure 2.2. SsrB expression inhibits motility and flagellar production in STM but activates motility in SBG.

(A) SsrB differentially affects swimming motility in STM and SBG. Data are mean ± SEM from n=3, ****P<0.0001 for comparison to $SsrB^-$ within each species. (B) Transmission electron micrograph images of STM and SBG expressing SsrB ($SsrB^+$) or not ($SsrB^-$). Scale bars represent 1 μm. (C) Quantification of surface flagella from fifty individual bacteria per strain. ****P<0.0001 for comparison to $SsrB^-$ within each species. (D) Protein levels of FliC. Lysates from $SsrB^-$ and $SsrB^+$ STM and SBG were analyzed by western blot with α-FliC. α-DnaK was used as a loading control. (E) Western blot showing SsrB-FLAG is equally expressed in STM and SBG under SPI-2-inducing conditions. DnaK was used as a loading control.

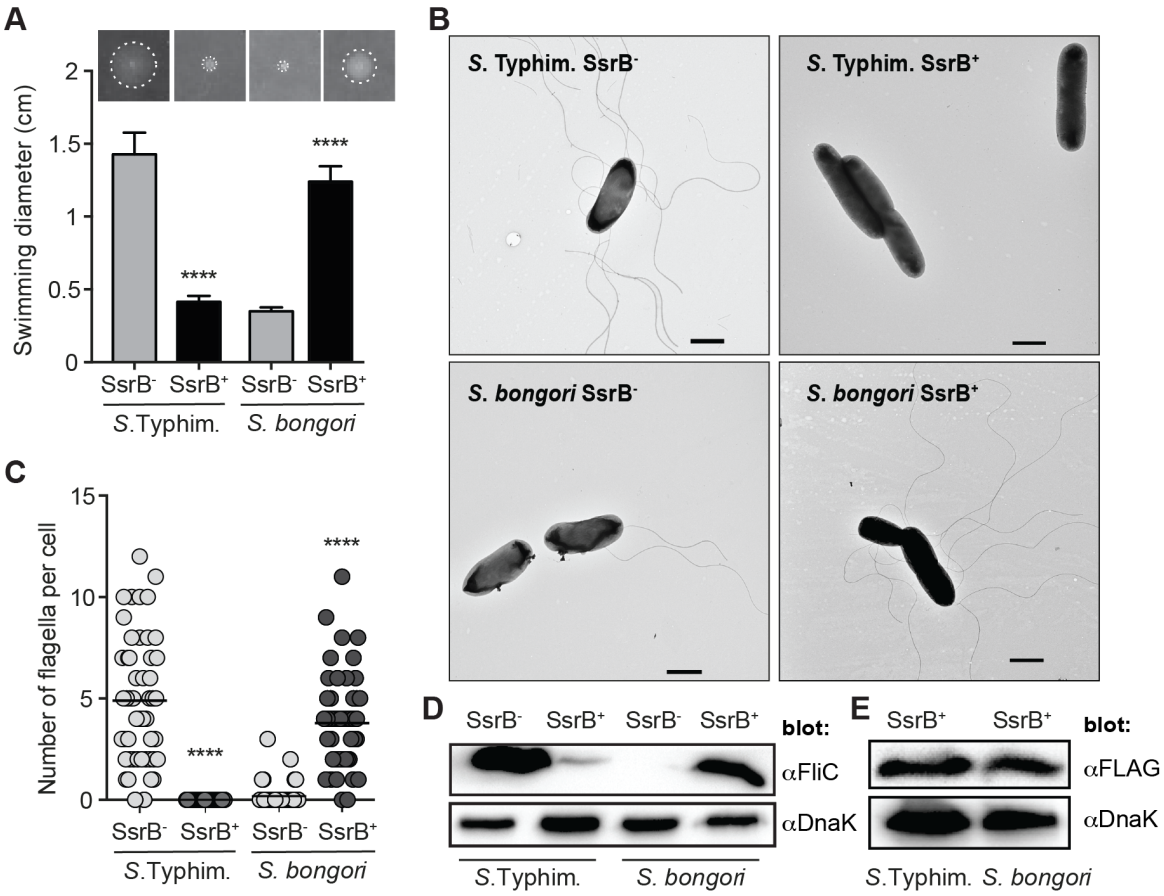
A
Swimming diameter (cm)
2
1.5
1
0.5
0
****
****
SsrB- SsrB+ SsrB- SsrB+
S.Typhim.
S. bongori
B
S. Typhim. SsrB-
S. Typhim. SsrB+
S. bongori SsrB-
S. bongori SsrB+
C
Number of flagella per cell
15
10
5
0
****
****
SsrB- SsrB+ SsrB- SsrB+
S.Typhim.
S. bongori
D
SsrB- SsrB+ SsrB- SsrB+
blot:
αFliC
αDnaK
S.Typhim.
S. bongori
E
SsrB+ SsrB+
blot:
αFLAG
αDnaK
S.Typhim. S. bongori

Figure 2.3. SsrB antagonizes transcription of flagellar genes in STM by binding P*flhDC*.

(A) Transcription of flagellar genes is repressed by SsrB in STM and activated in SBG, measured by RT-qPCR and expressed as $\log_2$ fold change in transcript levels relative to *rsmC*. Data are mean ± SEM from n=3. (B) Expression of P$flhDC_{SBG}$ does not show SsrB-dependent repression of STM *flhD*, measured by RT-qPCR. Data are mean ± SEM from n=6, *P=0.03 by Wilcoxon test. (C) EMSA of SsrBc incubated with P*flhDC* from either STM or SBG. Protein-DNA complexes are indicated with an asterisk. (D) EMSA showing that SsrBc does not bind to P*fliA* or P*fliC* from STM. (E) EMSA of SsrBc with truncations of P*flhDC* shows SsrBc binding of P$flhDC_{-328+83}$ but not of P$flhDC_{-197+83}$. Coordinates are relative to the *flhD* translational start site (+1). See also Fig. 2.S3.

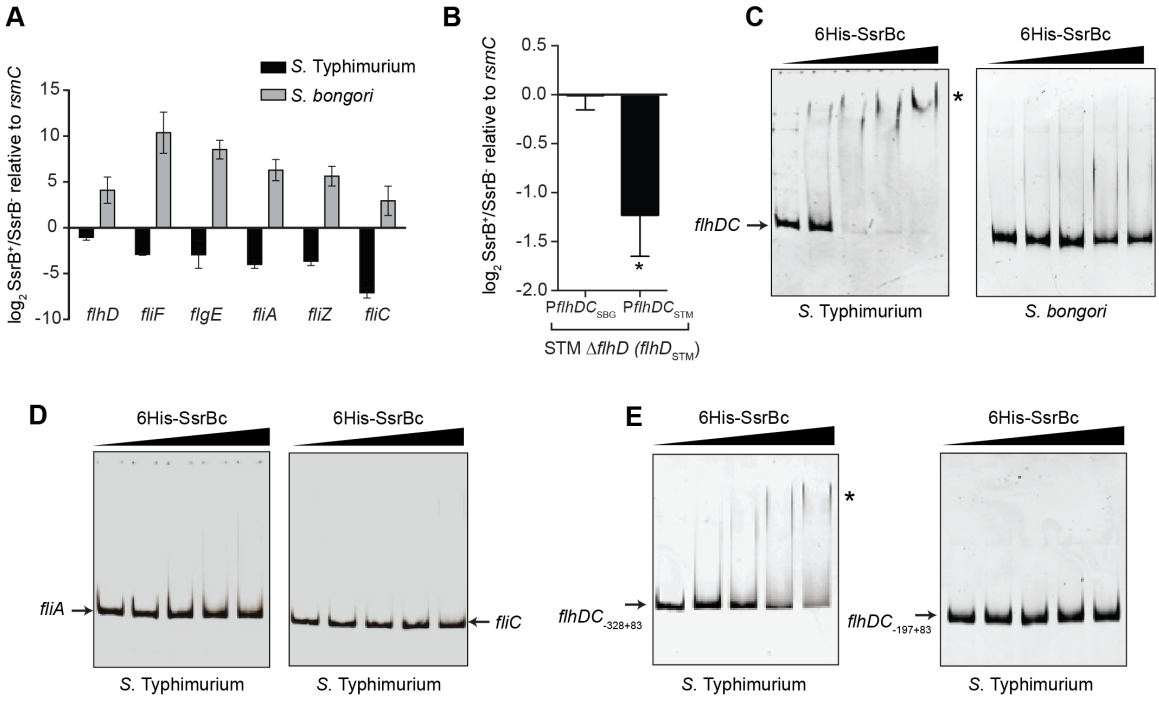
A
S. Typhimurium
S. bongori
log2 SsrB+/SsrB- relative to rsmC
15
10
5
0
-5
-10
flhD
fliF
flgE
fliA
fliZ
fliC
B
log2 SsrB+/SsrB- relative to rsmC
0.5
0.0
-0.5
-1.0
-1.5
-2.0
*
PflhDCSBG
PflhDCSTM
STM ΔflhD (flhDSTM)
C
6His-SsrBc
6His-SsrBc
flhDC
*
S. Typhimurium
S. bongori
D
6His-SsrBc
6His-SsrBc
fliA
fliC
S. Typhimurium
S. Typhimurium
E
6His-SsrBc
6His-SsrBc
*
flhDC-328+83
flhDC-197+83
S. Typhimurium
S. Typhimurium

Figure 2.4. STM evades inflammasome activation through SsrB-dependent repression of *fliC*.

(A) FliC protein levels are attenuated by $SsrB^+$ STM during macrophage infection. FliC western blots of RAW264.7 cell lysates following infection with $SsrB^-$ or $SsrB^+$ STM and SBG. DnaK and GAPDH indicate bacterial and host cell loading controls, respectively. (B) STM $SsrB^+$ reduces caspase-1 secretion (~50kDa) and proteolytic cleavage (~20kDa) during BMM infection. Caspase-1 measured by western blot from culture supernatants of infected BMM treated with either the vehicle control (left) or the caspase-1 inhibitor (right). (C) SsrB expression by STM attenuates IL-1β secretion from BMM. IL-1β was measured in culture supernatants of infected BMM by ELISA. Data are mean ± SEM from n=4. *P<0.05, ***P<0.001, ****P<0.0001, *nd* (not detected, below the limit of detection of the assay). See also Fig. 2.S4.

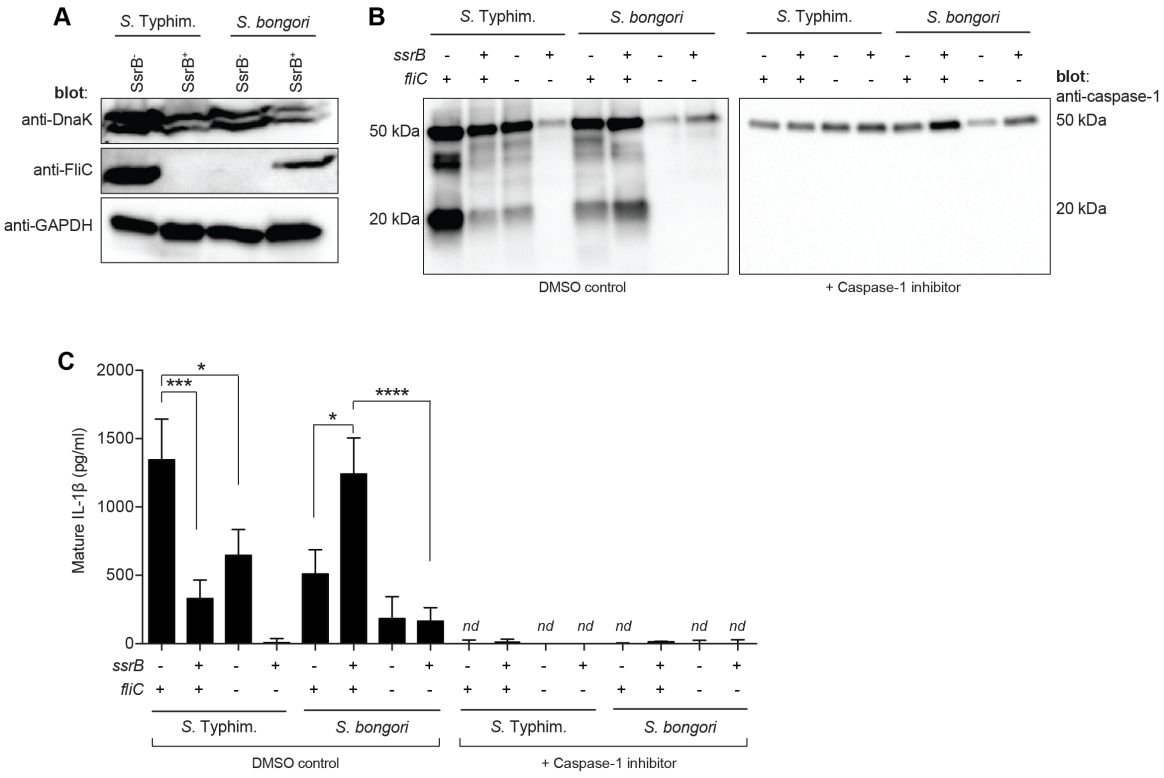
A
S. Typhim.
S. bongori
SsrB-
SsrB+
SsrB-
SsrB+
blot:
anti-DnaK
anti-FliC
anti-GAPDH
B
S. Typhim.
S. bongori
S. Typhim.
S. bongori
ssrB
fliC
blot:
anti-caspase-1
50 kDa
20 kDa
50 kDa
20 kDa
DMSO control
+ Caspase-1 inhibitor
C
Mature IL-1β (pg/ml)
2000
1500
1000
500
0
*
***
*
****
nd
ssrB
fliC
S. Typhim.
S. bongori
S. Typhim.
S. bongori
DMSO control
+ Caspase-1 inhibitor

Figure 2.S1. SsrB changes global gene expression in *S.* Typhimurium and *S. bongori.*

(A) Genome-wide fold change in gene expression ($SsrB^+/SsrB^-$) in *S.* Typhimurium (outer track) and *S. bongori* (inner track). Genomes are represented on a circular genomic plot. Green peaks indicate up-regulated genes (log_2 fold change >1), red peaks indicate down-regulated genes (log_2 fold change <1), and black lines represent genes that had over 100 reads in one expression condition but did not meet this priority cut-off. The *S. bongori* track only denotes orthologs present in *S.* Typhimurium. The name and genomic location of known *S.* Typhimurium genes that are significantly up-regulated by SsrB are labeled in green and those significantly down-regulated shown in red on the outer track of the Circos plot, with genomic islands highlighted. Best viewed in the electronic file with software magnification. (B) Summary of species-specific genes transcriptionally modified by SsrB. Horizontal bars show the total number of genes significantly modified in each set. (C) Functional characterization of genes identified in each regulatory set from B. All differentially expressed genes were assigned to a COG category (outer ring) and by functional group through manual curation (inner ring). (D) Fold change of *S.* Typhimurium specific genes, categorized by functional group (colored circles).

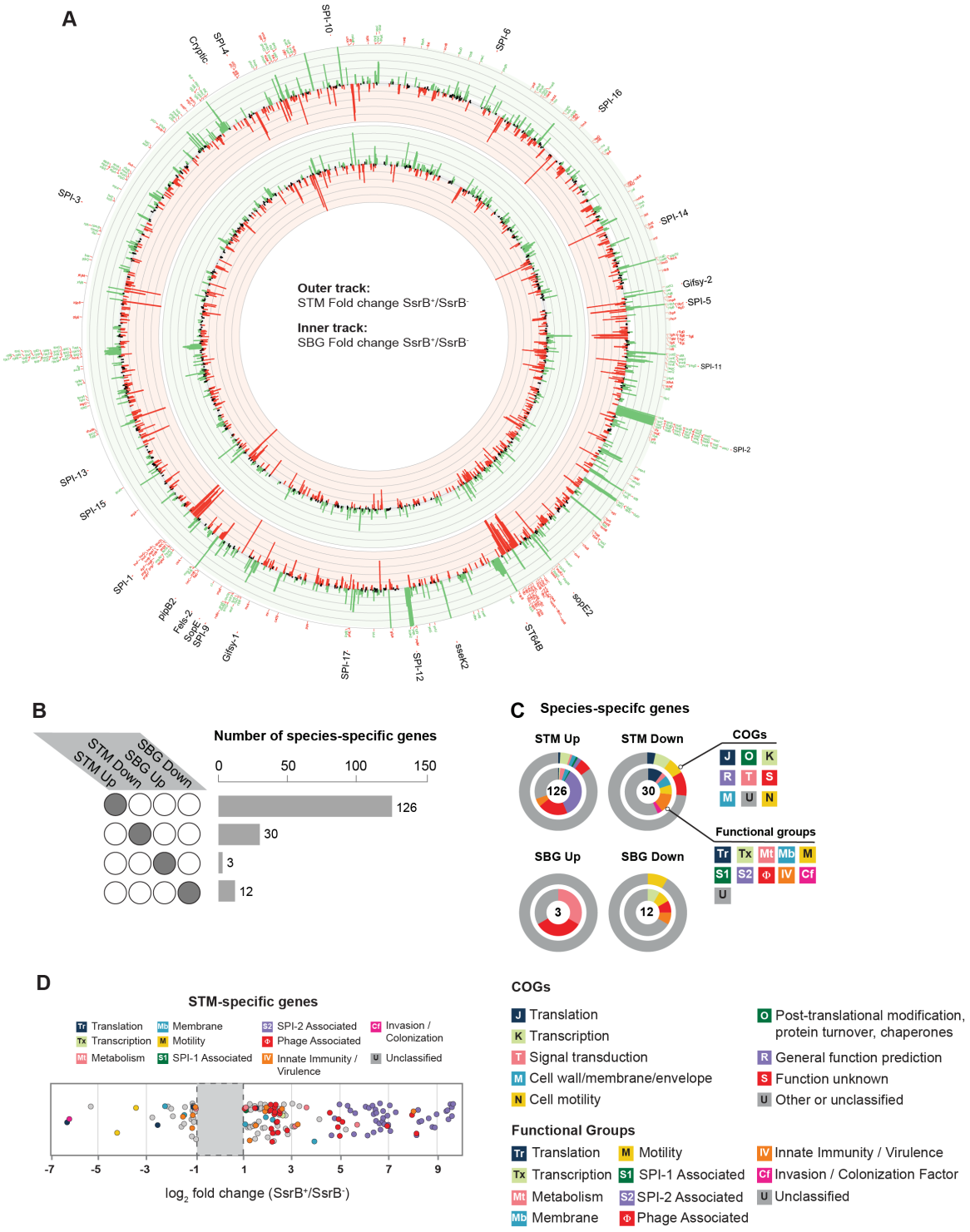
A
Outer track:
STM Fold change SsrB+/SsrB-
Inner track:
SBG Fold change SsrB+/SsrB-
SPI-10
SPI-6
SPI-16
SPI-14
Gifsy-2
SPI-5
SPI-11
SPI-2
SPI-13
SPI-15
SPI-1
pipB2
Fels-2
SopE
SPI-9
Gifsy-1
SPI-17
SPI-12
sseK2
ST64B
sopE2
SPI-3
SPI-4
Cryptic
B
Number of species-specific genes
SBG Down
SBG Up
STM Down
STM Up
0
50
100
150
126
30
3
12
C
Species-specifc genes
STM Up
STM Down
SBG Up
SBG Down
126
30
3
12
COGs
Functional groups
D
STM-specific genes
Translation
Transcription
Metabolism
Membrane
Motility
SPI-1 Associated
SPI-2 Associated
Phage Associated
Innate Immunity / Virulence
Invasion / Colonization
Unclassified
-7
-5
-3
-1
1
3
5
7
9
log2 fold change (SsrB+/SsrB-)
COGs
J Translation
K Transcription
T Signal transduction
M Cell wall/membrane/envelope
N Cell motility
O Post-translational modification, protein turnover, chaperones
R General function prediction
S Function unknown
U Other or unclassified
Functional Groups
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 Factor
U Unclassified

Figure 2.S2. Expression heat-map of orthologous genes regulated by SsrB in *S.* Typhimurium and *S. bongori.*

Heat maps depicting the fold change in gene expression for all orthologs regulated by SsrB, grouped by functional category as defined in Figure 1. Heat maps were generated in Excel, and fold change was cut off at a maximum of $\log_2$ +/- 5. Where gene names were not defined in *S.* Typhimurium, the SL1344 locus ID numbers are used.

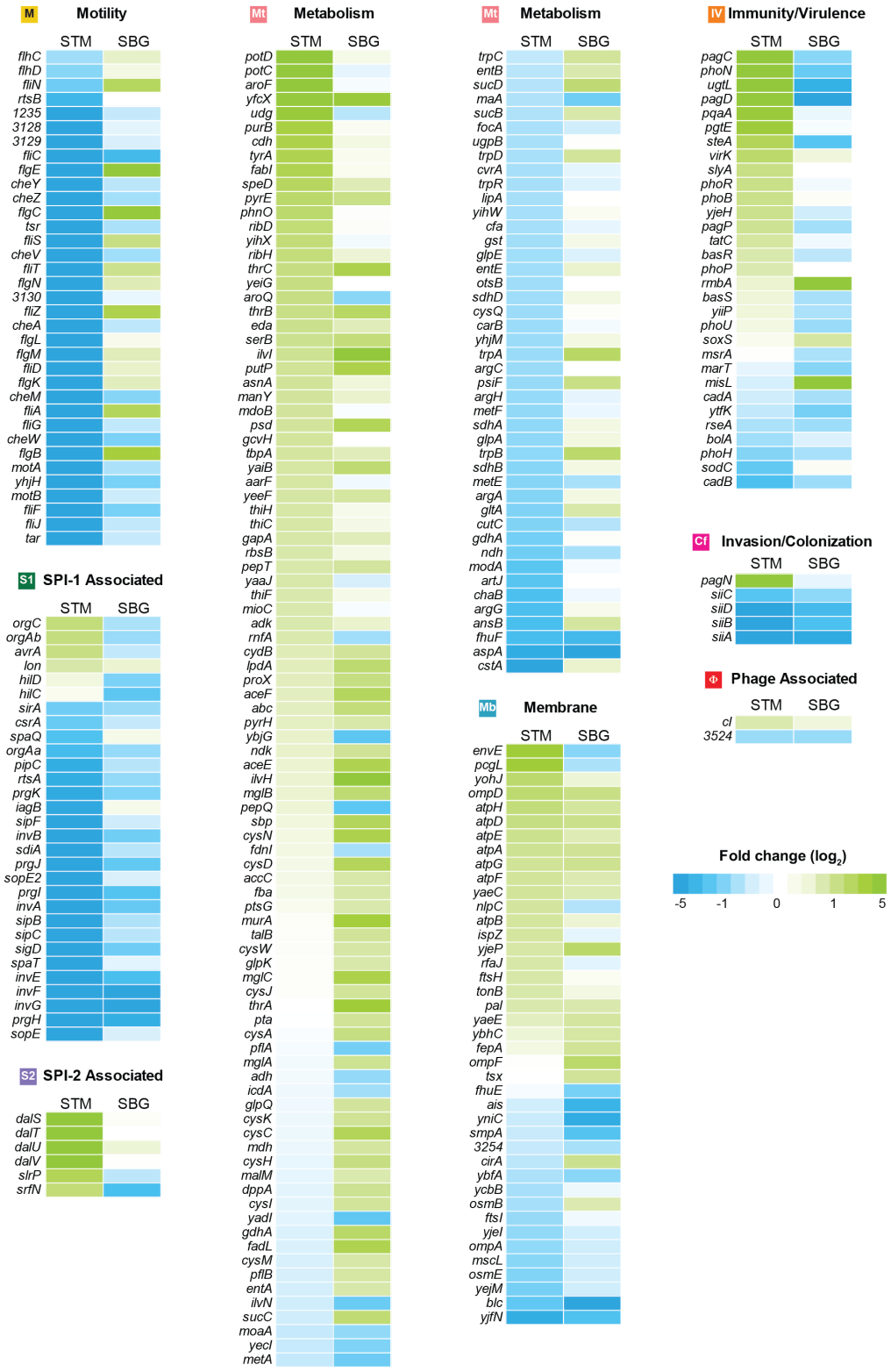
M Motility
STM SBG
flhC flhD fliN rtsB 1235 3128 3129 fliC flgE cheY cheZ flgC tsr fliS cheV fliT flgN 3130 fliZ cheA flgL flgM fliD flgK cheM fliA fliG cheW flgB motA yhjH motB fliF fliJ tar
S1 SPI-1 Associated
STM SBG
orgC orgAb avrA lon hilD hilC sirA csrA spaQ orgAa pipC rtsA prgK iagB sipF invB sdiA prgJ sopE2 prgI invA sipB sipC sigD spaT invE invF invG prgH sopE
S2 SPI-2 Associated
STM SBG
dalS dalT dalU dalV slrP srfN
Mt Metabolism
STM SBG
potD potC aroF yfcX udg purB cdh tyrA fabI speD pyrE phnO ribD yihX ribH thrC yeiG aroQ thrB eda serB ilvI putP asnA manY mdoB psd gcvH tbpA yaiB aarF yeeF thiH thiC gapA rbsB pepT yaaJ thiF mioC adk rnfA cydB lpdA proX aceF abc pyrH ybjG ndk aceE ilvH mglB pepQ sbp cysN fdnI cysD accC fba ptsG murA talB cysW glpK mglC cysJ thrA pta cysA pflA mglA adh icdA glpQ cysK cysC mdh cysH malM dppA cysI yadI gdhA fadL cysM pflB entA ilvN sucC moaA yecI metA
Mt Metabolism
STM SBG
trpC entB sucD maA sucB focA ugpB trpD cvrA trpR lipA yihW cfa gst glpE entE otsB sdhD cysQ carB yhjM trpA argC psiF argH metF sdhA glpA trpB sdhB metE argA gltA cutC gdhA ndh modA artJ chaB argG ansB fhuF aspA cstA
Mb Membrane
STM SBG
envE pcgL yohJ ompD atpH atpD atpE atpA atpG atpF yaeC nlpC atpB ispZ yjeP rfaJ ftsH tonB pal yaeE ybhC fepA ompF tsx fhuE ais yniC smpA 3254 cirA ybfA ycbB osmB ftsI yjeI ompA mscL osmE yejM blc yjfN
IV Immunity/Virulence
STM SBG
pagC phoN ugtL pagD pqaA pgtE steA virK slyA phoR phoB yjeH pagP tatC basR phoP rmbA basS yiiP phoU soxS msrA marT misL cadA ytfK rseA bolA phoH sodC cadB
Cf Invasion/Colonization
STM SBG
pagN siiC siiD siiB siiA
Φ Phage Associated
STM SBG
cI 3524
Fold change (log2)
-5 -1 0 1 5

Figure 2.S3. SsrB regulation of motility is independent of SlyA and HilD and is centered around the P1 transcriptional start site.

(A) A Δ*slyA* strain of *S.* Typhimurium shows SsrB-dependent repression of swimming motility. Data are mean ± SEM of three biological replicates, **** P<0.0001. (B) Purified HilD (MBP-HilD) can bind P*flhDC* of STM and SBG, indicated by a shift in DNA migration on a gel (asterisk). *ppK* is used as a negative control as it is known to not shift when incubated with HilD. (C) Co-incubation of P*flhDC* with SsrBc and MBP-HilD at the same time; with HilD first followed by SsrB; or with SsrB first followed by HilD (indicated by competition order) show similar DNA-binding abilities of both proteins; indicating independent binding sites for SsrBc and HilD. (D-F) Transcriptional reporters of truncated fragments of the full length *flhDC* intergenic region ($PflhDC_{-827+83}$) show SsrB-dependent repression of *flhD* transcription with P$flhDC_{-328+83}$, while a transcriptional reporter lacking the P1 transcriptional start site (P$flhDC_{-197+83}$) loses transcription and SsrB- dependent repression. Coordinates are relative to the translational start site of *flhD* (+1). Data are mean ± SEM of three biological replicates.

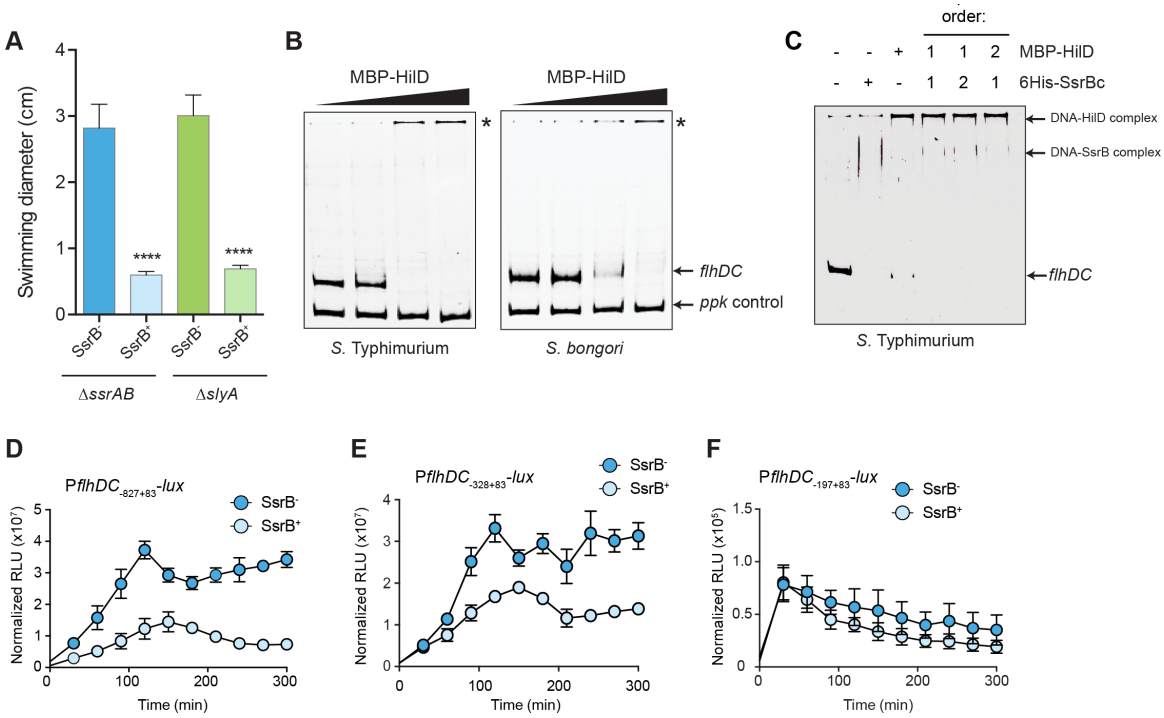
A
Swimming diameter (cm)
SsrB-
SsrB+
SsrB-
SsrB+
ΔssrAB
ΔslyA
****
****
B
MBP-HilD
MBP-HilD
S. Typhimurium
S. bongori
flhDC
ppk control
C
order:
MBP-HilD
6His-SsrBc
DNA-HilD complex
DNA-SsrB complex
flhDC
S. Typhimurium
D
PflhDC-827+83-lux
E
PflhDC-328+83-lux
F
PflhDC-197+83-lux
Normalized RLU
Time (min)

Figure 2.S4. SsrB represses P*fliC* during mouse infection.

(A) *In vivo* imaging of luciferase activity from the *fliC* promoter in *S.* Typhimurium expressing SsrB ($SsrB^+$) or not ($SsrB^-$) following infection of mice for the indicated times. (B) Quantification of total luminescence at each time point of the murine infection averaged from four mice per strain. Data are mean ± SEM, * $P<0.05$; ** $P< 0.01$.

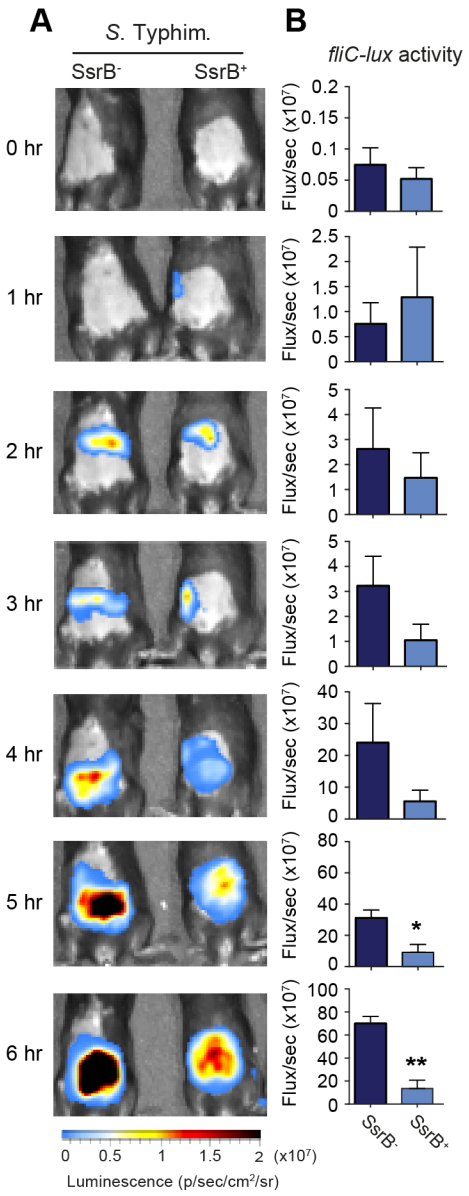
A
S. Typhim.
SsrB-
SsrB+
0 hr
1 hr
2 hr
3 hr
4 hr
5 hr
6 hr
0 0.5 1 1.5 2 (x10⁷)
Luminescence (p/sec/cm²/sr)
B
fliC-lux activity
Flux/sec (x10⁷)
*
**
SsrB-
SsrB+

Table 2.1 Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-DnaK	Enzo Life Sciences	Cat# ADI-SPA-880-J; RRID:AB_11179615
Anti-FliC	BioLegend	Cat# 629701; RRID:AB_572080
Anti-FLAG	SigmaAldrich	Cat# A1205; RRID:AB_2687448
anti-mouse-HRP	Jackson Immunoresearch	Cat# 115-035-003; RRID:AB_10015289
anti-GAPDH	R&D Systems Inc.	Cat# AF5718; RRID:AB_2278695
anti-goat-HRP	Santa Cruz Biotechnology	Cat# sc-2020; RRID:AB_631728
anti-caspase-1	Adipogen Life Sciences	Cat# AG-20B-0042; RRID:AB_2490248
Bacterial and Virus Strains		
Salmonella enterica serovar Typhimurium (*S.* Typhimurium) SL1344	Coombes et al., 2003	N/A
Salmonella bongori 66:z41	Osborne et al., 2009	ATCC #43975
S. Typhimurium SL1344 Δ*ssrAB*	Mulder et al., 2015	N/A
S. Typhimurium SL1344 Δ*slyA*	Osborne et al., 2009	N/A
S. Typhimurium SL1344 Δ*ssrAB* Δ*flhD*	This study	N/A
S. Typhimurium SL1344 Δ*ssrAB* Δ*fliC::cat*	This study	N/A
S. bongori Δ*fliC::cat*	This study	N/A
E. coli TOP10	Invitrogen	Cat#C404010
E. coli DH10B	Invitrogen	Cat#18297010
E. coli BL21(DE3)	Agilent Technologies	Cat#200131
Recombinant DNA		
pWSK129	Wang and Kushner, 1991	N/A
pWSK129-P*ssrA-ssrB* D56E	Pérez-Morales et al., 2017	N/A
pWSK129-P*ssrA-ssrB*-FLAG D56E	This study	N/A
pKD46	Datsenko and Wanner, 2000	N/A
pKD3	Datsenko and Wanner, 2000	N/A
pFlp2	Choi and Schweizer, 2005	N/A
pACYC184	NEB	Cat#E4152
pACYC184-P*flhDC*$_{SBG}$-*flhD*$_{STM}$	This study	N/A

pACYC184-P*flhDC*$_{STM}$-f*lhD*$_{STM}$	This study	N/A
pGEN-*luxCDABE*	Lane et al., 2007	N/A
pGEN-P*flhDC*$_{-827+83}$-*lux*	This study	N/A
pGEN-P*flhDC*$_{-328+83}$-*lux*	This study	N/A
pGEN-P*flhDC*$_{-197+83}$-*lux*	This study	N/A
pGEN-P*fliC*$_{STM}$-*lux*	This study	N/A
pK6-HIS-SsrBc	Pérez-Morales et al., 2017	N/A
pMAL-HilD	Bustamante et al., 2008	N/A

Chemicals, Peptides, and Recombinant Proteins

Trizol	Invitrogen	Cat#15596026
Turbo DNA-free kit	Invitrogen	Cat#AM1906
S. minnesota LPS R595	EMD Millipore	Cat# **437628**
Z-YVAD-FMK	BioVision Inc.	Cat#1141

Critical Commercial Assays

IL-1β ELISA Kit	Thermo Fisher Scientific	Cat# 88-7013-22; RRID:AB_2574942
LDH Cytotoxicity Kit	Pierce	Cat# 88953

Deposited Data

Raw RNA sequencing data	NCBI Gene Expression Omnibus	Accession number GSE120043

Experimental Models: Cell Lines

RAW264.7 Cell line		ATCC#TIB-71
L929 Fibroblasts		ATCC#CCL-1

Experimental Models: Organisms/Strains

C57BL/6 Mouse	Charles River Laboratories	N/A

Oligonucleotides

Refer to Table 2.2		

Software and Algorithms

Circos	Krzywinski et al., 2009	http://circos.ca/software/
UpSet	Lex et al., 2014	http://caleydo.org/tools/upset/
Rockhopper	McClure et al., 2013; Tjaden, 2015	https://cs.wellesley.edu/~btjaden/Rockhopper/

Table 2.2 List of primers used in this study

Primer name	Primer sequence (5′-3′)	Target gene
BIp66F	GGAATTGGTACCTTACTTGTCGTCATCGTCTTTGTAG TCTCCAGAATACTCTATTAACCTCATTCT	STM *SsrB* with C-terminal FLAG tag from pWSK129
BIp66R	ACGCGAGCTCAAATGGGAGTTTCTATCAAA	STM P*ssrA* from pWSK129
BIp96F	AGTTGGTTATTCTGGATGGGAACAATGCATACATCC GAGTTGCTGTGTAGGCTGGAGCTGCTTCG	STM *flhD::cat*
BIp96R	TTTTTTCACTCATTATCATGCCCTTTTCTTACGCGCCG TATCGTCCAATATGAATATCCTCCTTA	STM *flhD::cat*
BIp89F	ATAGAGACCGCTTTAGCCAGTAGCTGGTGACTTTCA GGGGTAACGGCAACGG	P*flhDC*$_{SBG}$, with BIp90F for SOE PCR with *flhD*
BIp90F	CTCCTTGCACAGCGTTTGATCGTCCAGGACAAAGCAT CTGCGATGTTCCGC	*flhD*$_{STM}$, with BIp89R for SOE PCR with P*flhDC*$_{SBG}$
BIp95R	GGAATTAAGCTTGATATCGCGAGCTTCCTGAAC	*flhD*$_{STM}$, to clone P*flhDC*$_{SBG}$-*flhD* or P*flhD*$_{STM}$-*flhD* into pACYC184 with BIp86F
BIp97F	CCCATACGATATAAGTTGTAA	pACYC184-P*flhDC-flhD*
BIp97R	GGTGATGTCGGCGATATAGG	pACYC184-P*flhDC-flhD*
RTF2	GAAAAGCAGCCGCAGTTTAG	STM *rsmC* for RT-qPCR
RTR2	CAGTTGGCTACCAACATCCA	STM *rsmC* for RT-qPCR
BIp60F	CGGCAGCCAACTGTTACTTT	SBG *rsmC* for RT-qPCR
BIp60R	ATCAAGGCTGGTTTGCATTC	SBG *rsmC* for RT-qPCR
BIp22F	TACTCCTTGCACAGCGTTTG	STM and SBG *flhD* for RT-qPCR
BIp22R	GCCGTATCGTCCACTTCATT	STM and SBG *flhD* for RT-qPCR
BIp23F	CGGACCCTGTACCGTCTAAA	STM and SBG *flgE* for RT-qPCR
BIp23R	AAGGAAGCTGAGGGAGAAGG	STM and SBG *flgE* for RT-qPCR
BIp24F	AGTTCAGCGAGCAGGTGAAT	STM and SBG *fliF* for RT-qPCR
BIp24R	GAGCAGATGACCGGATTGAT	STM and SBG *fliF* for RT-qPCR
BIp51F	GATGGTCAGGGTGTTGTCCT	STM and SBG *fliC* for RT-qPCR
BIp51R	CTCAGGCTTCCCGTAAC	STM and SBG *fliC* for RT-qPCR
BIp76F	GCTGGATGAATTACGCAGCCGC	STM and SBG *fliA* for RT-qPCR
BIp76R	TGCTATCGCCATGCTCTTCC	STM and SBG *fliA* for RT-qPCR
BIp78F	ATGACGGTGCAGCAACCTAAA	STM and SBG *fliZ* for RT-qPCR

BIp78R	TTGAAACCGATAATATCGAAAAAATC	STM and SBG *fliZ* for RT-qPCR
BIp86F	TATGCGGATCCGGGGTGACGGCAACGG	P*flhDC*$_{-827+83}$-*lux* for pGEN, use with BIp86R
BIp92F	TATGCGGATCCGTAAAAGCCATGCTGATGGT	P*flhDC*$_{-328+83}$-*lux* for pGEN, use with BIp86R
BIp94F	TATGCGGATCCGGAAAAATCTTAGGCATTTG	P*flhDC*$_{-197+83}$-*lux* for pGEN, use with BIp86R
BIp86R	GTCATATACGTAATCAAACGCTGTGCAAGGAG	P*flhDC*$_{-n+83}$-*lux* for pGEN
DTM64F	AAAACTGCCAGCATGAATCC	STM Δ*ssrAB* screening
DTM66R	ATGCTAAGCGCTTGTCGAAT	STM Δ*ssrAB* screening
flhD-RV11	TCCAAGCTTATCAAACGCTGTGCAAGGAG	*flhDC* intergenic region for EMSA (STM and SBG)
flhD-FW22	TGAGGATCCGGGGTGACGGCAACGG	P*flhDC*$_{-826+83}$ intergenic region for EMSA (STM and SBG)
flhDC-328F	GTAAAAGCCATGCTGATGGTT	STM P*flhDC*$_{-328}$ for EMSA. Use with flhD-RV11
flhDC-197F	GGAAAAATCTTAGGCATTTGTAA	STM P*flhDC*$_{-197}$ for EMSA. Use with flhD-RV11
fliA-FW44	GGAGGATCCAAAGCACCAGCTTGAAGAGG	*fliA* intergenic region for EMSA (STM and SBG)
fliA-RV33	GCAAAGCTTCGTGACGCACCAGCG	*fliA* intergenic region for EMSA (STM)
fliC-RVI-BH	GTTGGATCCCACACCTAATGATG	*fliC* intergenic region for EMSA (STM)
fliC-FWI-Hd	GACAAGCTTACAGACGCTCGATAGCGGTG	*fliC* intergenic region for EMSA (STM)
PPK-Rv1	ATCGGATCCTCTGATTCCGAACAGCGTG	*ppK* intergenic region for EMSA (STM)
PPK-Fw1	ATCGGATCCTCTGATTCCGAACAGCGTG	*ppK* intergenic region for EMSA (STM)
BIp52F	TATGCGGATCCGCCTTCTTCCTTTTTGATTGCAAA	STM P*fliC-lux* for pGEN
BIp52R	GTCATATACGTAGATCTTTTCCTTATCAATTACAAC	STM P*fliC-lux* for pGEN
BIp67F	ATGGCACAAGTCATTAATACAAACAGCCTGTCGCTG TTGACCCAGAATGTGTAGGCTGGAGCTGCTTCG	STM *fliC::cat*
BIp67R	TTAACGCAGTAAAGAGAGGACGTTTTGCGGAACCTG GTTCGCCTGCGCATATGAATATCCTCCTTA	STM *fliC::cat*
BIp69F	ATGGCACAAGTCATTAATACAAACAGCCTGTCGCTG TTGACCCAGAATGTGTAGGCTGGAGCTGCTTCG	SBG *fliC::cat*
BIp69R	TTAACGCAGTAAAGAGAGGACGTTTTGCGGAACCTG GTTAGCCTGTGCATATGAATATCCTCCTTA	SBG *fliC::cat*
BIp53F	TATGCGGATCCGCCTTCTTCCTTTTGATTGCAAAC	SBG *fliC::cat* screening
BIp73R	AGGCAGGTTCAGTGACGGTGATTTCTTTCA	*fliC::cat* screening, use with BIp52F (STM) or BIp53F (SBG)

CHAPTER 3: SALMONELLA TYPHIMURIUM SENSES HOST-DERIVED REACTIVE OXYGEN SPECIES TO LAUNCH AN INTRACELLULAR VIRULENCE PROGRAM

Chapter 3: Co-authorship statement

The chapter was written by BI. The following experiments were performed by collaborators other than myself:

(1) Antibiotic disk diffusion assays and *in vivo* infections were performed by SEO (Figure 3.1 and 3.3B).

(2) RT-qPCR on SPI-2 genes was performed by LM (Figure 3.2B).

(3) pet28a-SUMO-SsrA 681-920 was generated by DJL.

Salmonella senses reactive oxygen species to launch an intracellular virulence program

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Abstract

Niche-adapted gene expression in bacteria is coordinated by environmental sensing mediated by two-component systems that relay extracellular signals to direct transcriptional responses. During mammalian infection by the enteric pathogen *Salmonella* Typhimurium, the host anti-microbial defenses serve a secondary function as chemical cues for bacterial two component systems, which trigger changes in gene expression that promote survival and virulence within the host niche. Here, we identify a novel host cue, reactive oxygen species that are generated by the NADPH oxidase enzyme complex following bacterial phagocytosis, which serve as a signal to promote the expression of genes in *Salmonella* Typhimurium (STM) that are required for intracellular survival. Transcription of genes in the *Salmonella* Pathogenicity Island 2 (SPI-2), a cluster of genes necessary for bacterial intracellular replication and systemic dissemination, was promoted in the presence of small molecules that produce reactive oxygen species as well as within host cells or mice with a functional NADPH oxidase. This transcriptional activation was found to depend on the sensor kinase SsrA of the two-component system SsrA-SsrB that regulates SPI-2 gene expression. Taken together, these data indicate that STM has evolved to integrate the anti-bacterial defense of reactive oxygen species generation, into its transcriptional regulatory program to promote survival and dissemination.

Introduction

Salmonella enterica serovar Typhimurium (STM) is an enteric pathogen that infects a wide range of mammalian hosts causing gastroenteritis. During a mouse infection, STM targets the small intestine where it enters through epithelial cells and survives in neutrophils before spreading to more permissive host cells like macrophages (Carter and Collins, 1974; Geddes et al., 2007; Jones et al., 1994). Once within macrophages, bacterial replication occurs and bacteria are shuttled to systemic sites via the lymphatic system (Mastroeni and Sheppard, 2004). This intracellular lifestyle is vital for successful infection because it creates a site where replication can occur without competition for nutrients and resources from the commensal microbiome (Dunlap et al., 1992).

Although the intracellular environment provides several advantages, STM is also exposed to numerous antibacterial defenses within this niche. Following phagocytosis by neutrophils and macrophages, STM is exposed to several antimicrobial stresses: the vacuole is acidified, NADPH oxidase is assembled to produce reactive oxygen species (ROS), metal transporters limit nutrients, and antimicrobial peptides (AMPs) target the bacteria-containing vacuole (Flannagan et al., 2009). Survival within this niche is driven the activation of genes in the SPI-2 genomic island that encodes a type 3 secretion system (T3SS-2), which secretes effectors into the cytosol that modify the vacuole to make it amenable for bacterial survival and eventual propagation within the host (Haraga et al.,

2008; Jennings et al., 2017). These effectors modulate various aspects of the host response to phagocytosis such as dampening the immune response, inducing cytoskeletal changes, and controlling the localization of the *Salmonella* containing vacuole (Agbor and McCormick, 2011; Waterman and Holden, 2003). Coupling the sensing of the immune response to the activation of genes that protect against these challenges is a particularly effective strategy to defend against host attacks.

The sensing of these environmental cues and concomitant genetic response is driven by protein complexes called two component systems (TCS) that are composed of a sensor kinase protein that recognizes cues and transfers the signal to a response regulator protein to affect gene expression (Stock et al., 2000). The SPI-2 genes involved in intracellular virulence are regulated by a number of these TCS both directly and through cross-talk between the TCS (Erhardt and Dersch, 2015). The SsrA-SsrB TCS, present on the SPI-2 genomic island, is the master regulator of genes in SPI-2 and its associated effectors, but the activity of this system is modulated by inputs from EnvZ-OmpR and PhoQ-PhoP (Fass and Groisman, 2009; Garmendia et al., 2003). These TCS sense cues such as antimicrobial peptides, low pH, and low osmolarity (Anderson and Kendall, 2017; Bader et al., 2005; Chakraborty et al., 2015; Mulder et al., 2015; Wang et al., 2012). In addition to these TCS, the transcription factor SlyA has been shown to bind to the promoter of some SPI-2 genes and contribute to the expression of SPI-2 genes, directly and in a PhoP-dependent manner (Linehan et al., 2005; Navarre et al., 2005; Osborne and Coombes, 2011). In response, virulence genes that modify *Salmonella* LPS,

increase acid tolerance, and increase outer membrane porins are activated in a timely manner.

One of the first anti-microbial products produced by neutrophils and macrophages are reactive oxygen species (ROS) (VanderVen et al., 2009). Phagocytosis induces assembly of the NADPH oxidase protein complex to the phagosome membrane and its subsequent activation. Within seconds of phagocytosis, the NADPH oxidase complex produces superoxide anions. Superoxide is then rapidly converted to microbicidal intermediates, leading to a respiratory burst that kills invading microbes, both within the cell and in the extracellular environment (Flannagan et al., 2009; Mastroeni and Sheppard, 2004; Winterbourn and Kettle, 2013). Since ROS are produced rapidly following phagocytosis, they might present as a host cue to denote the intracellular environment. It has been shown that certain proteins in *Salmonella* can sense ROS to mount a protective program against oxidative stress (Cremers and Jakob, 2013; Kobayashi et al., 2013; Pardo-Esté et al., 2018; Zheng and Storz, 2000). The mechanism of this sensing relies on the ability of ROS to transiently modify cysteine residues in proteins, making them useful as reversible modifiers in signal transduction.

The abundance and timing of ROS production immediately upon phagocytosis into the intracellular environment led us to hypothesize that *Salmonella* may rely on ROS as an environmental signal to activate the T3SS-2 intracellular survival program. Here, we demonstrate that ROS are indeed a signal for SPI-2 gene expression during a STM

infection, and that thiol-reactive cysteine residues in SsrA contribute to this transcriptional response. These data highlight a novel pathway for transcriptional activation of an essential virulence program that promotes bacterial pathogenesis.

Results

Reactive oxygen species contribute to transcription of the SPI-2 gene *sseA*

To investigate how reactive oxygen species may be activating the transcription of SPI-2 genes in STM, we took advantage of the fact that bactericidal, but not bacteriostatic, antibiotics produce reactive oxygen species as a mechanism of killing (Acker and Coenye, 2017; Hassett and Imlay, 2007; Kohanski et al., 2007). We investigated the activation of SPI-2 genes in response to antibiotics that generate hydroxyl radicals. STM expressing a SPI-2 promoter P*sseA* upstream of the luciferase operon (P*sseA*-*lux*) was used as a transcriptional readout of SPI-2 activation in disk diffusion assays with a variety of bacteriostatic and bactericidal antibiotics. Following 24 h of growth in the presence of these antibiotics, the total luminescence around the zone of inhibition was measured as a readout of *sseA* activation (Fig. 3.1A and B). We found that bactericidal antibiotics had higher luminescence around the zone of inhibition relative to bacteriostatic ones. The anti-cancer drug mitoxantrone and the anti-fungal drug flucytosine were used as controls to ensure the transcriptional response we were measuring was specific to antibacterial drugs.

To confirm that the transcriptional response detected was indeed ROS mediated, the disk diffusion assay was repeated with the antibiotics that showed the highest response, rifampin and rifamixin, with and without supplementation of the ROS scavenger thiourea in the media (Wasil et al., 1987) (Fig. 3.1C). In the presence of thiourea, total luminescence around the zone of inhibition of these antibiotics was reduced. Additionally, measuring luminescence activity in response to the oxidiser hydrogen peroxide showed similar levels of P*sseA* activation, and a concomitant reduction in transcriptional activity with thiourea supplementation. The zone of inhibition of hydrogen peroxide was also dramatically reduced in the presence of thiourea, confirming that thiourea in the media can scavenge ROS (Fig. 3.1C). These data confirm that the luminescence observed in response to bactericidal antibiotics is, at least in part, mediated by STM responding to reactive oxygen species.

Transcription of multiple genes in the SPI-2 genomic island is promoted by ROS

To further explore the ROS dependent induction of genes in STM, we evaluated the transcriptional response of *sseA* to hydrogen peroxide during liquid growth. Following growth of STM to mid-log phase in glucose-rich minimal media at neutral pH, a condition in which SPI-2 gene expression is typically low, bacteria were exposed to various sub-inhibitory concentrations of hydrogen peroxide for ten minutes then lysed for RNA extraction. The relative expression of *sseA* was compared between hydrogen peroxide (H_2O_2) exposed and untreated STM by RT-qPCR, normalized to the housekeeping gene *rsmC*, to determine the fold induction by H_2O_2. As a control,

expression of the OxyR-dependent gene *ahpC*, whose transcription is known to be induced by ROS, was also measured (Fig. 3.2A). Across all three concentrations, *ahpC* transcription was significantly upregulated by hydrogen peroxide. In contrast, *sseA* induction was only seen at 0.5 mM and 1 mM hydrogen peroxide. These results confirm that *sseA* transcription is hydrogen peroxide sensitive.

Further, we queried whether this phenotype was specific for *sseA*, or if it could be extended to the other operons within SPI-2. There are six predicted transcriptional start sites in the SPI-2 gene cluster, which includes genes involved in regulation of this island (P*ssrA*), type 3 secretion system apparatus genes (P*ssaB,* P*ssaG,* P*ssaM,* and P*ssaR*), and chaperone and translocon genes (P*sseA*) (Tomljenovic-Berube et al., 2010). Testing the hydrogen peroxide induced activity of genes in each of these operons with 0.5 mM hydrogen peroxide revealed upregulation of genes at all these transcriptional start sites (mean fold induction >1), although the fold induction for all the genes tested was lower than the induction seen for *sseA* (Fig. 3.2B). Thus, the SPI-2 genomic island is transcriptionally responsive to hydrogen peroxide treatment, and *sseA* transcription is a useful proxy for this transcriptional response.

Reactive oxygen species promote transcription of *sseA* during an STM **infection**

Having established that hydrogen peroxide can induce the transcription of SPI-2 genes, we next wanted to characterize this transcriptional response in response to host-generated ROS. gp91^{phox} is an essential subunit of the phagocytic NADPH oxidase, which

is the primary source of reactive oxygen species generation in mice (Lambeth, 2004; Yu et al., 1998). NADPH oxidase knockout mice (gp91$^{phox-/-}$) mice lack the ability to produce reactive oxygen species in response to a bacterial infection. To evaluate the transcriptional response of STM to ROS *in vivo*, we infected primary peritoneal neutrophils from wild-type mice or gp91$^{phox-/-}$ mice with STM expressing the P*sseA* transcriptional reporter (P*sseA-lux*). Neutrophils are the primary niche for STM within the first 24 hours of infection, and survival in this niche is a determinant for successful infection in mice (Cheminar et al., 2004; Dunlap et al., 1992; Geddes et al., 2007). P*sseA* was induced during a STM infection in primary neutrophils, however, this transcriptional activation was reduced in gp91$^{phox-/-}$ neutrophils, indicating that this transcriptional activation is in part driven by the activity of NADPH oxidase (Fig. 3.3A). A similar trend was observed for infection with STM expressing P*ahpC-lux* (Fig. 3.S1A). The *sseA* transcriptional activity was dependent on the presence of SsrA, the master sensor kinase that is involved in activation of SPI-2 genes (Fig. 3.S1B). Further, pre-treatment of neutrophils from wild-type mice with the ROS scavenger thiourea reduced the activity of P*sseA* to a level lower than in a gp91$^{phox-/-}$ background. Neutrophils express a second enzyme that can produce hydrogen peroxide, D-amino acid oxidase (DAO), which is a source of ROS stress even in a gp91$^{phox-/-}$ mutant background (Tuinema et al., 2014). The low P*sseA* transcription activity detected in gp91$^{phox-/-}$ neutrophils could be mediated by activation of DAO, whereas treatment with thiourea would scavenge the H_2O_2 from this source as well, leading to even lower *sseA-lux* levels in this condition.

Infection of mice with this P*sseA* reporter strain of STM showed a similar phenotype, where *sseA* transcription within the first hour of an intraperitoneal infection is largely driven by the presence of a functional NADPH oxidase (Fig. 3.3B and C). Together, these data confirm that ROS generated by the host can act as a transcriptional cue for SPI-2 genes during an STM infection.

The cysteine residue in SlyA is not required for hydrogen peroxide-dependent *sseA* transcription

The regulatory hierarchy that drives SPI-2 gene expression is complex and relies on interplay between several two-component sensory systems (TCS) and transcription factors. This complex network involving input from PhoQ-PhoP, EnvZ-OmpR, and SsrA-SsrB TCS, as well as transcriptional regulation by the DNA binding protein SlyA, allows for fine-tuning of gene expression and the integration of numerous environmental cues (Erhardt and Dersch, 2015). To investigate whether any of the known SPI-2 regulators played a role in the STM response to reactive oxygen species, we tested *sseA* transcription in STM single gene knockouts of these proteins. Disk diffusion assays with hydrogen peroxide (Fig. 3.4A) and RT-qPCR for *sseA* transcriptional activation (Fig. 3.4B) revealed a decrease in hydrogen peroxide-induced *sseA* transcription in each of these mutants compared to wild-type. However, *sseA* transcription is known to be strongly controlled by these genes (Osborne and Coombes, 2011), and *sseA* expression levels in these mutants was lower than wild-type STM even in the untreated samples (Fig. 3.S2A).

To take a more directed approach at evaluating the role the known regulatory mutants play in *sseA* transcription, we explored the known mechanisms for how proteins sense reactive oxygen species. Of the regulatory proteins evaluated in Fig. 3.4, we primarily focused on SsrA and SlyA because of their direct role in *sseA* transcription. SlyA, a DNA binding protein, has been shown to bind to P*sseA,* and *sseA* transcription is reduced in a *slyA* mutant (Navarre et al., 2005) (Fig. 3.S3B). SsrA is the sensor kinase of the SsrA-SsrB two-component system that activates transcription of genes on the SPI-2 genomic island. We aimed our investigation at cysteine residues in these proteins, because the thiol (-SH) side chain can be reversibly oxidised, and this can impact protein structure and function, thereby making the protein a redox switch (Cremers and Jakob, 2013; Hillion and Antelmann, 2015). SlyA has one cysteine residue that is in a flexible loop region of the winged helix domain that binds DNA. However, directed mutagenesis of this residue to serine, which maintains the overall amino acid structure but eliminates the oxidative thiol group, did not modify the transcriptional activation of *sseA* in response to hydrogen peroxide (Fig. 3.S3A). SlyA C81S can drive *sseA* transcription equally well as SlyA without hydrogen peroxide induction (Fig S3.3B), confirming that mutagenesis of this residue does not alter the native SlyA function.

SsrA has thiol residues that contribute to the hydrogen peroxide induced transcription of *sseA*

As a sensor kinase, modification of cysteine residues in SsrA could affect SsrA function that can be transduced to the response regulator SsrB, which promotes

transcription of *sseA* through direct binding at the *sseA* promoter. While the structure of SsrA has not been determined, protein modeling based on sequence homology allowed us to predict that SsrA is an inner membrane protein with a periplasmic domain that has previously been shown to be involved in sensing acidification (Mulder et al., 2015). Since the periplasmic domain of sensor kinase proteins are typically involved in sensing, we targeted the three cysteine residues (C82, C108, and C312) in the predicted periplasmic domain of SsrA for directed mutagenesis. Previous research has shown that the RCK motif enhances the nucleophilic nature of cysteine, making the C between these two positively charged residues more readily oxidised (Swem et al., 2003). The SsrA protein has one cysteine that matches this motif, C782, and another residue that is flanked by R and S, C826, both in the C-terminal cytoplasmic domain. Thus, these residues were also selected for targeted mutagenesis. Previous work on the periplasmic cysteine residues C82 and C108 has shown that mutation of these residues to alanine affects SsrA dependent *sseA* transcription at neutral but not acidic pH levels (Mulder et al., 2015). Since these constructs were being used to test the role of these residues in hydrogen peroxide sensing, the hydrogen peroxide induction assay was performed in acidic, SPI-2 inducing media – low phosphate and magnesium minimal media (LPM) at pH 5.8.

Wild-type STM grown in LPM pH 5.8 showed hydrogen peroxide dependent induction of *ahpC* and *sseA*, confirming that these conditions could be used to investigate the SsrA cysteine variants (Fig. 3.5A). Investigation of a STM *ssrA* mutant complemented in trans with wild-type SsrA or SsrA with mutations in the indicated

cysteine residues showed a reduction in *sseA* induction in certain cysteine variant strains relative to wild-type. Specifically, the C82A C108A double mutant and a C782S mutant had a mean fold induction of around 0.5, relative to a fold induction of 2 for the wild-type strain (Fig. 3.5B). However, the SsrA complemented strain also did not show complete complementation to wild-type levels (mean fold induction of 1.1), so the decrease in induction with these cysteine variants was not significant. Complementation of Δ*ssrA* with these cysteine variants activated *sseA* transcription in the untreated samples equal to wild-type levels (mean *sseA* transcription >1 relative to wild-type), except for in the C826S and C782S C826S strains (Fig. 3.S4A). This confirmed that the decrease in hydrogen peroxide promoted activity for C82A C108A and C782S was not due to these point mutations affecting native SsrA function. The mean *sseA* transcription in the C826S and C782S C826S SsrA variants was as low as in a Δ*ssrA* strain in the untreated samples, suggesting that residue C826S is important for SsrA function independent of hydrogen peroxide sensing. All of these SsrA complemented strains also had wild-type levels of hydrogen peroxide dependent *ahpC* induction, confirming that any effects on *sseA* induction were not due to global effects on transcription or hydrogen peroxide sensing in these strains (Fig. 3.S4B). Overall, the reduced hydrogen peroxide activity of SsrA C82A C108A and C782S implicated two domains of SsrA that may be involved in ROS sensing.

Attempts to purify the SsrA periplasmic domain have been unsuccessful, however we were able to purify a C-terminal portion (residues 681-920) that contains residue

C782. Purified protein is amenable to molecular labeling, wherein reactive thiol groups can be identified with tagged probes by click chemistry. Briefly, reactive thiol groups are oxidized with iodoacetamide attached to an alkyne that subsequently reacts with a labelled azide by copper-catalyzed click chemistry. This creates a triazole linker that is chemically attached to the thiol group and has a detectable tag for identification by western blot or mass spectrometry (Leichert and Jakob, 2004; Presolski et al., 2011). The C-terminal soluble domain of SsrA, SsrA 681-920, was expressed and purified in the wild-type form or as a C782S variant. The presence of reactive cysteine residues in these proteins was tested with click chemistry using a biotin labelled azide, allowing for detection of the thiol reactivity by western blotting against biotin (Fig. 3.5C). Intriguingly, wild-type SsrA 681-920 as well as the C782S variant both appear to be biotinylated to a similar degree. This domain has six cysteine residues, and one or multiple of these could be labeled by this technique. In contrast, purified full-length SlyA and the SlyA C81S variant are not biotinylated in a similar reaction, further suggesting that the cysteine residue is not oxidation sensitive (Fig. 3.S3C).

To identify whether any of the other five cysteines in SsrA 681-920 were being tagged with biotin by click chemistry, we performed mass spectrometry on the biotin-labelled wild-type and C782S variant of SsrA 681-920. Following tryptic digestion and liquid chromatography-mass spectrometry (LC-MS/MS), a single peptide in SsrA 681-920 appeared to have a cysteine residue with a biotin tag (Table 3.1). Sample preparation for mass spectrometry involved oxidation of methionine residues and alkylation of

cysteine residues. All unmodified cysteine residues are detected as a carbidomethyl cysteine or cysteine, except for C765 (peptide indicated with ◆ in table 3.1). However, although the C782S variant of SsrA appeared to be biotinylated by western blot, the biotin tag was not detected on the peptides containing C765 in this sample by mass spectrometry (peptides indicated with ∗ in table 3.1). This discrepancy could be due to the incomplete reaction of all the protein molecules with the biotin azide, leading to only a subset of the molecules being biotinylated. This leads to a reduced ability for detection by mass spectrometry. Indeed, two variants of the peptide that contains the biotinylated cysteine residue are detected in the wild-type SsrA sample, where only one has the molecular weight change that indicates the presence of a biotin tag, suggesting incomplete modification of this wild-type sample as well (unmodified peptide marked with ∗ in table 3.1). Unfortunately, due to C782 being flanked by R and K, tryptic digest prior to mass spectrometry created a peptide fragment that was too small for detection, so it was not possible to determine whether C782 was tagged in the wild-type SsrA 681-920 variant.

Since C765S had a biotin tag in SsrA by mass spectrometry, the thiol reactivity of this residue was investigated with targeted mutagenesis. C765S and a C765S C782S double mutant of SsrA 681-920 were expressed and purified for click chemistry with biotin azide. However, both these protein samples were also detected by western blot against biotin following the chemical labeling reaction (Fig. 3.5C). A C765S variant of SsrA also showed wild-type levels of hydrogen peroxide dependent *sseA* induction (mean

fold induction of 1.3 compared to 1.4 for wild-type), indicating that while this residue may be reactive by click chemistry, this residue alone is not sufficient for ROS sensing by SsrA to induce *sseA* transcription (Fig. 3.5D). SsrA C765S is able to transcribe *sseA* to wild-type levels in the absence of hydrogen peroxide treatment as well, and the hydrogen peroxide dependent induction of *ahpC* is also not impaired in this strain (Fig. 3.S4C and D). Together, these data demonstrate that certain cysteine residues in SsrA (C82, C108, and C782) may be important for *sseA* transcription in the presence of hydrogen peroxide, and that the C-terminal domain of SsrA has reactive thiol groups. Without improvements in the ability to detect modified residues by mass spectrometry, the exact thiol residues that are labeled by biotin and therefore considered reactive cannot be determined. Thus, whether direct oxidation of cysteine residues in SsrA affects the signal transduction cascade that leads to *sseA* transcription following hydrogen peroxide sensing remains an open question.

Discussion

Salmonella Typhimurium rapidly adapts to new environments by modifying gene expression in different niches to promote survival and infection. This is controlled by the coordinated activity of numerous TCS, each of which recognize different environmental cues within a host. Previous work on the TCS involved in SPI-2 activation has shown that a single sensor kinase can respond to multiple host cues, suggesting that the activation of SPI-2 virulence genes can be fine-tuned by the accumulation of host signals. Reactive

oxygen species are a potent anti-microbial response generated within seconds of phagocytosis, and the ability to sense and detoxify ROS is a major virulence determinant for *Salmonella* Typhimurium (Aussel et al., 2011). Our data show that in addition to sensing ROS for this detoxifying process, ROS acts as a chemical cue to promote transcription of the intracellular virulence program in STM.

Our preliminary investigations into the link between ROS and SPI-2 transcription took advantage of the finding that bactericidal antibiotics stimulate production of hydroxyl radicals through the activation of metabolic pathways that drive the electron transport chain. These findings have been topic of some debate in the field, with contradictory results and biological arguments put forth against the ROS hypothesis (Acker and Coenye, 2017; Keren et al., 2013). Indeed, the classes of antibiotics that were best known to promote this response, the aminoglycosides (kanamycin and cinoxacin) and fluoroquinolones (norfloxacin), had less *sseA-lux* activation than the rifampin family, which are RNA synthesis inhibitors. However, recent research has identified that the rifampin family can produce hydroxyl radicals in *M. tuberculosis* (Nandakumar et al., 2014; Piccaro et al., 2014), and we verified that the transcriptional response to this family of antibiotics was ROS-dependent by supplementing the media with the ROS scavenger thiourea. Thus, although there has been some uncertainty as to whether bactericidal antibiotics are a valid source of ROS stress, we observed transcription of the SPI-2 gene *sseA* in response to bactericidal antibiotics that appears to be ROS-dependent.

Analysis of the transcriptional response of the SPI-2 genomic island to hydrogen peroxide showed that *ssrA* and *ssaB* had the highest fold induction following hydrogen peroxide treatment after *sseA*. Previous work in our lab on the transcriptional activity of the different SPI-2 promoters showed that *ssaB* and *sseA* had the most SsrB dependence, where transcription of these promoters in LPM was completely eliminated in a *ssrB* mutant background (Osborne and Coombes, 2011). The other promoters had low levels of activity even in this mutant due to input from the other regulators that feed into SPI-2 gene expression. Thus, if SsrA is indeed the ROS sensor for this system, *ssaB* and *sseA* would be the most transcriptionally sensitive to this signal.

SlyA is part of the MarR family of proteins, whose members have been shown to regulate virulence gene expression in a ROS-dependent manner in several Gram-positive bacterial species (Zheng and Storz, 2000). Although the cysteine residue shared between these proteins is not present in SlyA, previous work has shown that SlyA is important for STM survival against ROS, and that SlyA modifies STM gene expression in response to ROS (Buchmeier et al., 1997; Cabezas et al., 2018). We confirmed that SlyA is important in protecting STM against ROS through a competitive infection between a *slyA* mutant and wild-type *Salmonella* in NADPH oxidase deficient mice. In wild-type mice, a *slyA* mutant is less fit than wild-type, however, this fitness defect is significantly lessened in NADPH oxidase mutant mice (Fig. 3.S3D). This suggests that part of the virulence contribution of *slyA* is in response to ROS produced by NADPH oxidase. However, while we were able to confirm that *slyA* is important to STM in a ROS context, we found that

this is not through direct sensing of ROS by the cysteine residue in SlyA. Wild-type SlyA did not have an oxidation sensitive thiol as detected by click chemistry, and targeted mutagenesis of the only cysteine residue did not modify the induction of *sseA* by hydrogen peroxide (Fig. 3.S3).

The sensor kinase SsrA and response regulator SsrB were horizontally acquired on the SPI-2 genomic island along with the apparatus, chaperone, and translocon components of the SPI-2 type 3 secretion system. Although SsrA-SsrB have been shown to be required for transcription of SPI-2 genes, the signals that drives activation of SsrA and subsequent phosphorylation of SsrB to its DNA-binding active form have not been identified. Previous work in our lab has shown that histidine residues in the periplasmic domain can be protonated in the low pH environment of the *Salmonella* containing vacuole, which promotes SsrA activity. Here we show that in addition to low pH, ROS may act as a second signal to promote SsrA activity. We identified an oxidation sensitive cysteine residue in the cytoplasmic C-terminal domain of SsrA (C765) but the mutation of this residue alone did not reduce *sseA* induction in response to hydrogen peroxide. The incomplete molecular labelling of SsrA led to differences in the identification of this or other potential oxidation-sensitive residues by mass spectrometry. This can be overcome using isotopically labelled tags with cleavable linkers (ICAT), allowing for immunoprecipitation of the biotin tag with streptavidin beads and subsequent cleavage of this biotin linker to release only the tagged proteins that are then digested and analyzed by mass spectrometry. The use of different isotopes for different samples allows for

comparison of the relative abundance of tagged peptides between samples as well (Murray and Van Eyk, 2012). These ICAT reagents are not commercially available but can be chemically synthesised (Weerapana et al., 2007). The use of a protease that does not target lysine and arginine would also allow for the detection of C782, thereby being able to probe whether this residue was biotin labeled.

The inability to purify full-length SsrA also restricted analysis of oxidation-sensitive cysteine residues to the C-terminal domain alone. An alternative approach to identify the residues that are oxidised during exposure of STM to hydrogen peroxide is through mass spectrometry of immunoprecipitated native SsrA. This was attempted with a chromosomally-encoded C-terminal FLAG tagged copy of SsrA that was pulled down with anti-FLAG beads following treatment of STM with or without hydrogen peroxide. This immunoprecipitated protein was used for mass spectrometry. However, although the full-length protein was precipitated successfully and visualized by western blot, only a portion of the protein was detected by mass spectrometry (data not shown). Improvements in sample preparation and handling would allow for identification of oxidation-sensitive cysteine residues throughout the protein.

Intriguingly, while exploring cysteine residues important for ROS sensing, we identified a mutation that completely abrogated SsrA-dependent *sseA* transcription under all conditions tested. The SsrA C826S variant, located in the putative histidine phosphotransfer domain, behaved like a Δ*ssrA* strain, suggesting that it plays a central

role in the function of SsrA. The histidine phosphotransfer domains of other sensor kinases have been shown to be involved in intramolecular interactions (Marina et al., 2005). This residue may be involved in intermolecular disulfide bonding. Future work will be required to explore the nature of this role in the structure and function of SsrA.

Taken together, our data identify a new signal that contributes to SPI-2 transcriptional activation that may be transduced via the sensor kinase involved in regulating this genomic island. Future work will focus on exploring how this signal is integrated with other intracellular cues, to gain a better understanding of the fine-tuning of gene expression that drives virulence in *Salmonella* Typhimurium.

Materials and methods

Ethics statement

All animal experiments were performed according to guidelines from the Canadian Council on Animal Care using protocols approved by the Animal Research Ethics Board at McMaster University.

Bacterial strains

Salmonella enterica serovar Typhimurium SL1344 and isogenic mutants were used in this study. A full list of strains used in this study are provided in table 3.2.

Bacteria were grown in for routine propagation supplemented with antibiotics at the following concentrations: streptomycin (50 mg/ml) ampicillin (100 mg/ml), chloramphenicol (34 mg/ml), or kanamycin (50 mg/ml). Bacteria were grown in M9 pH 7.4 or LPM pH 5.8 for hydrogen peroxide inducing assays.

Plasmid generation

All plasmids used in this study are listed in Table 3.2. P*sseA-lux* was generated in Osborne and Coombes, 2011 and P*ahpC-lux* was generated in Tuinema et al., 2014 on the pGEN-*luxCDABE* backbone from Lane et al., 2007.

pWSK129-P*ssrA-ssrA* C82A, C108A, C82AC108A, C312A, and the wild-type variant were generated in (Mulder et al., 2015). pWSK129-P*ssrA-ssrA* C782S, C826S, C782S C826S, C765S, and C765S C782S were generated by site-directed mutagenesis (SDM) of wild-type pBluescript-P*ssrA-ssrA* using primers described in table 3.3. Sequence verified SDM mutants were sub-cloned into the low copy vector pWSK129 to generate the corresponding *ssrA* constructs in pWSK129. These were transformed into STM Δ*ssrA* (Mulder et al., 2015).

pWSK129-P*slyA-slyA* was generated by amplification of *slyA* and 1000 bp upstream of the gene by PCR. The gene product was ligated into pWSK129 and transformed into TOP10 cells for amplification of the plasmid. Sequence verified plasmids was transformed into STM Δ*slyA* (Osborne and Coombes, 2011). pWSK129-

P*slyA-slyA* C81S was generated by site-directed mutagenesis of pWSK129-P*slyA-slyA*. The mutation was verified by sequencing prior to transformation into STM Δ*slyA*.

Pet28a-6HIS-SUMO was generated by sub-cloning the SUMO tag from pet15b-SUMO. Pet28a-6HIS-SUMO-SsrA 681-920 was generated by PCR amplification of the region from STM *ssrA* and ligation into pet28a-SUMO. Pet28a-6HIS-SUMO-SsrA 681-920 C782S, Pet28a-6HIS-SUMO-SsrA 681-920 C765S, and Pet28a-6HIS-SUMO-SsrA 681-920 C765S C782S were generated by PCR amplification of the 681-920 region from the respective pBluescript-P*ssrA-ssrA* variants and ligation into pet28a-SUMO. All plasmids were transformed into BL21 DE3 cells for protein expression and purification.

Pet28a-6HIS-SlyA was generated by amplification of the *slyA* coding sequence from STM and ligation into pet28a. Pet28a-6HIS-SlyA C81S was generated by site-directed mutagenesis of the wild-type plasmid. Both plasmids were transformed into BL21 DE3 cells for protein expression and purification.

Antibiotic and hydrogen peroxide disk diffusion assays

STM expressing pGEN-P*sseA-lux* was spread on M9 pH 7.4 agar plates. Sterile disks were placed in the center of the plate and antibiotic or hydrogen peroxide was spotted on the disk. Plates were incubated at 37 °C overnight and imaged on a Perkin Elmer *In vivo* Imaging System to visualize growth and luminescence. Luminescence

around the zone of inhibition was quantified using manually defined regions of interest. For experiments with thiourea, M9 agar plates were supplemented with thiourea.

In vitro hydrogen peroxide assay

Overnight cultures of STM were sub-cultured into M9 pH 7.4 or LPM pH 5.8 and grown with shaking to mid-log (OD_{600} 0.4-0.6) at 37 °C. Bacteria were then centrifuged and resuspended in fresh media. Hydrogen peroxide, diluted in water, was added to the "treatment" samples at the specified concentration and all samples were returned to the shaker for ten minutes. Following this, samples were centrifuged again and bacterial pellets were lysed in Trizol (Invitrogen) for RNA extraction. Samples were stored in Trizol at -20 °C prior to processing.

RNA extraction and RT-qPCR

RNA was extracted by chloroform (BioShop) separation following the manufacturer's protocol, precipitated with 100% isopropanol (BioShop) and washed with 75% ethanol (Sigma), and treated with Dnase I (Invitrogen). RNA was then re-precipitated in isopropanol following Dnase I inactivation (Dnase inactivation beads, Ambion), washed with ethanol, and resuspended in DEPC water (Invitrogen). RNA concentrations were quantified by nanodrop prior to downstream analysis. cDNA was synthesized from purified RNA using qScript cDNA Supermix (Quantabio), diluted 1:10 and used in quantitative PCR reactions for SPI-2 genes, *ahpC*, and the housekeeping gene

rsmC in a LightCycler 480 (Roche) with Quanta Perfecta SYBR Green Supermix (Quantabio). Primers for each gene are listed in table 3.3. Quantification of hydrogen peroxide induced activity was measured by calculating the relative gene expression (treatment-control), normalized to *rsmC*, using the Pfaffl method. Relative *sseA* gene expression in isogenic mutants was calculated with the Pfaffl method compared to the average gene expression in wild-type STM as the control, normalized to *rsmC.*

Isolation of murine peritoneal neutrophils and neutrophil infections

Peritoneal neutrophils were stimulated with Biogel and isolated as described previously (Tuinema et al., 2014). Briefly, female 8-10-week-old C57BL/6 or B6.129S6-*CYBB*^{tm1Din}/J (gp91$^{phox-/-}$) mice were peritoneally injected with 1 ml of 2% Biogel (Bio-Rad) in PBS. Twelve to sixteen hours later, mice were sacrificed and neutrophils were isolated by peritoneal lavage with 6 ml ice-cold RPMI supplemented with 10% FBS, 1× HEPES, 1× β-mercaptoethanol, 1× sodium pyruvate, and 1× essential amino acids. The cell suspension was passed through a 40 μm strainer and neutrophils were collected by centrifugation at 500 *g* for five minutes. Cells were counted and seeded in black-well clear bottom 96 well plates at 5×10^5 cells per well. Cells were allowed to attach for two hours prior to infection at 37 °C in a humidified incubator containing 5% CO_2. Cells were treated with 150 mM thiourea during this attachment period where indicated. Overnight cultures of STM expressing pGEN-P*sseA-lux* or pGEN-P*ahpC-lux* were sub-cultured in LB to mid-log in LB and used to infect neutrophils at a multiplicity of infection of 100:1 in RPMI. Following 30 minutes of infection, cells were washed five times and media was

replaced with fresh RPMI. Luminescence from intracellular bacteria was read every thirty minutes. At end-point, the cells were once again washed with PBS and lysed with 1% Triton-X, 0.1% SDS in PBS. Lysates were serial diluted and plated for enumeration of intracellular bacteria. Luminescence from each well was normalized to end-point bacterial colony forming units (CFU).

In vivo imaging

Abdomens of female 8-10-week-old C57BL/6 or B6.129S6-CYBBtm1Din/J (gp91$^{phox-/-}$) mice were depilated with Nair hair removal cream one day prior to infection. Mice were infected intraperitoneally with 1×10^7 cfu STM carrying pGEN-P*sseA*-*lux* and anesthetized with 2% isofluorane one hour post infection for imaging. Mice were imaged with a Perkin Elmer *In vivo* Imaging System with a 30 s exposure for luminescence. Mice were then euthanized and spleens were isolated for *ex vivo* imaging. Luminescence was quantified in the region of interest using the Living Image software. Spleens were homogenized, serially diluted, and plated for CFU enumeration. Luminescence was normalized to CFU from each mouse.

Protein expression and purification

BL21 DE3 cells containing the relevant plasmids were sub-cultured into LB at 37 °C with shaking to mid-log phase, then induced with 0.5 mM IPTG to stimulate protein expression. Induced cultures were grown at 30 °C for a further 3-4 hours, when bacteria

were harvested by centrifugation. Bacterial pellets were frozen at -20 °C for future protein purification.

SsrA 681-920 and its variants were lysed by sonication in ice-cold lysis buffer (50 mM Tris pH 7.5, 300 mM NaCl, 10 mM imidazole, 2 mM β-mercaptoethanol, EDTA-free protease inhibitor tablet). SlyA and SlyA C81S were lysed by sonication in ice-cold lysis buffer (10 mM Tris pH 7.5, 500 mM NaCl, 2 mM β-mercaptoethanol, 20 mM imidazole, EDTA-free protease inhibitor tablet). Cell lysates were harvested by centrifugation at 30,000 *g* for thirty minutes. Lysates were then run on a Ni-NTA column for protein purification. Lysis buffer was used as the wash buffer for the column, and proteins were eluted from the column with their respective buffers containing 250 mM imidazole. β-mercaptoethanol was not included in the elution buffer. Proteins were then dialyzed into 10 mM Tris pH 7.5 and 500 mM NaCl overnight with 0.5 mg/ml TEV protease to cleave the SUMO tag. Proteins were run on a nickel column to purify the proteins from the cleaved tag. Protein concentrations were quantified with a Bradford assay. Aliquots were frozen at -80 °C for future use.

Click chemistry

Purified proteins were diluted to 1 mg/ml in PBS and 20 μM iodoacetamide alkyne probe (Thermo Fisher Scientific) was added to 500 μl of this protein solution. Equal volume of DMSO was added instead of iodoacetamide alkyne for the negative control. The mixture was vortexed and let stand at room temperature for 1 h. Two

milimolar biotin azide (Thermo Fisher Scientific) was added to the solution, the solution was vortexed, followed by 10 mM ascorbic acid, further vortexing, and 1 mM of copper (II) sulphate. This mixture was vortexed and allowed to incubate at room temperature for 1 h, with a pulse vortex at 30 min. Samples were then centrifuged at 6500 *g* for five min at 4 °C. Five hundred microliters of ice-cold methanol was added to the pellet, which was sonicated for 5 s at an amplitude of 5 to resuspend the pellet. Following sonication, samples were rotated on ice for 10 min and pelleted at 6500 *g* for five min. The ice-cold methanol wash and sonication was repeated. Samples were one again rotated and pelleted. Pellets were either air-dried and frozen for mass spectrometry or resuspended in 1 ml of 1.2% SDS in PBS. This was sonicated for 5 s and heated at 85 °C for 5 min, then diluted in PBS with 0.2% SDS. These samples were used for western blot against biotin.

Mass spectrometry

Sample preparation and mass spectrometry were performed by the Alberta Proteomics and Mass Spectrometry Facility. Protein pellets were dissolved in 100 mM ammonium bicarbonate, reduced with 5 µl of 200 mM dithiothreitol (DTT) in 50 mM ammonium bicarbonate for 1 h, and alkylated with 20 µl of 200 mM iodoacetamide for 1 h in the dark. Twenty microliters of 200 mM DTT was then added to consume any extra iodoacetamide for 1 h in the dark, then samples were digested with a 1:50 trypsin:protein ratio of 2 µg/µl trypsin overnight at room temperature. Formic acid was then added to acidify the sample to a pH of 3-4 and tryptic peptides were resolved and ionized with nanoflow high performance liquid chromatography (HPLC, Easy-nLC II, Thermo

Scientific) with a PicoFrit fused silica capillary column (New Objective ProteoPepII, C18, 100 μM ID, 300 Å, 5 μM) coupled to an LTQ XL-Orbitrap hybrid mass spectrometer (Thermo Scientific). Samples were injected on the column with a flow rate of 3000 nl/min and resolved at 500 nl/min using a 60 min linear gradient from 0 to 35% acetonitrile in 0.2% formic acid. The mass spectrometer was operated in a data-dependent acquisition mode, recording high-accuracy and high-resolution survey Orbitrap spectra using external mass calibration with a resolution of 30,000 and a mass/charge (m/z) range of 400–2000. The fourteen most intense multiply charged ions were sequentially fragmented by collision induced dissociation, and spectra of their fragments were recorded in the linear ion trap; after two fragmentations all precursors selected for dissociation were dynamically excluded for 60 s. Data was processed using Proteome Discoverer 1.4 (Thermo Scientific) and searched against the supplied sample sequences using SEQUEST (Thermo Scientific). Search parameters included a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.8 Da.

Western blot

Protein samples following click chemistry were separated on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane with a semi-dry transfer apparatus. Membranes were blocked in Tris-buffered saline containing 0.2% Tween (TBST) with 5% bovine serum albumin (BSA) for one hour. The membrane was washed three times with TBST and HRP-conjugated anti-biotin antibody (Cell Signaling, 1:1000 in TBST) was added for one hour. The membrane was washed and HRP was activated with BioRad

ECL reagent and imaged on a a Chemidoc XRS+ (Bio-Rad). The membrane was then stripped with Thermo Scientific Restore stripping buffer, blocked with 5% skim milk in TBST for 30 min, and incubated with mouse anti-HIS6 antibody (GE Healthcare, 1:1000). The membrane was washed and incubated in goat anti-mouse HRP conjugated secondary antibody (Jackson Labs, 1:5000). The membrane was washed five times and activated with BioRad ECL reagent for imaging on a Chemidoc XRS+ (Bio-Rad).

Competitive infections

Female 8-10-week-old C57BL/6 or transgenic B6.129S6-CYBBtm1Din/J (gp91$^{phox-/-}$) mice were intraperitoneally infected with a 1:1 ratio of STM *ushA::cat* (neutral mutant) (Coombes et al., 2005) and STM Δ*slyA*. Mice were monitored for body weight daily and euthanized when they reached 20% body weight loss. Spleens and livers were isolated, homogenized, and serially diluted for CFU enumeration. Colonies were then replica plated onto LB-chloramphenicol and LB-streptomycin to determine the ratio of wild-type (*ushA::cat*) to mutant colonies.

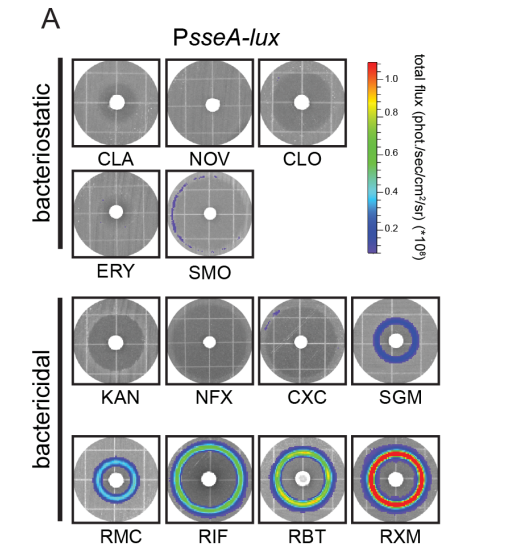
Statistical analysis

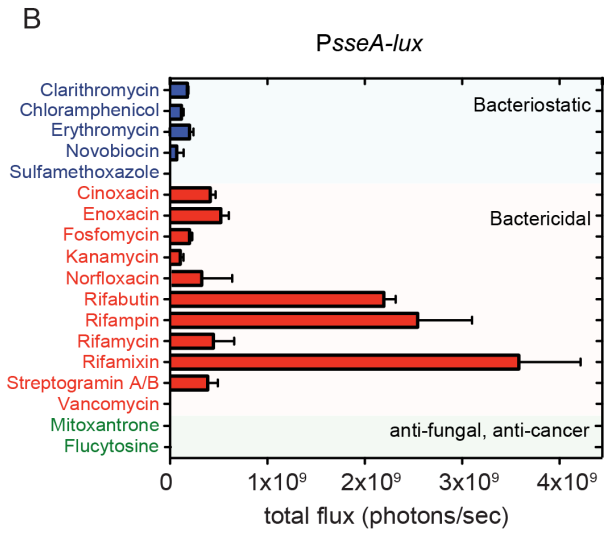
Data were analyzed using GraphPad Prism v 6.0. Statistical parameters such as number of replicates and statistical tests are included in the figure legends. P values of <0.05 were considered significant.

Figures and Tables

Figure 3.1. Bactericidal, but not bacteriostatic, antibiotics activate the *sseA* promoter.

A. pP*sseA*-*lux* lawns on M9 plates were exposed to various bacteriostatic or bactericidal antibiotics via diffusion from a disk, and luminescence was measured after overnight growth. CLA- clarithromycin, NOV – novobiocin, CLO – chloramphenicol, ERY – erythromycin, SMO – sulfamethaxozole, KAN – kanamycin, NFX – enoxacin, CXC – cinoxacin, SGM – streptogramin A/B, RMC – rifamycin, RIF – rifampin, RBT – rifabutin, RXM – rifamixin. B. Quantification of luminescence around the zone of inhibition from the disk diffusion assays shows increased transcriptional activation by bactericidal antibiotics but not bacteriostatic. Data are mean ± SEM from three biological replicates. C. P*sseA* induction by rifampin (RIF), rifaximin (RXM), or hydrogen peroxide is quenched upon free radical scavenging by thiourea supplemented in the media.


A
PsseA-lux
bacteriostatic
CLA
NOV
CLO
ERY
SMO
bacteridical
KAN
NFX
CXC
SGM
RMC
RIF
RBT
RXM
total flux (phot./sec/cm²/sr) (*10⁸)


B
PsseA-lux
Clarithromycin
Chloramphenicol
Erythromycin
Novobiocin
Sulfamethoxazole
Cinoxacin
Enoxacin
Fosfomycin
Kanamycin
Norfloxacin
Rifabutin
Rifampin
Rifamycin
Rifamixin
Streptogramin A/B
Vancomycin
Mitoxantrone
Flucytosine
Bacteriostatic
Bactericidal
anti-fungal, anti-cancer
0
1x10⁹
2x10⁹
3x10⁹
4x10⁹
total flux (photons/sec)

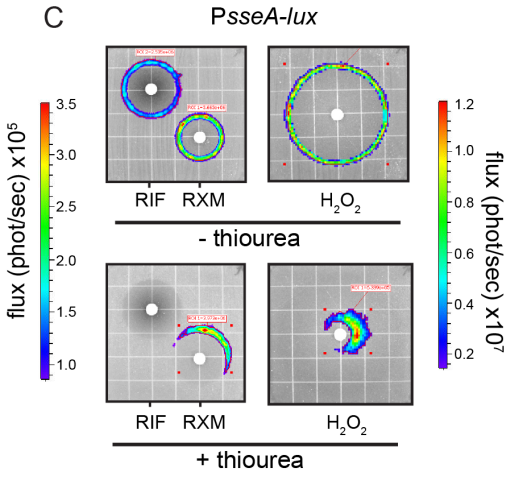

C
PsseA-lux
flux (phot/sec) x10⁵
flux (phot/sec) x10⁷
RIF
RXM
H₂O₂
- thiourea
+ thiourea

Figure 3.2. Hydrogen peroxide induces multiple SPI-2 genes *in vitro*.

A. Sub-inhibitory concentrations of hydrogen peroxide (0.25 mM, 0.5 mM, 1 mM) induce gene expression of *sseA* in wild-type STM measured by RT-qPCR. *ahpC* gene expression was measured as a positive control. Data are mean ± SEM normalized to untreated RNA, relative to *rsmC* from six biological samples. * $p < 0.05$ relative to *rsmC* (no change, dotted line) by *t*-test. B. Genes from each operon within the SPI-2 genomic island are transcribed in response to treatment with 0.5 mM hydrogen peroxide relative to untreated controls. Data are mean ± SEM of five biological samples, * $p < 0.05$ by one-way ANOVA with Holm-Sidak correction for multiple comparisons relative to *rsmC* (dotted line).

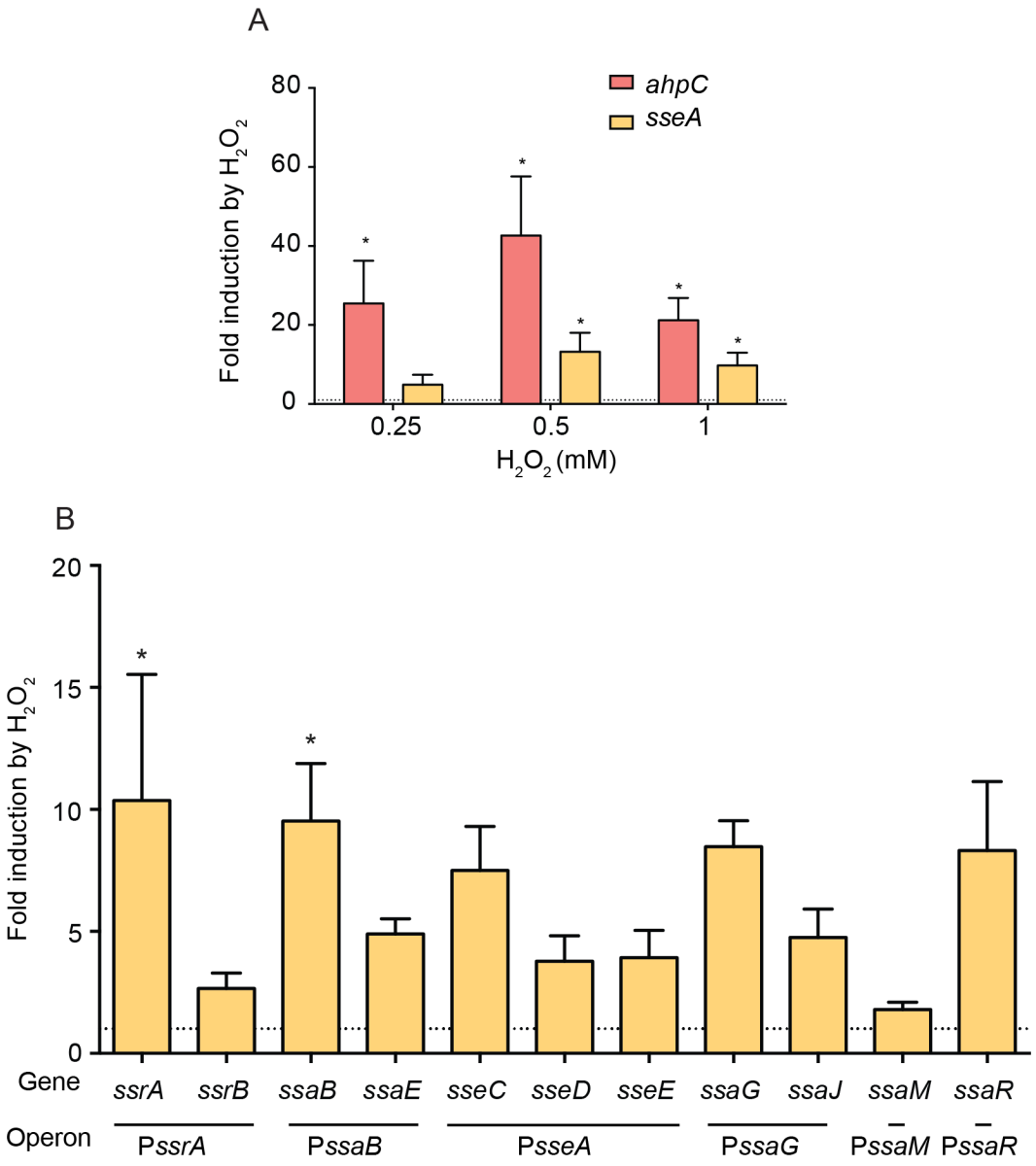

A
ahpC
sseA
Fold induction by H2O2
80
60
40
20
0
0.25
0.5
1
H2O2 (mM)
B
Fold induction by H2O2
20
15
10
5
0
Gene
ssrA
ssrB
ssaB
ssaE
sseC
sseD
sseE
ssaG
ssaJ
ssaM
ssaR
Operon
PssrA
PssaB
PsseA
PssaG
PssaM
PssaR

Figure 3.3. P*sseA* is induced during STM **infection of neutrophils or whole mice expressing NADPH oxidase.**

A. Infection of neutrophils from wild-type or gp91$^{phox-/-}$ (lacking NADPH oxidase) mice with STM expressing P*sseA-lux* shows increased transcription of the promoter in wild-type neutrophils over the course of an infection. Data are mean ± SEM of five biological replicates. Treatment of neutrophils with thiourea inhibits P*sseA-lux* transcription during a STM infection. Data are mean ± SD from three replicates. B. Intraperitoneal infection of wild-type or gp91$^{phox-/-}$ mice reveals increased transcriptional activation of P*sseA-lux* in wild-type mice. C. Quantification of luciferase activity relative to bacterial loads from spleens of wild-type or NADPH oxidase knockout mice infected with STM P*sseA-lux* for one hour. Data are mean ± SEM. * $p < 0.05$.

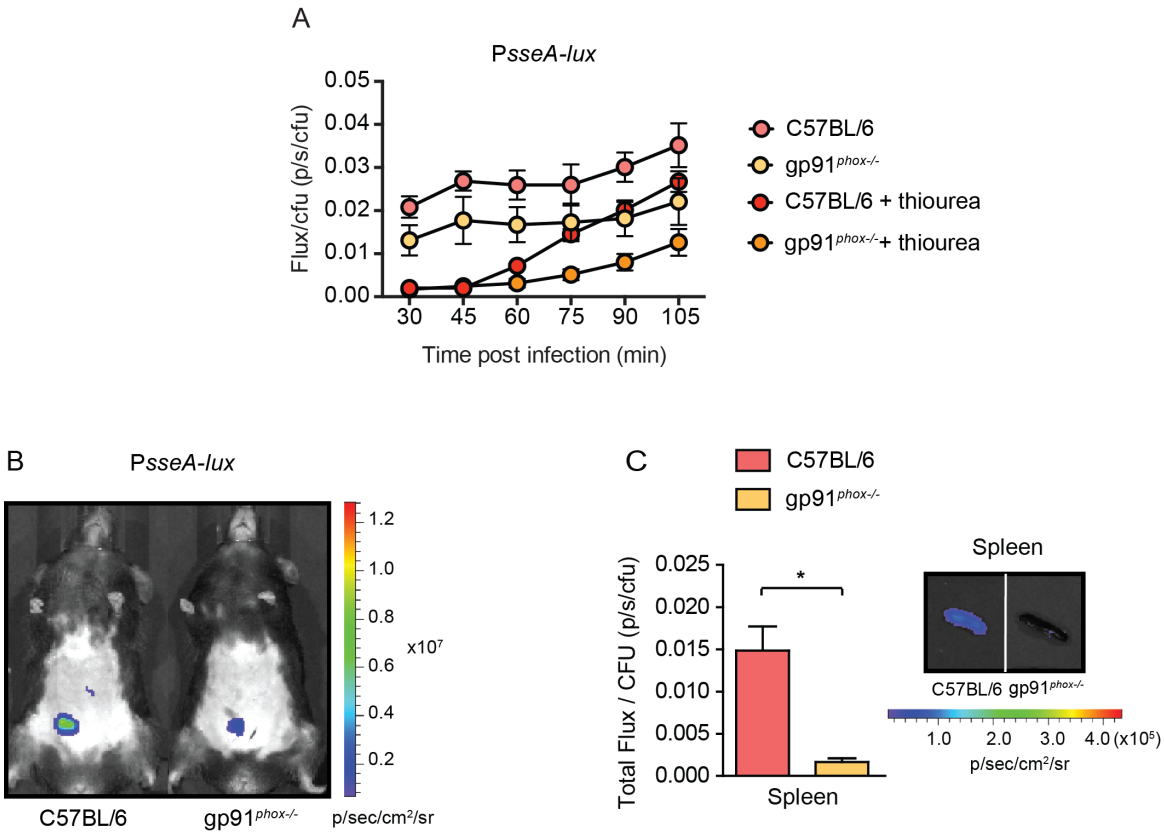

A
PsseA-lux
Flux/cfu (p/s/cfu)
0.05
0.04
0.03
0.02
0.01
0.00
30
45
60
75
90
105
Time post infection (min)
C57BL/6
gp91phox-/-
C57BL/6 + thiourea
gp91phox-/- + thiourea
B
PsseA-lux
1.2
1.0
0.8
0.6
0.4
0.2
x10^7
C57BL/6
gp91phox-/-
p/sec/cm²/sr
C
C57BL/6
gp91phox-/-
Total Flux / CFU (p/s/cfu)
0.025
0.020
0.015
0.010
0.005
0.000
*
Spleen
Spleen
C57BL/6 gp91phox-/-
1.0
2.0
3.0
4.0 (x10^5)
p/sec/cm²/sr

Figure 3.4. Hydrogen peroxide induced transcription of *sseA* varies in SPI-2 regulatory mutants of STM**.**

A. Diffusion of hydrogen peroxide from a disk shows reduced P*sseA-lux* activation in STM mutants of SPI-2 regulatory genes compared to wild-type STM. Data are mean ± SEM from three independent experiments. B. RT-qPCR of *sseA* from different STM strains with 0.5 mM hydrogen peroxide induction shows reduced levels of *sseA* induction in several mutant strains. Data are mean ± SEM from one to three biological replicates per mutant strain. * $p < 0.05$ compared to wild-type (WT) by one-way ANOVA with Holm-Sidak correction for multiple comparisons.

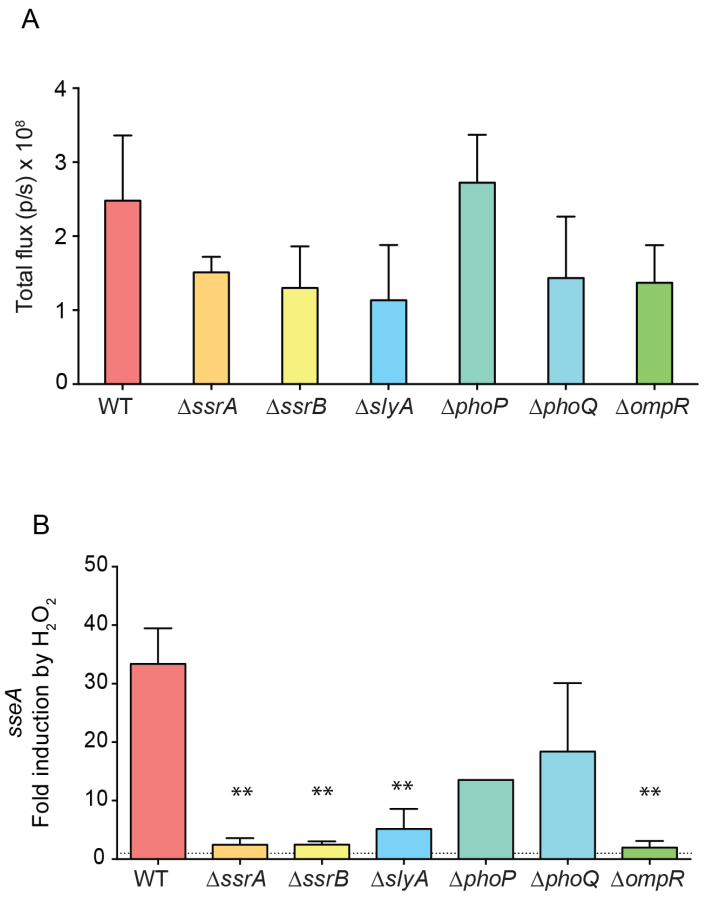

A
Total flux (p/s) x 10^8
4
3
2
1
0
WT
ΔssrA
ΔssrB
ΔslyA
ΔphoP
ΔphoQ
ΔompR
B
sseA
Fold induction by H2O2
50
40
30
20
10
0
**
**
**
**
WT
ΔssrA
ΔssrB
ΔslyA
ΔphoP
ΔphoQ
ΔompR

Figure 3.5. Cysteine residues in SsrA contribute to hydrogen peroxide dependent *sseA* activation under SPI-2 inducing conditions.

A. Treatment with 0.5 mM hydrogen peroxide induces *sseA* transcription when STM is grown under SPI-2 inducing conditions (LPM pH 5.8). Data are normalized to untreated RNA levels and are relative to *rsmC*. Data are mean ± SEM of six biological replicates. * $p < 0.05$ relative to *rsmC* by *t*-test. B. STM *ssrA* mutants complemented in trans with wild-type SsrA or site-directed cysteine variants show differences in *sseA* induction in response to hydrogen peroxide. Data are mean ± SEM from four biological replicates. C. Wild-type SsrA 681-920 and the indicated variants have reactive thiol groups, detected by click-reactive labeling of the proteins with a biotin-linked triazole and visualized with an anti-biotin western blot. The negative control was wild-type SsrA 681-920 that was incubated with biotin azide but not the iodoacetamide alkyne. D. SsrA C765S does not affect *sseA* transcription in response to hydrogen peroxide. Data are mean mean ± SEM from six biological replicates, normalized to *rsmC*.

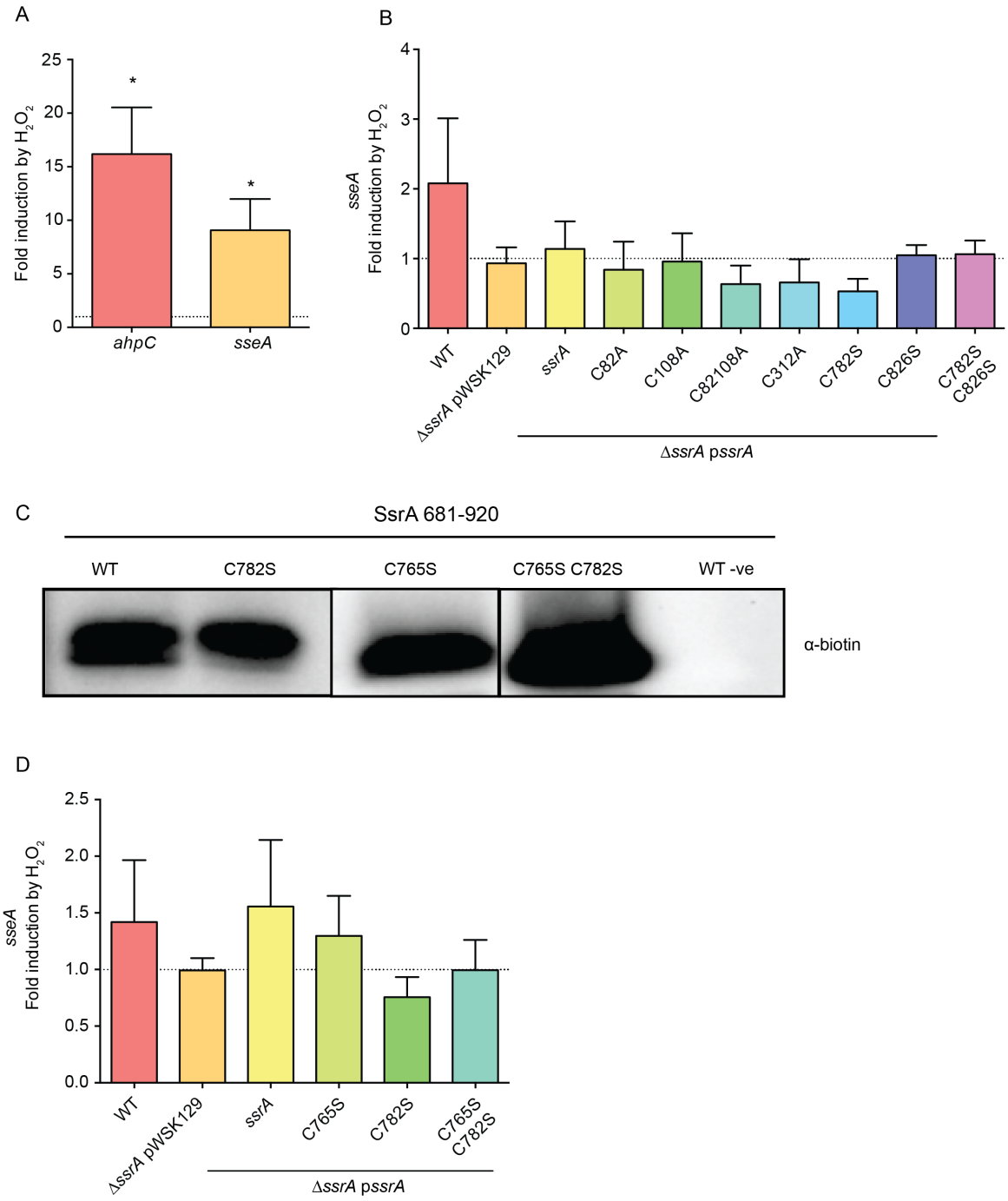

A
Fold induction by H_2O_2
25
20
15
10
5
0
*
*
ahpC
sseA
B
sseA
Fold induction by H_2O_2
4
3
2
1
0
WT
ΔssrA pWSK129
ssrA
C82A
C108A
C82108A
C312A
C782S
C826S
C782S C826S
ΔssrA pssrA
C
SsrA 681-920
WT
C782S
C765S
C765S C782S
WT -ve
α-biotin
D
sseA
Fold induction by H_2O_2
2.5
2.0
1.5
1.0
0.5
0.0
WT
ΔssrA pWSK129
ssrA
C765S
C782S
C765S C782S
ΔssrA pssrA

Figure 3.S1. P*sseA* transcription during a neutrophil infection is dependent on *ssrA*.

A. Infection of murine neutrophils with STM expressing P*ahpc-lux* shows reduced *ahpC* transcription in neutrophils lacking a functional NADPH oxidase (gp91$^{phox-/-}$). Data are mean ± SEM from three biological replicates. B. Infection of murine neutrophils with a *ssrA* mutant of STM shows reduced transcription of P*sseA* and no difference between transcription levels in wild-type and NADPH oxidase mutant neutrophils. Data are mean ± SEM from one to three biological replicates.

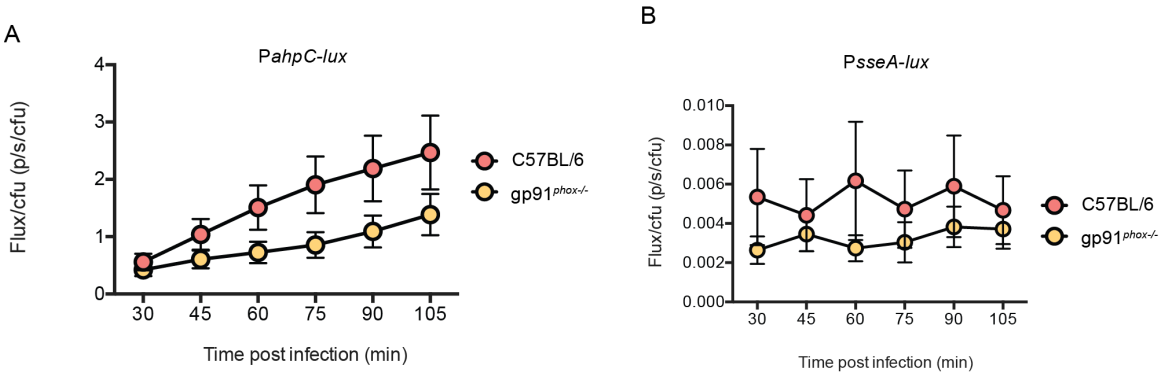

A
PahpC-lux
Flux/cfu (p/s/cfu)
4
3
2
1
0
30
45
60
75
90
105
Time post infection (min)
C57BL/6
gp91phox-/-
B
PsseA-lux
Flux/cfu (p/s/cfu)
0.010
0.008
0.006
0.004
0.002
0.000
30
45
60
75
90
105
Time post infection (min)
C57BL/6
gp91phox-/-

Figure 3.S2. STM **SPI-2 regulatory mutants have reduced *sseA* transcription independent of hydrogen peroxide treatment.**

A. Measurement of *sseA* expression relative to wild-type (dotted line) in uninduced samples relative to *rsmC* shows reduced *sseA* transcription in *ssrA*, *ssrB*, and *slyA*, mutants. Data are mean ± SEM from three biological replicates.

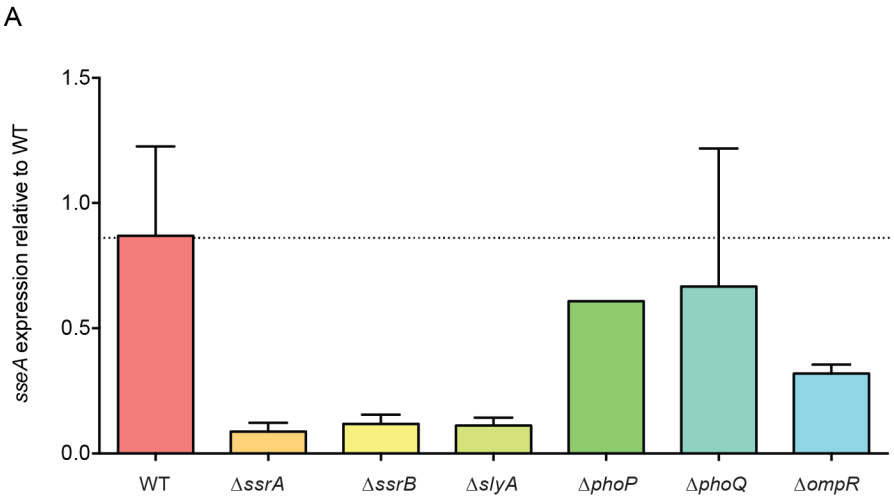

A
sseA expression relative to WT
1.5
1.0
0.5
0.0
WT
ΔssrA
ΔssrB
ΔslyA
ΔphoP
ΔphoQ
ΔompR

Figure 3.S3. The cysteine residue in SlyA is not involved in hydrogen peroxide-dependent induction of *sseA*.

A. *sseA* induction is not reduced in a *slyA* mutant expressing a C81S variant in trans. B. Transcription of *sseA* is not affected by the SlyA C82S variant compared to wild-type STM (dotted line). C. 6HIS-SlyA and 6HIS-SlyA C81S do not have thiol reactive cysteines that can be detected by click-reactive biotin labelling. D. A *slyA* mutant is less fit than wild-type STM (*ushA::cat* neutral marked mutant) in a competitive murine infection and this fitness defect is reduced in NADPH oxidase deficient mice. Competitive index of Δ*slyA*/*ushA::cat*. * $p < 0.05$ by two-way ANOVA with Holm-Sidak correction for multiple comparisons.

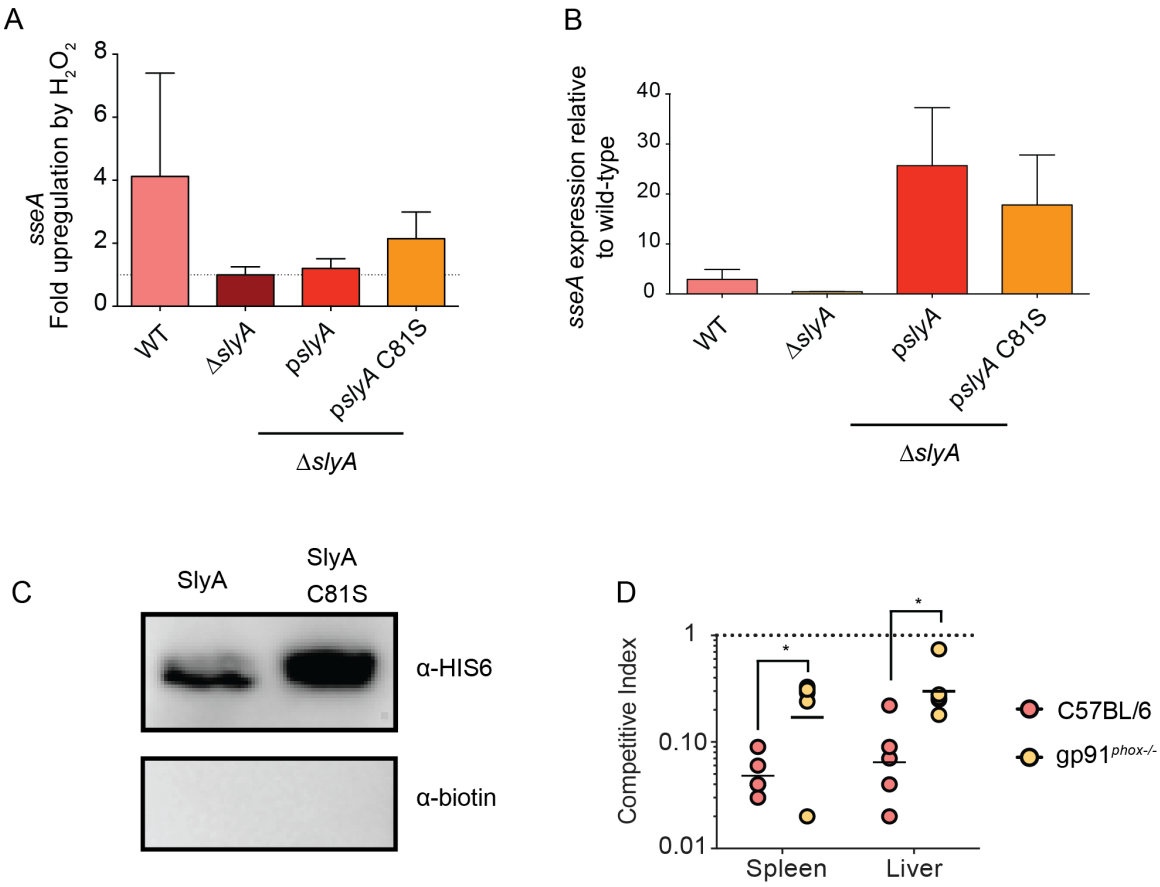

A
sseA
Fold upregulation by H2O2
8
6
4
2
0
WT
ΔslyA
pslyA
pslyA C81S
ΔslyA
B
sseA expression relative to wild-type
40
30
20
10
0
WT
ΔslyA
pslyA
pslyA C81S
ΔslyA
C
SlyA
SlyA C81S
α-HIS6
α-biotin
D
Competitive Index
1
0.10
0.01
*
*
Spleen
Liver
C57BL/6
gp91phox-/-

Figure 3.S4. Certain SsrA cysteine variants are defective for *sseA* transcriptional activation under SPI-2 conditions.

A. Measurement of *sseA* expression relative to wild-type STM relative to *rsmC* reveals decreased levels of *sseA* transcription in SsrA C826S and C782S C826S in untreated samples grown in LPM (dotted line – average *sseA* transcription in wild-type sample). B. Hydrogen peroxide induction of *ahpC* is not impaired in STM *ssrA* cysteine variants in LPM, relative to untreated samples and normalized to *rsmC* (dotted line). Data are mean ± SEM of four biological replicates. C. SsrA C765S and C765S C782S variants are equally able to promote *sseA* transcription compared to wild-type (dotted line) under SPI-2 inducing conditions. Data are mean ± SEM from six biological replicates normalized to *rsmC*. D. *ahpC* transcription is promoted by hydrogen peroxide treatment in SsrA C765S and C765S C782S variants to the same level as wild-type SsrA. Data are mean ± SEM from six biological replicates normalized to *rsmC* (dotted line).

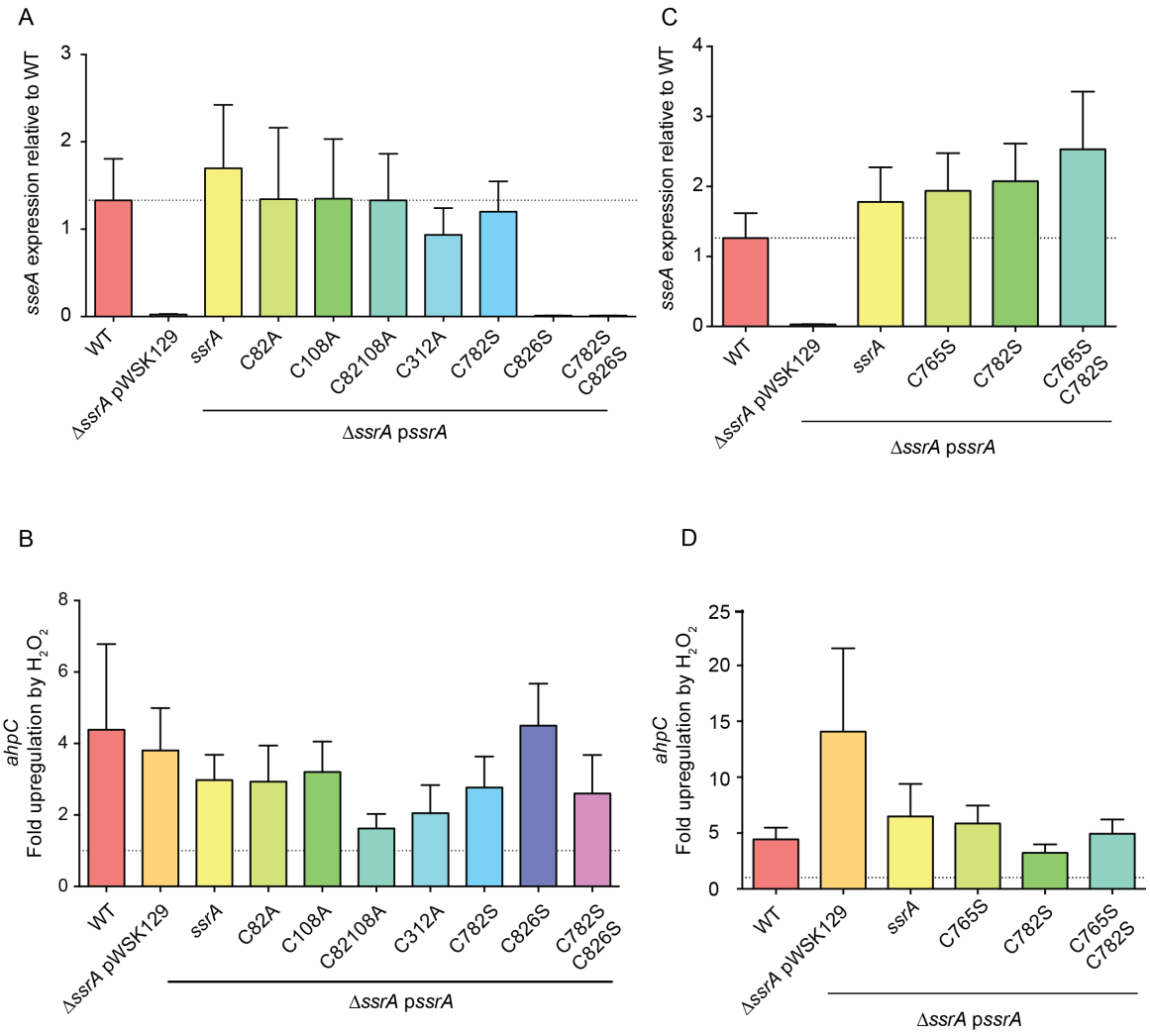

A
sseA expression relative to WT
0
1
2
3
WT
ΔssrA pWSK129
ssrA
C82A
C108A
C82108A
C312A
C782S
C826S
C782S C826S
ΔssrA pssrA
C
sseA expression relative to WT
0
1
2
3
4
WT
ΔssrA pWSK129
ssrA
C765S
C782S
C765S C782S
ΔssrA pssrA
B
ahpC Fold upregulation by H2O2
0
2
4
6
8
WT
ΔssrA pWSK129
ssrA
C82A
C108A
C82108A
C312A
C782S
C826S
C782S C826S
ΔssrA pssrA
D
ahpC Fold upregulation by H2O2
0
5
10
15
20
25
WT
ΔssrA pWSK129
ssrA
C765S
C782S
C765S C782S
ΔssrA pssrA

Table 3.1 List of SsrA peptides and associated modifications identified by mass spectrometry

Sample	Peptide	Modifications
SsrA 681-920	IFQSLDLLLADIENAVSAGEK	
	YISIAAEYQLLR	
	NGIHHYITKPVTLATLARYISIAAEYQLLR	
	MLVSLGQHVTIAASSNEALTLSQQQR	
	cSALLATDDMVINSK	C1(Carbamidomethyl)
	mLVSLGQHVTIAASSNEALTLSQQQR	M1(Oxidation)
	NGIHHYITKPVTLATLAR	
	IIALEELTDLR	
	LWHDEPNNLDPDcMFVALSASVATEDIHR	C13(Carbamidomethyl)
	GcLGQIGQTELVcYVIDIENR	C2(Carbamidomethyl); C13(Carbamidomethyl)
	NIELQEQDPSR	
	CSALLATDDmVINSK	M10(Oxidation)
	cSALLATDDmVINSK	C1(Carbamidomethyl); M10(Oxidation)
	IIALEELTDLRQK	
	FDLVLIDIR	
	LWHDEPNNLDPDcmFVALSASVATEDIHR✱	C13(Carbamidomethyl; M14(Oxidation)
	IDQLIHTLK	
	KNGIHHYITKPVTLATLAR	
	CSALLATDDMVINSK	
	GSHMNNLLPPWQLQILLVDDADINR	
	MGKIIALEELTDLR	
	NGIHHYITKPVTLATLARYISIAAEYQLLRNIELQEQ DPSR	
	MPEIDGIEcVR	C9(Carbamidomethyl)
	GCLGQIGQTELVCYVIDIENR	
	mPEIDGIEcVR	M1(Oxidation); C9(Carbamidomethyl)
	LWHDEPNNLDPDcMFVALSASVATEDIHR◆	C13(B-biotintag)
	MPEIDGIECVR	
	MIFKNYTIT	
	mPEIDGIECVR	M1(Oxidation)
SsrA 681-920 C782S	IFQSLDLLLADIENAVSAGEK	
	MLVSLGQHVTIAASSNEALTLSQQQR	
	YISIAAEYQLLRNIELQEQDPSR	
	NGIHHYITKPVTLATLARYISIAAEYQLLR	

mLVSLGQHVTIAASSNEALTLSQQQR	M1(Oxidation)
YISIAAEYQLLR	
IIALEELTDLR	
GcLGQIGQTELVcYVIDIENR	C2(Carbamidomethyl); C13(Carbamidomethyl)
cSALLATDDMVINSK	C1(Carbamidomethyl)
NGIHHYITKPVTLATLAR	
cSALLATDDmVINSK	C1(Carbamidomethyl); M10(Oxidation)
KNGIHHYITKPVTLATLARYISIAAEYQLLR	
LWHDEPNNLDPDcMFVALSASVATEDIHR✱	C13(Carbamidomethyl)
IIALEELTDLRQK	
CSALLATDDmVINSK	M10(Oxidation)
LWHDEPNNLDPDcmFVALSASVATEDIHR✱	C13(Carbamidomethyl; M14(Oxidation)
CSALLATDDMVINSK	
NIELQEQDPSR	
FDLVLIDIR	
IDQLIHTLK	
NGIHHYITKPVTLATLARYISIAAEYQLLRNIELQEQ DPSR	
KNGIHHYITKPVTLATLAR	
MGKIIALEELTDLR	
mPEIDGIEcVR	M1(Oxidation); C9(Carbamidomethyl)
MPEIDGIEcVR	M1(Oxidation); C9(Carbamidomethyl)
IIALEELTDLRQKIR	C9(Carbamidomethyl)
GCLGQIGQTELVCYVIDIENR	
MPEIDGIECVR	
GSHMNNLLPPWQLQILLVDDADINR	
mPEIDGIECVR	M1(Oxidation)
CKKNGIHHYITKPVTLATLAR	
MGKIIALEELTDLRQK	
MIFKNYTIT	
LWHDEPNNLDPDCMFVALSASVATEDIHR✱	
mIFKNYTIT	M1(Oxidation)

Table 3.2 List of strains and plasmids used in this study

Strain	Genotype	Source
S. Typhimurium SL1344	Wild type (Sm^R)	Our collection
S. Typhimurium SL1344	Δ*ssrA*	Mulder et al., 2015
S. Typhimurium SL1344	Δ*ssrB*	Our collection
S. Typhimurium SL1344	Δ*slyA*	Our collection
S. Typhimurium SL1344	Δ*phoP*	Our collection
S. Typhimurium SL1344	Δ*phoQ*	Our collection
S. Typhimurium SL1344	Δ*ompR*	Our collection
S. Typhimurium SL1344	*usha::cat*, Cm^R	Our collection
Plasmid		
pWSK129	Low copy-number vector for complementation, Kan^R	(Mulder et al., 2015)
pGEN-*luxCDABE*	Low copy plasmid carrying the *luxCDABE* operon downstream of the constitutive em7 promoter, Amp^R	Lane et al., 2007
pGEN-P*sseA-lux*	pGEN-*luxCDABE* derivative containing intergenic region upstream of the *S.* Typhimurium *sseA* translational start site in place of em7	Osborne et al., 2011
pGEN-P*ahpC-lux*	pGEN-*luxCDABE* derivative containing intergenic region upstream of the *S.* Typhimurium *ahpC* translational start site in place of em7	Tuinema et al., 2014
pWSK129-P*ssrA-ssrA*	pWSK129 containing *ssrA* under native *ssrA* promoter	Mulder et al., 2015
pWSK129-P*ssrA-ssrA* C82A	pWSK129 containing *ssrA* C82A under native *ssrA* promoter	Mulder et al., 2015
pWSK129-P*ssrA-ssrA* C108A	pWSK129 containing *ssrA* C108A under native *ssrA* promoter	Mulder et al., 2015
pWSK129-P*ssrA-ssrA* C82A C108A	pWSK129 containing *ssrA* C82A C108A under native *ssrA* promoter	Mulder et al., 2015
pWSK129-P*ssrA-ssrA* C312A	pWSK129 containing *ssrA* C312A under native *ssrA* promoter	Mulder et al., 2015
pWSK129-P*ssrA-ssrA* C782S	pWSK129 containing *ssrA* C782S under native *ssrA* promoter	This study
pWSK129-P*ssrA-ssrA* C826S	pWSK129 containing *ssrA* C826S under native *ssrA* promoter	This study
pWSK129-P*ssrA-ssrA* C782S C826S	pWSK129 containing *ssrA* C782S C826S under native *ssrA* promoter	This study
pWSK129-P*ssrA-ssrA* C765S	pWSK129 containing *ssrA* C765S under native *ssrA* promoter	This study

pWSK129-P*ssrA*-*ssrA* C765S C782S	pWSK129 containing *ssrA* C765S C782S under native *ssrA* promoter	This study
pWSK129-P*slyA*-*slyA*	pWSK129 containing *slyA* under native *slyA* promoter	This study
pWSK129-P*slyA*-*slyA* C81S	pWSK129 containing *slyA* C81S under native *slyA* promoter	This study
Pet28a-6HIS-SUMO	Protein expression vector with T7 promoter, SUMO solubility tag, and N-terminal hexa-histidine tag	This study
Pet28a-6HIS-SUMO-SsrA 681-920	*S.* Typhimurium SsrA 681-920 expressed from the T7 promoter, Kan^R	This study
Pet28a-6HIS-SUMO-SsrA C782S 681-920	*S.* Typhimurium SsrA C782S 681-920 expressed from the T7 promoter, Kan^R	This study
Pet28a-6HIS-SUMO-*ssrA* C765S 681-920	*S.* Typhimurium SsrA C765S 681-920 expressed from the T7 promoter, Kan^R	This study
Pet28a-6HIS-SUMO-*ssrA* C765S C782S 681-920	*S.* Typhimurium SsrA C765S C782S 681-920 expressed from the T7 promoter, Kan^R	This study
Pet28a	Protein expression vector with T7 promoter and N-terminal hexa-histidine tag	Novagen
Pet28a-6HIS-SlyA	*S.* Typhimurium SlyA expression vector with T7 promoter and N-terminal hexa-histidine tag	This study
Pet28a-6HIS-SlyA C81S	*S.* Typhimurium SlyA expression vector with T7 promoter and N-terminal hexa-histidine tag	This study

Sm^R – Streptomycin resistance, Kan^R – kanamycin resistance, Cm^R – chloramphenicol resistance, Amp^R – ampicillin resistance

Table 3.3 List of primers used in this study

Primer name	Sequence (5′-3′)	Target gene
DTM_333_F	AAATCCGGGCTAAGGTGAGT	*sseA* RT-qPCR
DTM_333_R	TAAATCCTTCTCGGCCTCCT	*sseA* RT-qPCR
BIp08F	TGCGTGAAGATGAAGGTCTG	*ahpC* RT-qPCR
BIp08R	CGACCAGGTCTAAGGATGGA	*ahpC* RT-qPCR
rsmCRTF_2	GAAAAGCAGCCGCAGTTTAG	*rsmC* RT-qPCR
rsmCRTR_2	CAGTTGGCTACCAACATCCA	*rsmC* RT-qPCR
DTM_332_F	ATCTGTGCTTACCGCAGCTT	*ssrA* RT-qPCR
DTM_332_R	CGTCACGTTCAGCTTCTTCA	*ssrA* RT-qPCR
DTM_334_F	CGGAAATCATAAGCGTCCAT	*sseC* RT-qPCR
DTM_334_R	GCTGTTGGCATAACGATTGA	*sseC* RT-qPCR
DTM_330_F	AATCACCGACGTTCTGGATG	*sseE* RT-qPCR

DTM_330_R	AACGTCGCTGGATAAGATGC	*sseE* RT-qPCR
DTM_331_F	AGTGGATATGCTCTCCCACA	*ssaG* RT-qPCR
DTM_331_R	GCGCTTTAATCATCGATTCTG	*ssaG* RT-qPCR
LM01	GTCTGAGGAGGGATTCATGC	*ssaB* RT-qPCR
LM02	TGCTGCAAGCAGTAGTGTCA	*ssaB* RT-qPCR
LM03	GTGAAGAGGCCAAAGGCATA	*ssaE* RT-qPCR
LM04	GTGCGCTGTTATGGTAACGA	*ssaE* RT-qPCR
LM05	GCCAGAAGATGAAGCGAATC	*ssaJ* RT-qPCR
LM06	AAACATCTTATCCGCCGTTG	*ssaJ* RT-qPCR
LM07	ACGGCCTTTAGCAACCAATA	*ssaM* RT-qPCR
LM08	TTGAGGAATACCCTGGAACG	*ssaM* RT-qPCR
LM09	GATTCGCCTTTGCAACTGAT	*ssaR* RT-qPCR
LM10	TCGGCCCCATAATGAATAAG	*ssAR* RT-qPCR
LM11	AGCTAATGGAGCTTGCCAAA	*sseD* RT-qPCR
LM12	TCGTCAGTACACCCGACAAC	*sseD* RT-qPCR
BIp21F	GCGTTGGCCAGCAATGAATA	*ssrB* RT-qPCR
BIp21R	TTGCAATGCCGCTAACAGAA	*ssrB* RT-qPCR
BIp32F	TATGCGAGCTCCATTACATAAAATGAATGGCTATC	*slyA* for ligation into pWSK129
BIp32R	GTCATGGTACCTCAATCGTGAGAGTGCAATTCCATA	*slyA* for ligation into pWSK129
BIp33F	TCGCGGCAAACCTCCGCCAGCGATCGT	SDM of C81 to serine in *slyA*
BIp33R	ATCGCTGGCGGAGGTTTGCCGCGAAATTAG	SDM of C81 to serine in *slyA*
BIp45F	GATATTCATCGTAGTAAAAAAAATGGG	SDM of C782 to serine in *ssrA*
BIp45R	GATATTCATCGTAGTAAAAAAAATGGG	SDM of C782 to serine in *ssrA*
BIp48F	GAGCAGGATCCGAGTCGCAGCTCAGCGCTACTGGCG	SDM of C826 to serine in *ssrA*
BIp48R	TGTCGCCAGTAGCGCTGAGCTGCGACTCGGATCCTG	SDM of C826 to serine in *ssrA*
BIp108F	GATCCTGACTCCATGTTTGTG	SDM of C765 to serine in *ssrA*
BIp108R	TAAATTATTCGGCTCATCATG	SDM of C765 to serine in *ssrA*
DJL14	GGGCATATGAATAATTTGTTACCACCC	*ssrA* 681-920 for pet28a-SUMO
DJL17	GGCTCGAGTTAAGTAATGGTGTAGTT	*ssrA* 681-920 for pet28a
BIp64F	GTCATCATATGGAATCGCCACTAGGTTCTGATCTGGCA	*slyA* for pet28a
BIp64R	TATGCCTCGAGTCAATCGTGAGAGTGCAATTCCATAAT	*slyA* for pet28a

CHAPTER 4: DISCUSSION

The data presented in the previous chapters describe novel regulatory connections that promote *S.* Typhimurium virulence in an intracellular niche. *S.* Typhimurium has an evolved regulatory network that links the sensing of environmental cues to adaptive gene expression, and the results presented in the previous two chapters expand our understanding of this network by characterizing a new environmental cue and identifying a novel adaptive response that occurs *in vivo*. However, there remain unexplored questions and future avenues for investigation regarding *S.* Typhimurium virulence, some of which will be expanded on below.

Revealing intracellular regulatory adaptations regulated by SsrB

Microarray and chromatin immunoprecipitation experiments with SsrB have previously shown that SsrB can bind and regulate gene expression outside of the SPI-2 genomic island, and that some of these inputs occur at genes that have orthologs in *S. bongori* (Tomljenovic-Berube et al., 2010). The RNA-seq dataset presented here expands on these findings, to demonstrate that the introduction of SsrB into *S. bongori* often results in divergent expression patterns of orthologs shared between *S. bongori* and *S.* Typhimurium. Ten percent of the ~3400 orthologs shared between these two species are divergently regulated by SsrB, including genes from diverse functional groups like metabolism, translation, membrane-associated proteins, motility, and genes of unknown function (Fig 2.1). Using this comparative transcriptomic approach, we were able to recapitulate the changes in gene expression that occur following acquisition of a

transcription factor, as these divergently regulated genes point to genes that are under selection for regulation by SsrB. From these data, we characterized the evolutionary basis behind a well-studied immune evasion strategy. This research revealed the evolution of a regulatory node upstream of the flagellar transcription factor, *flhDC,* specifically in *S.* Typhimurium, which promotes flagellar gene repression by SsrB to allow for immune evasion in an intracellular environment.

A new *S.* Typhimurium lineage, called sequence type (ST) 313, has emerged recently in Africa that causes disease phenotypically similar to *S.* Typhi, with limited gastroenteritis but invasive systemic disease in humans. This strain appears to have an intermediate phenotype between the broad host range, gastroenteritis inducing *S.* Typhimurium ST19 and the human-restricted *S.* Typhi. Phenotypic characterization of strains from this lineage indicate that they can infect mice and non-human primates similar to *S.* Typhimurium ST19, but this infection results in less fecal shedding, induces less invasion and inflammation, and reduces inflammasome activation compared to ST19 (Carden et al., 2015). A comparison of the ST313 and ST19 genomes reveals that the ST313 genome has gained prophage islands and plasmids and has lost some genes compared to ST19, but the core genome of these two strains has under 1000 single nucleotide polymorphisms (SNPs). Some of the phenotypic differences between ST313 and ST19 can be explained by gene gain or loss, such as the pseudogenization of a SPI-1 effector that promotes systemic dissemination, however these changes are not sufficient to explain the extent of the phenotypic differences between the two strains, suggesting

that regulatory changes might be involved (Canals et al., 2019; Carden et al., 2017; Owen et al., 2017). Indeed, a SNP in the cis-regulatory domain of a known serum-resistance gene, *pgtE,* promoted PhoP-dependent transcription in ST313 and allowed for protection against serum killing *in vitro* (Hammarlöf et al., 2018). The extent of regulatory rewiring in ST313 could be revealed through comparative RNA-seq between this strain and ST19 in different regulatory mutant backgrounds such as an *ssrB* mutant versus wild-type. Understanding the regulatory network in this emergent strain of *S.* Typhimurium could be used to understand the phenotypic differences observed and elucidating the infection biology of ST313.

While the emergence of antibiotic-resistant and increasingly virulent strains of *S.* Typhimurium has made research into this pathogen a clinically relevant topic, *S.* Typhimurium has long been used as a model organism to understand aspects of bacterial and host biology (Broz et al., 2012; Garai et al., 2012). A good example of this is the finding that the flagellin from *S.* Typhimurium can activate the inflammasome, which led to the characterization of a new bacterial clearance mechanism termed PITS – pore-induced intracellular traps. Artificially up-regulated *fliC* can activate the inflammasome and associated pyroptosis during a *S.* Typhimurium infection, and a recent study demonstrated that pyroptosis of macrophages can trap the intracellular bacteria, leading to efferocytosis by neutrophils that are recruited by the pro-inflammatory cytokines (Jorgensen et al., 2016b). This pathway would likely never be encountered during an actual *S.* Typhimurium infection, but this immune response was subsequently

demonstrated to be relevant for defense against opportunistic pathogens (Maltez et al., 2015). The RNA-seq data presented in chapter 2 includes a set of ~70 unclassified genes that are down-regulated by SsrB in *S.* Typhimurium, which show a similar pattern of gene expression as motility genes (Fig. 2.1). Given the role of SsrB in intracellular survival, it is likely that these genes are repressed because they confer a fitness defect *in vivo*. This could be confirmed by generating overexpression constructs of these genes and evaluating their fitness *in vivo* against wild-type *S.* Typhimurium. These overexpression constructs could also be used to characterize the host response to the protein products of these genes, and the *in vivo* dynamics of *S.* Typhimurium when these genes are not repressed. Numerous cell culture and genetic mouse models can be harnessed to explore the functions of these genes and the relevance of their repression during a *S.* Typhimurium infection, both to the host response and to the bacteria.

Determining the environmental sensing involved in virulence gene expression in *S.* Typhimurium

The activation of SsrB is dependent on the activation of the SsrA sensor kinase and inputs from numerous other regulatory programs. The net result of this hierarchy is intracellular survival, which is driven largely by the SPI-2 T3SS. Understanding the contextual cues that drive this gene response helps to delineate the different stages of *S.* Typhimurium gene expression, and the overall effects on disease outcome. Mice and humans lacking a functional NADPH oxidase are more susceptible to *Salmonella*

infections, indicating the importance of this innate immune response (van den Berg et al., 2009; Mastroeni et al., 2000; Simonsen et al., 2009). Here, we demonstrated that reactive oxygen species generated by neutrophils can promote SPI-2 gene expression, and that this signaling is likely mediated by reactive residues on the sensor kinase SsrA. This finding integrates ROS into the signalling cascade that includes low pH, AMPs, and osmolarity, which all appear to work together to activate SPI-2 gene expression, although further details regarding the mechanism still need to be teased out.

SsrA has been shown to be responsive to pH and here we demonstrate that SsrA likely has ROS-reactive cysteine residues that contribute to its activation. However, SsrA is still able to function to promote *sseA* transcription independent of these two signals. Mutational and structural prediction analyses have indicated the presence of a *Salmonella*-specific ligand binding loop in the periplasmic domain of SsrA but the inability to stably purify the periplasmic domain of SsrA has led to difficulty in characterizing this ligand (Mulder et al., 2015). In the absence of purified protein, an alternative approach to identifying this ligand could be through identifying inhibitors of the SsrA sensor kinase and characterizing their target. High-throughput screening (HTS) has gained traction as an approach to develop anti-virulence drugs in several pathogens including *Salmonella*, and a number of these have been found to target two-component systems in these pathogens (Brannon and Hadjifrangiskou, 2016; Johnson and Abramovitch, 2017). Using a SsrA-SsrB dependent transcriptional reporter like P*sseA-lux* in an HTS would likely identify compounds that target SsrA-SsrB. The characterization

of this compound activity and target, along with counter-selection for mutants that suppress transcriptional inhibition by the compound, could yield insight into the compound target. If the compound does specifically target SsrA, the structure of the compound or mutations in SsrA that block compound activity would provide some functional information on SsrA, such as the type of ligand this sensor kinase might interact with. A similar approach was used to identify a cue that represses PhoQ, long chain fatty acids, which allows environment-specific gene expression (Viarengo et al., 2013).

Here, we identified yet another host antimicrobial cue that can drive adaptive gene expression in *S.* Typhimurium. However, the relative strengths of various cues and how they work together to contribute to *in vivo* fitness is still unclear. Analyses of *S.* Typhimurium gene expression in different media that approximate the environmental cues of the SCV, the lumen, or other stress conditions have been performed, but with the increasing accessibility of RNA-seq it has become apparent that gene expression in the intra-macrophage environment is different from even our closest *in vitro* approximate media (Srikumar et al., 2015). The role of the host genotype in modulating *S.* Typhimurium gene expression has been explored to a degree, such as the finding that an *S.* Typhimurium infection in *Nramp1*$^{+/+}$ macrophages increased SPI-2 gene expression relative to in *Nramp*$^{-/-}$ cells, and that different virulence factors are required in these different genetic backgrounds (Yoon et al., 2011; Zaharik et al., 2002). However, a genome-wide characterization of how host genotype modulates bacterial gene expression

is lacking. *gp91*$^{phox-/-}$ mice lack a functional NADPH oxidase, *Cramp*$^{-/-}$ mice are deficient for cathelicidin production, and, while wild-type C57BL/6 mice lack a functional Nramp-1, a *Nramp1*$^{+/+}$ congenic strain has been generated that can be used to probe the role of ion sequestration (Arpaia et al., 2011; Brodsky et al., 2005; Richards et al., 2012; Vazquez-Torres et al., 2000a). RNA-seq following infection in primary macrophages from each of these mouse lines, as well as in wild-type macrophages treated with a V-ATPase inhibitor, would reveal how individual host cues affect *S.* Typhimurium gene expression. An analysis of these genetic backgrounds in the context of different host cells, such as macrophages compared to neutrophils, would also be insightful to understand the fine-tuning of gene expression that occurs at different sites of an *S.* Typhimurium infection. For example, neutrophils have MPO that generates hypochlorous acid from H_2O_2, as well as a second source of H_2O_2 through the activity of DAO (Faurschou and Borregaard, 2003; Nakamura et al., 2012; Tuinema et al., 2014). As well, the specific proteases and defensins from neutrophils likely elicit a different genetic program in *S.* Typhimurium than macrophages.

ROS sensing is a common mechanism of activating the genes important for defense against ROS. However, proteome-wide analyses of other pathogenic bacteria has revealed that ~200 proteins in *Pseudomonas aeruginosa* or *Staphylococcus aureus,* including a majority that are conserved with other bacteria, have oxidation-sensitive cysteine residues that are important for metabolism and *in vivo* survival (Deng et al., 2013). More interesting, however, are the proteins that are modified within host cells in

response to the host NADPH oxidase. A recent study in *E. coli* demonstrated that the *E. coli* proteome is oxidised in response to phagocytosis by a neutrophil-like cell line (Xie et al., 2019). Given the rise in identification of redox-reactive proteins in *S.* Typhimurium that have been implicated in *in vivo* survival (Fu et al., 2017; Pardo-Esté et al., 2018; Yang et al., 2019), a similar mass spectrometry approach could be leveraged on the *S.* Typhimurium proteome following infection of wild-type or gp91$^{phox-/-}$ macrophages and neutrophils. This approach would identify proteins that are selectively modified within the host environment and would allow for a better understanding for how the host environment modifies the bacteria at a protein level during an infection, as well as expand our understanding of the relative importance of these modifications to bacterial pathogenesis.

While much of the focus on TCS has been on the activation of response regulators and their downstream effects, the role of de-phosphorylation of the response regulator on the tight control of gene expression cannot be understated (Kenney, 2010). Some sensor kinases, including PhoQ and EnvZ, have phosphatase domains that can dephosphorylate the cognate response receiver. Recent research has demonstrated that non-phosphorylated SsrB can have effects on gene expression, and this study suggests that the phosphorylation state allows *S.* Typhimurium undergo a lifestyle switch to survive extracellularly (Desai et al., 2016). It is possible that if SsrA has phosphatase activity, this activity would be important to SsrB when *S.* Typhimurium transitions from an intracellular to an extracellular environment. The sensor kinase PhoQ has been shown to

have phosphatase activity that is mediated by increasing magnesium ion concentrations. It is not clear whether increased magnesium is an *in vivo* cue or reflects the native state of inactive PhoQ when magnesium is not displaced by AMPs, but this is an example of a sensor kinase shutting off its cognate response regulator in the absence of activating cues (Castelli et al., 2000; Montagne et al., 2001). Transcriptional reporters for SsrA-SsrB activity, such as P*sseA-lux*, are an indirect way to measure the activation state of the TCS and are often complicated by other regulatory inputs at the promoter level. Traditional estimations of the phosphorylation state have involved radio-labeled inorganic phosphate. However, the ability to visualize the phosphorylation state of proteins directly by SDS-PAGE with a Phos-tag would allow for dynamic assays and characterization of residues important for regulating the phosphorylation state of SsrB by SsrA directly (Choi and Groisman, 2017; Gao and Stock, 2018). Unraveling the link between SsrA and SsrB towards regulating an SsrB-dependent lifestyle switch would increase our understanding of the role of this two-component system in the various environments that *S.* Typhimurium inhabits.

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