Correlation Between Physiological Fluid Shear and RpoS in Regulating the Stationary Phase Stress Response in *Salmonella*

by

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ABSTRACT

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a Gram-negative enteric pathogen that causes self-limiting gastroenteritis in healthy individuals and can cause systemic infections in those who are immunocompromised. During its natural lifecycle, *S.* Typhimurium encounters a wide variety of stresses it must sense and respond to in a dynamic and coordinated fashion to induce resistance and ensure survival. *Salmonella* is subjected to a series of stresses that include temperature shifts, pH variability, detergent-like bile salts, oxidative environments and changes in fluid shear levels. Previously, our lab showed that cultures of *S.* Typhimurium grown under physiological low fluid shear (LFS) conditions similar to those encountered in the intestinal tract during infection uniquely regulates the virulence, gene expression and pathogenesis-related stress responses of this pathogen during log phase. Interestingly, the log phase *Salmonella* mechanosensitive responses to LFS were independent of the master stress response sigma factor, RpoS, departing from our conventional understanding of RpoS regulation. Since RpoS is a growth phase dependent regulator with increased stability in stationary phase, the current study investigated the role of RpoS in mediating pathogenesis-related stress responses in stationary phase *S.* Typhimurium grown under LFS and control conditions. Specifically, stationary phase responses to acid, thermal, bile and oxidative stress were assayed. To our knowledge the results from the current study demonstrate the first report that the mechanical force of LFS globally alters the *S.* Typhimurium χ3339 stationary phase stress response independently of RpoS to acid and bile stressors but dependently on RpoS to oxidative and thermal stress. This indicates that fluid shear-dependent differences in acid and bile stress responses are regulated by alternative pathway(s) in *S.* Typhimurium, were the oxidative and thermal stress responses are regulated through RpoS in LFS conditions. Results from this study further highlight how bacterial mechanosensation may be important in promoting niche recognition and adaptation in the mammalian host during infection, and may lead to characterization of previously unidentified pathogenesis strategies.

DEDICATION

To my Mother and Father for their love and support. I would not have had this amazing opportunity without you. Thank you for instilling in me the thought that I have the ability to accomplish anything I put my mind to.

ACKNOWLEDGMENTS

This thesis would have never been possible without the help of my committee. Dr. Cheryl A. Nickerson, thank you for bringing me into your lab and allowing me to follow my passion. Dr. Barrila, Dr. Ott and Dr. Stout, thank you for all of your support and guidance.

TABLE OF CONTENTS

LIST OF FIGURES

Figure Page

CHAPTER 1

INTRODUCTION

Salmonella enterica serovar Typhimurium

Clinical Importance

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a Gram-negative, non-typhoidal, enteric bacterial pathogen. *S.* Typhimurium infections typically cause diarrhea and gastroenteritis in healthy individuals and can also cause systemic infections in individuals that are immunocompromised (Graham S. M., 2010; Majowicz et al. 2010; Parry et al. 2013; Varma et al. 2005; Vugia et al. 2004). The global health impact by non-typhoidal *Salmonella* serovars has been estimated to cause 93.8 million illnesses, and 155,000 deaths annually and *S.* Typhimurium is an important contributor to these numbers (Majowicz et al. 2010; Scallan et al. 2005). In 2004 the National Centers for Infectious Disease, Center for Disease Control and Prevention estimated that economic burden of *Salmonella* in the United States was an annual total cost of $2.8 billion (Adhikari et al. 2004). *S.* Typhimurium is known to cause illness when orally ingested, commonly through animal fecal contamination of food or water. This foodborne pathogen is also a health and safety concern of commercially produced food products (Alvarez-Ordonez et al. 2015; Painter et al. 2013).

During the *S.* Typhimurium life cycle, the pathogen enters the host and encounters a change in temperature and acidic environment of the stomach. From the stomach *Salmonella* moves to the alkaline environment of the small intestinal duodenum, where bile salts are introduced for the break down of fat-soluble molecules. *S.* Typhimurium then moves into the ileum where it adheres to the mucosa and invades epithelial cells. During cell invasion, *S.* Typhimurium experiences oxidative stress by host cells and low intraphagosomal pH of macrophages (Murray, H. W. 1988; Ohkuma, S., Poole, B. 1978). *S.* Typhimurium encounters mechanical fluid shear in the external environment and as *S*. Typhimurium moves through the gastrointestinal tract, which is a regulatory factor of *S*. Typhimurium pathogenesis (Nauman et al.

2007; Nickerson et al. 2007; Nickerson et al. 2000, 2001, 2003, 2004; Pacello et al. 2012; Wilson et al. 2002a, 2002b, 2007, 2008).

RpoS Regulation

RpoS is closely related to the major housekeeping sigma factor RpoD (sigma 70), encoded by the *rpoD* gene, which transcribes the majority of genes and encodes important products for exponentially growing bacteria in normal conditions (Gross et al. 1992; Gruber and Gross, 2003). These two sigma factors recognize similar promoter sequences and can regulate different sets of genes (Hengge-Aronis, 2002b). Structurally and functionally similar, these sigma factors compete to reversibly bind core RNA polymerase, to form the holoenzyme necessary for promoter recognition and initiation of transcription (Gaal et al. 2001; Hengge-Aronis, 2002b) Distinct regions, –10 and –35 sites, upstream of the start point of transcription (designated as +1) have been conserved for sigma factor promoter recognition (Hengge-Aronis, 2002b). RpoD and RpoS deviate in their promoter specificity, specifically elements surrounding the -10 and -35 regions, and length between the -10 and -35 regions, DNA topography and transcriptional regulators (Hengge-Aronis, 2002b). Expression of RpoS gene expression (mRNA and protein) is highly complex and involves regulation at multiple levels including transcriptional, translational, degradation and activity (Hengge-Aronis, 2002b). As it is not possible within the scope of this thesis to fully cover the complex regulation of RpoS however, there are some good reviews on the topic (Andrews-Polynebus et al. 2010; Audia et al. 2001; Dodd et al 2001, 2007; Rychlik et al. 2005). In *E. coli* transcription of *rpoS* is activated by the general stress alarmone ppGpp, when unfavorable conditions require RpoS regulation, but no evidence of ppGpp regulating RpoS in *S*. Typhimurium at either the mRNA or protein level (Hirsch and Elliott, 2002; Lange et al. 1995; Lange and Hengge-Aronis, 1991, 1994, Gentry et al, 1993; Pizarro-Cedra and Tedin, 2004). Although ppGpp may not regulate RpoS in *S.* Typhimurium, Fis a DNA-binding protein has been shown to repress *rpoS* at the transcriptional level during log phase (Hirsch and Elliott, 2004; Pizarro-Cedra and Tedin, 2004). The RNA-chaperone Hfq and small regulatory RNAs (sRNA) are involved in the activation and inhibition of *rpoS* translation (Brennan et al. 2007; Lange and

Hengge-Aronis 1994; Muffler et al. 1997b; Repoila et al. 2003). The Hfq-dependent sRNA, DsrA and RprA work to promote *rpoS* translation through altering the secondary structure of *rpoS* mRNA (Majdalani et al. 1998, 2001; Muffler et al. 1997b). The sRNA OxyS is also Hfq-dependent, which represses *rpoS* translation through binding of Hfq and altering Hfq activity (Zhang et al. 1998). The Hfq chaperone protein has also been implicated in regulating RpoS translational stability (Breannan R. G., 2007; Brown and Elliott, 1996; Muffler et al. 1997b) as well as in the control of pathogen virulence (Bajaj et al. 1996; Vogel et al. 2006; Wilson et al. 2007). Post-translational regulation was observed from studies that show increased stability of the RpoS sigma factor during stationary phase, or encountering stressful conditions (Bearson et al. 1996; Hengge-Aronis, 2000, 2002a; Lynch et al. 2004; Muffler et al. 1996, 1997a). The post-translational regulation of RpoS stability through proteases, ClpXP, and phosphorylated response regulator proteins, RssB, determine the turnover rate of RpoS (Becker et al. 1999, 2002; Bearson et al. 1996, Bouche et al. 1998; Hengge, R., 2009; Lange and Hengge-Aronis, 1994; Hengge, R. 2008; Klauck et al. 2001; Muffler et al. 1997a; Zhou et al. 2001). Negative regulation of RpoS is important to ensure that core RNA polymerase is available for proper sigma factor binding, during times RpoS is not essential for bacterial survival. The dynamic, complex and coordinated regulation of the RpoS sigma factor is important in establishing infection and survival of *S*. Typhimurium during its life cycle.

RpoS a Major Regulator of *Salmonella* Stress Response and Virulence

S. Typhimurium is exposed to a variety of stressors outside the host that are similar to those experienced within the host. Among these stressors include nutrient deprivation, oxidative, osmotic, and changes in pH and temperature (Alvarez-Ordonez et al. 2015; Hengge-Aronis 2000, 2002a; Rychlick et al. 2005). The ability of *S*. Typhimurium to dynamically regulate gene expression and pathogenesis-related stress responses is crucial for the survival of this bacterium outside and within the host environment. The alternative sigma factor RpoS is encoded by the *rpoS* gene and is the master regulator of the general stress response in various Gram-negative bacteria, including *Salmonella* (Lange and Hengge-Aronis, 1991, Hengge, R. 2011; Loewen, P.,

& Hengee-Aronis, R. 1994). RpoS plays a role in regulating the expression of genes in stationary phase and genes important to provide cross-protection to multiple environmental stresses in *Escherichia coli* (*E. coli*) and *S*. Typhimurium (Hengge-Aronis, 2000, 2002a). Although more stable in stationary phase, RpoS is active in log phase of growth (Lynch et al. 2004). Given the diverse function of genes regulated by RpoS, it is not surprising that *Salmonella* strains that carry a mutant *rpoS* allele display major alterations in their stress response phenotype and are highly attenuated for virulence. (Fang et al. 1992; Bang et al. 2005; Nickerson and Curtiss, 1997; Robbe-Saule et al. 1995; Wilmes-Riesenberg et al. 1997; Wilson et al. 2002a). Consistent with the central role for RpoS in *Salmonella* virulence, tissue distribution studies have shown a decreased ability for *rpoS* null mutant strains, as well as strains with *rpoS* allelic replacements, to colonize murine lymphoid Peyer's patch tissue, as well as to reach the deep tissues of the liver and spleen (Coynault et al. 1996; Nickerson and Curtiss, 1997; Wilmes-Riesenberg et al. 1997). Relevant to deep tissue colonization, RpoS is known to regulate the *Salmonella* plasmid virulence (*spv*) genes, which are important for *S*. Typhimurium to establish an infection beyond the Peyer's patches (Fang et al. 1992; Gulig and Doyle, 1993).

Importance of Fluid Shear

Fluid shear is the force of fluid across the surface of an object as it moves through the fluid (Nauman et al. 2006; Nickerson et al. 2004). Fluid shear is a physical force measured in dynes/cm^2, a measure of force per unit area, wherein one dyne equals one gram accelerated by 1 cm/second^2. It is important to understand what fluid shearing forces organisms are exposed to *in vivo*, in order to provide a better model for testing them *in vitro*. Although fluid shear is only one aspect of the bacterial microenvironment, it has been shown to play an important role in the global reprogramming of virulence, gene expression and/or pathogenesis-related stress responses of *S.* Typhimurium, *E. coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Allen et al. 2008; Castro et al. 2011; Crabbe et al. 2010; Fang et al. 1997; Lynch et al. 2004; Nickerson et al. 2000; Nickerson et al. 2004; Pacello et al. 2012; Soni et al. 2014Wilson et al. 2002a; Wilson et al. 2002b, Wilson et al. 2007).

It is important to mimic the fluid shear that is encountered by *S.* Typhimurium during its life cycle when performing *in vitro* studies, in order to collect data that best models interactions that occur *in vivo*. There are many ways to culture bacteria and not all represent the same environmental cues. In particular, *S*. Typhimurium encounters different physiologically relevant mechanical fluid shear forces, including high fluid shear (HFS) within the lumen of the small intestine and low fluid shear (LFS) between the brush border microvilli of epithelium (Beeson et al. 2000; Cai et al. 2000; Guo et al. 2000; Nickerson et al. 2004; Nauman et al. 2006). The mechanical forces sensed by *S.* Typhimurium *in vivo* should be considered when characterizing the bacteria for pathogenesis-related stress responses, but in order to mimic the fluid shear levels experienced by *S*. Typhimurium *in vivo* the proper culturing model and technique should be established.

Culturing Techniques

There are many methods that can be used to culture bacterial cells in order to identify cellular responses to stressful conditions, but it is important for *in vitro* models to mimic the *in vivo* fluid shear conditions that are relevant to the pathogen during its course of infection. Many well-known culturing techniques like shaking or static cultures in flasks have been used for years. These conventional culturing techniques have provided a wealth of knowledge, but are limited in the ways they mimic the low physiological fluid shear encountered by pathogens in certain areas of the infected host.

Rotating Wall Vessel

The rotating wall vessel bioreactor (RWV) created by NASA at the Johnson Space Center (Houston, TX) is an optimized suspension culture technology that allows for investigation of the effects of fluid shear forces on cells (Schwarz and Wolf, 1991; Wolf and Schwarz, 1991). Culturing in the RWV allows cells to grow in suspension, while reducing the fluid shear forces encountered by the cell (Gao et al. 1997; Lynch et al. 2004; Nauman et al. 2006; Nickerson et al. 2003, 2004; Schwarz and Wolf, 1991; Wilson et al. 2002a; Wolf and Schwarz, 1991). Within the

RWV bioreactor a hydrophobic membrane allows for oxygenation of the culture media (Nickerson et al. 2004, Schwarz and Wolf, 1991; Wolf and Schwarz, 1991). The RWV has been shown to culture cells under fluid shear conditions that are physiologically relevant to those encountered by pathogens in the infected host, including by *Salmonella* in the intestinal tract (Nauman et al. 2006; Nickerson et al. 2004)

The RWV bioreactor was used in this study (**Fig. 1**). The RWV can rotate on either a horizontal or vertical axis, to study low fluid shear (LFS) and control conditions, respectively (**Fig. 2**). When the RWV is rotating on a horizontal axis, it maintains a gentle fluid orbit of culture, which creates a low fluid shear (<0.01 dynes/cm^2) environment for bacterial cultures (Nauman et al. 2007). The sedimentation of cells within the RWV in the LFS orientation is offset by the solid body rotation of the media, allowing the cells to remain in suspension (Nickerson et al. 2004). All air bubbles are removed in order to minimize fluid shear disturbances to the cultures. The amount of fluid shear force that is experienced by the cells in the culture is based on the density of the cell relative to the media and the particles radius, as the radius and density increase, the fluid shear forces increase (Gao et al. 1997). When the RWV is rotated on a vertical axis (control orientation, **Fig. 2**) the cells sediment and experience increased fluid shear forces.

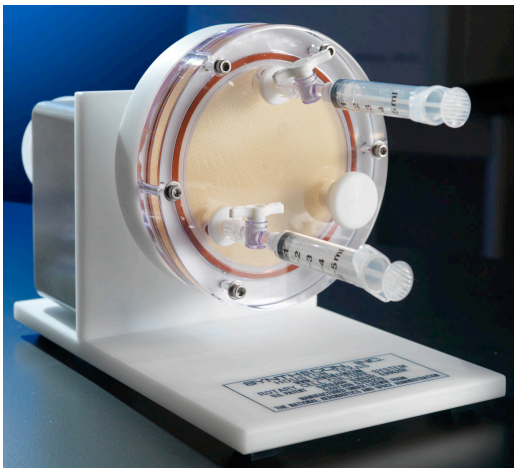

Figure 1. RWV Bioreactor
The NASA-engineered RWV bioreactor is an optimized suspension culture system that was developed to produce a physiologically relevant low fluid shear environment (LFS).

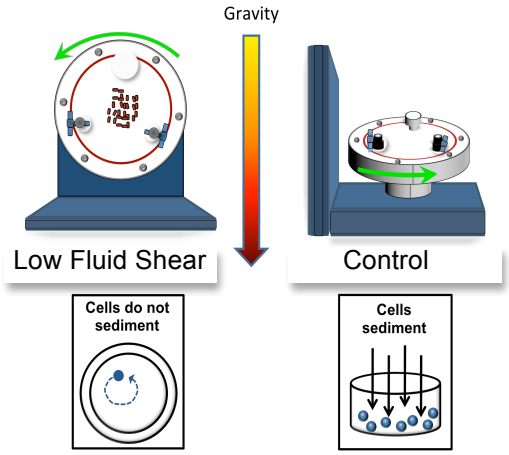

Figure 2. LFS and Control Orientations
During experiments, reactors are rotated in either the LFS or control positions. In the LFS orientation, cells remain in suspension and do not sediment. In the control orientation, bacteria sediment to the bottom of the vessel and are subjected to higher fluid shear conditions. (Modified from Dr. Jennifer Barrila).

Published Studies Using the RWV

Previous studies by our lab have shown that culturing *S*. Typhimurium χ3339 (the same strain used in this work) in the LFS condition to late log phase induced global changes in gene-expression, virulence, and pathogenesis-related stress responses (Nickerson et al. 2000; Wilson et al. 2002a, 2002b). Collectively, the results of these studies suggested that the sigma factor RpoS would be a logical candidate that might serve as a global regulator of the LFS-induced response of *S.* Typhimurium (Hengge-Aronis 2000, 2002a; Nickerson et al. 2000; Wilson et al. 2002a, 2002b). In follow up studies, it was demonstrated by our lab that LFS-dependent stress regulation is RpoS independent for *S.* Typhimurium χ3339 grown to late log phase (Wilson et al. 2002a). Based on our findings, Lynch et al. 2004 investigated the effects of LFS on *E. coli* stress regulation during log phase and stationary phase of growth, with subjection to a variety of environmental stressors. The LFS response of *E. coli* was found to be RpoS-independent in log phase, but RpoS-dependent in stationary phase (Lynch et al. 2004). A subsequent study by Pacello et al. 2012 using *S*. Typhimurium strain 14028 cultured to stationary phase (20 hours)

found RpoS-dependent regulation of the LFS acid stress response, but RpoS-independent regulation for the LFS oxidative stress response (Pacello et al. 2012). To our knowledge we are the first to study the relationship between mechanosensation of the LFS culture environment by *S*. Typhimurium χ3339 in stationary phase of growth, and its effects on RpoS regulation of the general stress response to a broad spectrum of pathogenesis related stresses.

Current Study

In this study, we test the hypothesis that low fluid shear stimulates regulation of multiple environmental stresses in *S.* Typhimurium χ3339 in an RpoS-independent manner at stationary phase of growth. Previous characterization of the same strain of *S*. Typhimurium in LFS conditions at late log phase has provided evidence for RpoS-independent regulation of the stress response through an alternative pathway(s), which we believe to also occur in stationary phase of growth (Wilson et al. 2002a). In this current study, we have identified stationary phase RpoS-independent regulation of χ3339 to acid and bile stress, in addition to RpoS-dependent regulation to oxidative and thermal stress during LFS culture. To our knowledge this is the first report that mechanosensation of the LFS stimulus by *S.* Typhimurium χ3339 induces RpoS independent and RpoS dependent stress regulation to multiple environmental stresses at stationary phase of growth. This study expands on the complex regulation of *S*. Typhimurium to sense and respond to physiological fluid shear.

CHAPTER 2

METHODS

Bacterial Strains

All studies were performed using *S.* Typhimurium strain χ3339 (wild type, CAN672), an animal-passaged isolate of SL1344 (Gulig, P., & Curtiss, R. 1987), and an isogenic *rpoS* mutant derivative of χ3339 (CAN968). Jiseon Yang performed construction of the rpoS mutant through deletion of the complete *rpoS* gene (+1 though +993 base pairs). A suicide plasmid was constructed and conjugated to the recipient's plasmid. The first cross over conjugants of χ3339::pYA4804 were selected on Lennox Broth (LB) with 30 µg/mL chloramphenicol (Cm). The second crossovers were induced and selected with 5% sucrose. The *ΔrpoS*-deletion mutant was verified by PCR and also verified as Cm-sensitive and catalase-negative. A catalase-negative phenotype would be present due to the absence of *rpoS*-dependent catalase gene expression.

Growth Conditions

Bacteria were grown in LB for all experiments (Lennox, E.S., 1955). Bacterial strains were inoculated into 5 mL of LB in a 15 mL snap-cap tube directly from frozen glycerol stock using a sterile pipette tip. Bacteria were cultured at 37^{o}C, shaking at 250 rpm for 15 hours (overnight culture). A 1:200 dilution was subsequently made by transferring 750 µL of overnight culture into fresh 150 mL of LB media in a 250mL flask. This 50 mL of culture was loaded into the RWV bioreactor, completely filling the vessel with media and all air bubbles removed. The RWVs were oriented for growth in the LFS and HFS orientations (**Fig. 2**). RWVs were placed into a 37^{o}C incubator rotated at a speed of 25 rpm. Cultures were harvested after 24 hours of growth for all experiments, unless indicated otherwise. At 24 hours, LFS and control cultures were in stationary phase of growth.

Growth Curve

Cultures of both strains of *S.* Typhimurium were grown in the RWV bioreactors in LB media in the LFS and HFS control orientations for 24 hours at 37^{o}C and 25 rpm. The growth

curve was performed by sampling from the RWVs, with removal and inversion of the HFS control bioreactor in order to thoroughly mix the culture (necessary due to sedimentation of bacteria in control RWV) (**Fig. 3**). Cultures were sampled every hour over the course of 12 hours and cultures serially diluted and plated for viable colony forming units (CFU/mL) on LB agar plates. The second half of the growth curve (remaining 12 hours) samples was taken every 2 hours. Two independent trials were performed for both the wild type and mutant strains.

Environmental Stress Assays

Strains were grown in the RWV bioreactors in the LFS and HFS control orientations at 37^{o}C and harvested after 24 hours of growth. Once harvested, the cultures were immediately subjected to the environmental stress condition being tested. With the exception of thermal stress, all cultures were incubated statically on the bench top at room temperature for the duration of the stress assay. Acid stress was subjected to the culture by lowering the pH of the culture to 3.5 with the addition of sterile 1M citrate buffer. During the acid stress assay, the pH was monitored using a pH probe on an additional culture sample to avoid contamination. Following the acid stress assay, the pH of the LFS and HFS control cultures was confirmed using a pH probe. The acid stress assay was performed for 60 minutes, with cultures sampled, serially diluted, and plated at 30, 45, and 60 minutes (**Fig. 4**). The bile stress assay was performed by the addition of 10% bile salts (1.18mM) (Sigma-Aldrich: B8756). Cultures were sampled, serially diluted and plated 15, 30, 60 and 90 minutes (**Fig. 5**). Oxidative stress using fresh hydrogen peroxide (stored at 4°C for less than a month) was added to the culture from a 30% stock solution to a final concentration of 0.09% for both strains. The assay was performed at room temperature for 30 minutes, serially diluted and plated at 15 and 30 minutes (**Fig. 6**). An additional oxidative stress assay using fresh 30% stock solution and adding a final concentration of 0.24% to the wild-type strain was performed for 30 minutes (**Fig. 7**). For thermal stress assay, a 2 mL aliquot was immediately transferred to heating blocks that were set at a temperature of 53^{o}C. The assay was performed statically for 60 minutes and cultures sampled, serially diluted and plated at 15, 30, 45, and 60 minutes (**Fig. 8**). For all of the stress assays, samples were removed at time zero before

the stress was added to the culture. In addition, samples were removed at the designated time points and plated on LB agar to determine the number of viable colony forming units (CFU/mL). For these data, the percent survival was calculated as the number of CFU/mL at each time point divided by the number of CFU/mL at time zero. For each assay, at least three independent trials were performed using independent cultures. p-values were calculated using two-tailed Student's t-test. Significance is indicated in graphs by * $p < 0.05$, ** $p < 0.01$, and *** and $p < 0.001$.

CHAPTER 3

RESULTS

Growth Curve

Previous studies by our laboratory demonstrated that wild type *S*. Typhimurium χ3339 displayed no differences in its growth profiles in L broth when grown in the LFS and control conditions (Nickerson et al. 2000). In the current study, growth curves were performed to confirm this trend with the wild type, as well as to determine if culturing in LFS affects the growth dynamics of the newly constructed *S.* Typhimurium χ3339 Δ*rpoS* mutant (**Fig. 3**). Both strains were cultured in LB media for 24 hours at 37^{o}C in the RWVs positioned in the LFS and control orientations and rotated at 25 rpm. Wild type *S.* Typhimurium χ3339 and the Δ*rpoS* mutant strain displayed no significant differences in growth between the LFS and control conditions. There is an observed jump in the growth curve at 12 hours and could be due to the start of the second part of the growth curve in a separate experiment were the incubating bioreactors were not disturbed and held at a constant temperature of 37 ^{o}C for 12 hours. The first part of the experiment requires the incubator be opened every hour to retrieve samples, changing the temperature of the incubator. This has an observed effect on the bacterial growth when cultures are disturbed. The data confirmed that both strains were in stationary phase of growth at 24 hours and shared similar growth profiles when cultured in LFS and control conditions. The 24 hour time point was selected for the environmental stress assays.

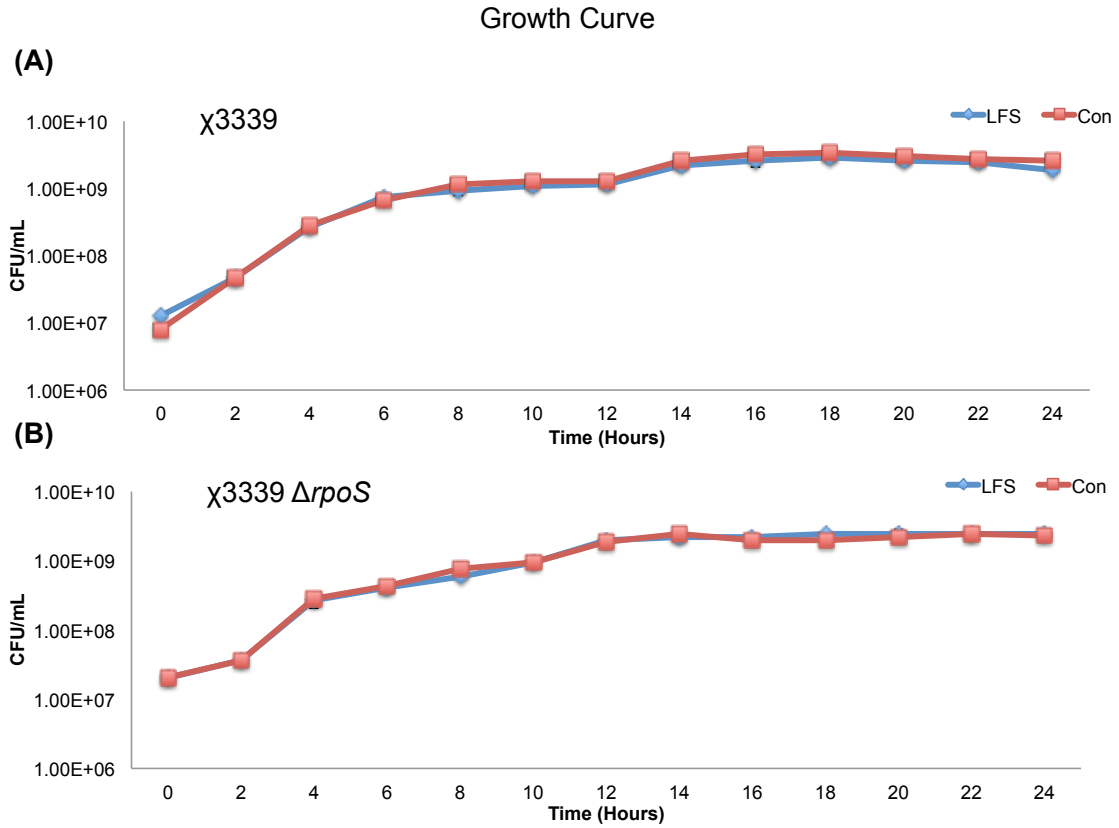

Figure 3. Growth Curve
Growth curve for (A) wild type *Salmonella* χ3339 (CAN968) and (B) χ3339 *ΔrpoS* (CAN672) cultured in low fluid shear (LFS), blue bar, and control (Con), red bar, conditions for 24 hours at 25 rpm at 37°C. The percentage of number of bacteria present was determined through plating dilutions on LB agar. There were two independent trials run. The p-value was calculated through a two-tailed t-test. P-value is significant if (<0.05).

Environmental Stress Assays

S. Typhimurium χ3339 and *ΔrpoS* strains were cultured in LB media for 24 hours in RWV bioreactors, rotating at 25 r.p.m. in the LFS and control orientations, and incubated at 37°C. At 24 hours of growth (stationary phase) the RWV bioreactors were harvested and immediately subjected to environmental stress. This culture technique was used for all environmental stress assays. These assays were used to establish whether RpoS plays a role in regulating select pathogenesis-related stress responses of *S*. Typhimurium following culture in the LFS environment.

To test the response of *S.* Typhimurium to pathogenesis-related stress, χ3339 and *ΔrpoS* strains were subjected to acid or bile stress. After challenging *S*. Typhimurium χ3339 and *ΔrpoS* strains with acid pH 3.5 (**Fig. 4**), a comparison was made between the survival profiles for each strain cultured in the LFS and control orientations. Consistent with previous studies performed by our laboratory using stationary phase cultures of χ3339 (Wilson et al. 2007, Wilson et al. 2008), we found in this study that LFS culture reduced the resistance of *S*. Typhimurium to acid stress (**Fig. 4a**). A similar trend was observed with the isogenic *rpoS* mutant when cultured in the LFS condition, wherein the LFS cultures were more sensitive to acid stress relative to the control cultures (**Fig. 4b**). The *rpoS* mutant strain displayed increased sensitivity to this stress compared to the wild type. These similar trends in survival profiles between the LFS and control condition were also observed, when each strain was challenged with bile salts at 10% concentration (**Fig. 5**).

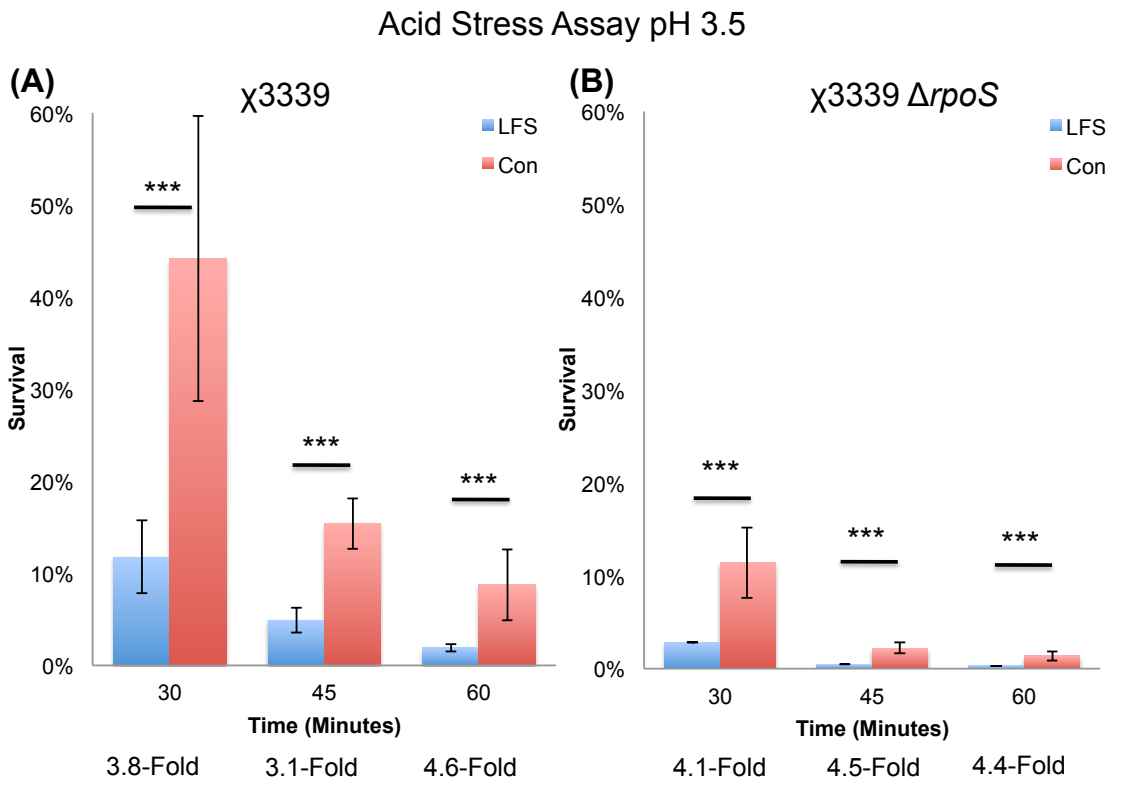

Figure 4. Acid Stress Assay
Salmonella strains (A) χ3339 (CAN968) and (B) *ΔrpoS* (CAN672) were cultured in LFS (blue bars) and control (Con, red bars) growth conditions for 24 hours at 37°C, and Percent survival

was assessed at each time point by sampling each culture, performing serial dilutions and plating on LB agar to determine CFU/ml. Bacterial counts at each time point were normalized to the counts obtained prior to the addition of the stress. Results shown represent three biological replicates, with three technical replicates per experiment. The p value was calculated using a Student's t-test. P-value is considered significant if $p < 0.05$. The error bars correspond to the standard error of the mean.

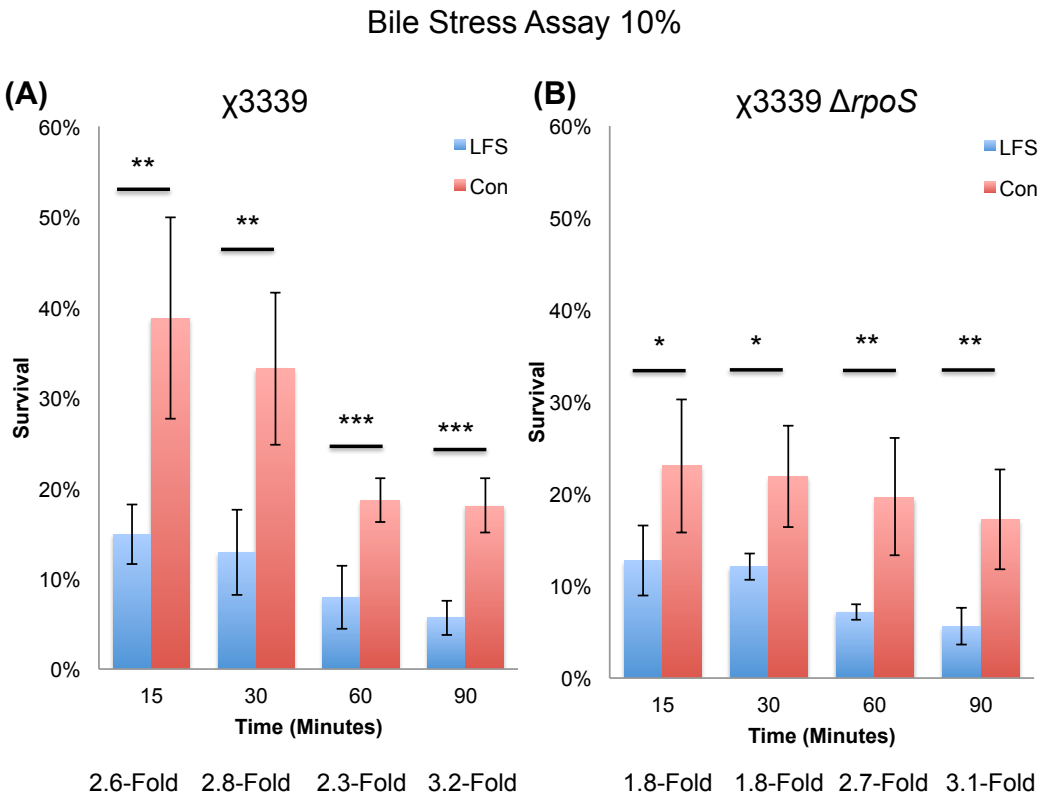

Figure 5. Bile Stress Assay
Salmonella strains (A) χ3339 (CAN968) and (B) *ΔrpoS* (CAN672) were cultured in the RWV oriented in the LFS (blue bars) and control (Con, red bars) orientations for 24 hours at 37°C, and then subjected to a 10% bile salt concentration. Percent survival was assessed at each time point by sampling each culture, performing serial dilutions and plating on LB agar to determine CFU/ml. Bacterial counts at each time point were normalized to the counts obtained prior to the addition of the stress. Results shown represent three biological replicates, with three technical replicates per experiment. The p value was calculated using a Student's t-test. P-value is considered significant if $p < 0.05$. The error bars correspond to the standard error of the mean.

S. Typhimurium wild type and *rpoS* mutant strains were subjected to 0.09% hydrogen peroxide (H_2O_2) oxidative stress following RWV culture (**Fig. 6**). Each strain showed no significant difference in response to oxidative stress after culturing in LFS and control conditions in the 0.09% concentration of H_2O_2. The *rpoS* mutant displayed enhanced sensitivity compared to the wild type. The wild type and *rpoS* mutant strains shared similar trends in response to 0.09%

H_2O_2 oxidative stress for both the LFS and control growth orientations. Since the wild type response to 0.09% H_2O_2 oxidative stress showed little decrease in survival, additional assays were performed with 0.24% H_2O_2 oxidative stress (**Fig. 7**). The results showed an increased sensitivity of the wild type in LFS conditions exposed to 0.24% H_2O_2.

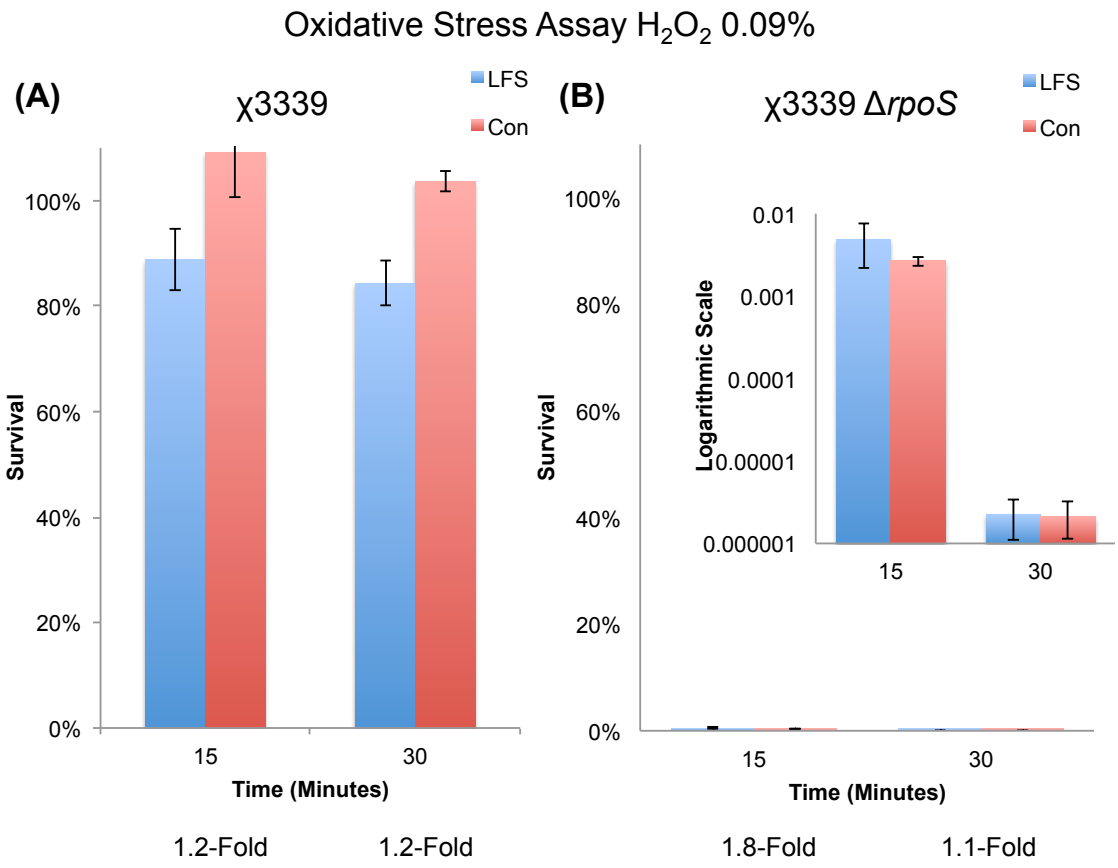

Figure 6. Oxidative Stress Assay 0.09%
Salmonella strains (A) χ3339 (CAN968) and (B) *ΔrpoS* (CAN672) were cultured in the RWV oriented in the LFS (blue bars) and control (Con, red bars) orientations for 24 hours at 37°C, and then subjected to 0.09% hydrogen peroxide. Percent survival was assessed at each time point by sampling each culture, performing serial dilutions and plating on LB agar to determine CFU/ml. Bacterial counts at each time point were normalized to the counts obtained prior to the addition of the stress. Results shown represent three biological replicates, with three technical replicates per experiment. The p value was calculated using a Student's t-test. P-value is considered significant if $p < 0.05$. The error bars correspond to the standard error of the mean.

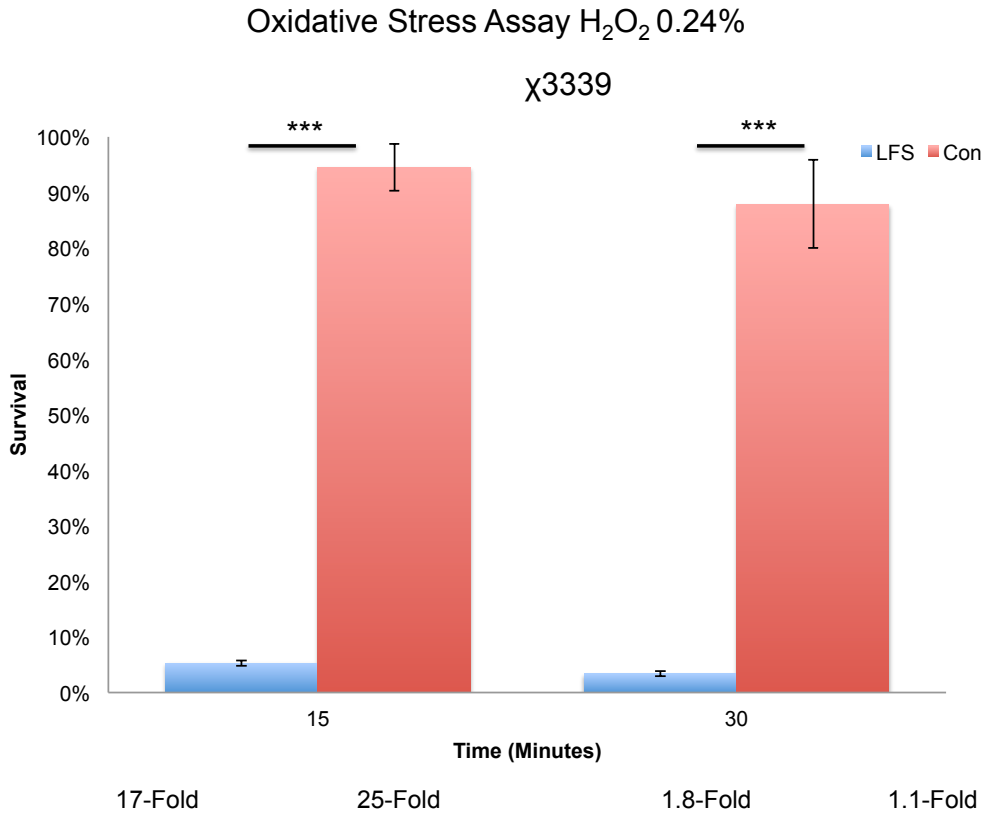

Figure 7. Oxidative Stress Assay 0.24%
Salmonella strains (A) χ3339 (CAN968) was cultured in the RWV oriented in the LFS (blue bars) and control (Con, red bars) orientations for 24 hours at 37°C, and then subjected to 0.24% (WT χ3339) and (χ3339 *ΔrpoS)* hydrogen peroxide. Percent survival was assessed at each time point by sampling each culture, performing serial dilutions and plating on LB agar to determine CFU/ml. Bacterial counts at each time point were normalized to the counts obtained prior to the addition of the stress. Results shown represent three biological replicates, with three technical replicates per experiment. The p value was calculated using a Student's t-test. P-value is considered significant if $p < 0.05$. The error bars correspond to the standard error of the mean.

S. Typhimurium wild type and *rpoS* mutant strains were subjected to thermal stress (53^{o}C) following RWV culture (**Fig. 8**). Interestingly, the wild type and *rpoS* mutant strains showed opposite trends in response to thermal stress. The wild type strain displayed an increased sensitivity for cultures grown under low fluid shear relative to the control cultures. Conversely, the *rpoS* mutant strain was more resistant to thermal stress following low fluid shear culture relative to the control. In accordance with observations from a previous study, the *rpoS* mutant was more sensitive to thermal stress relative to the wild type (Wilson et al. 2002a).

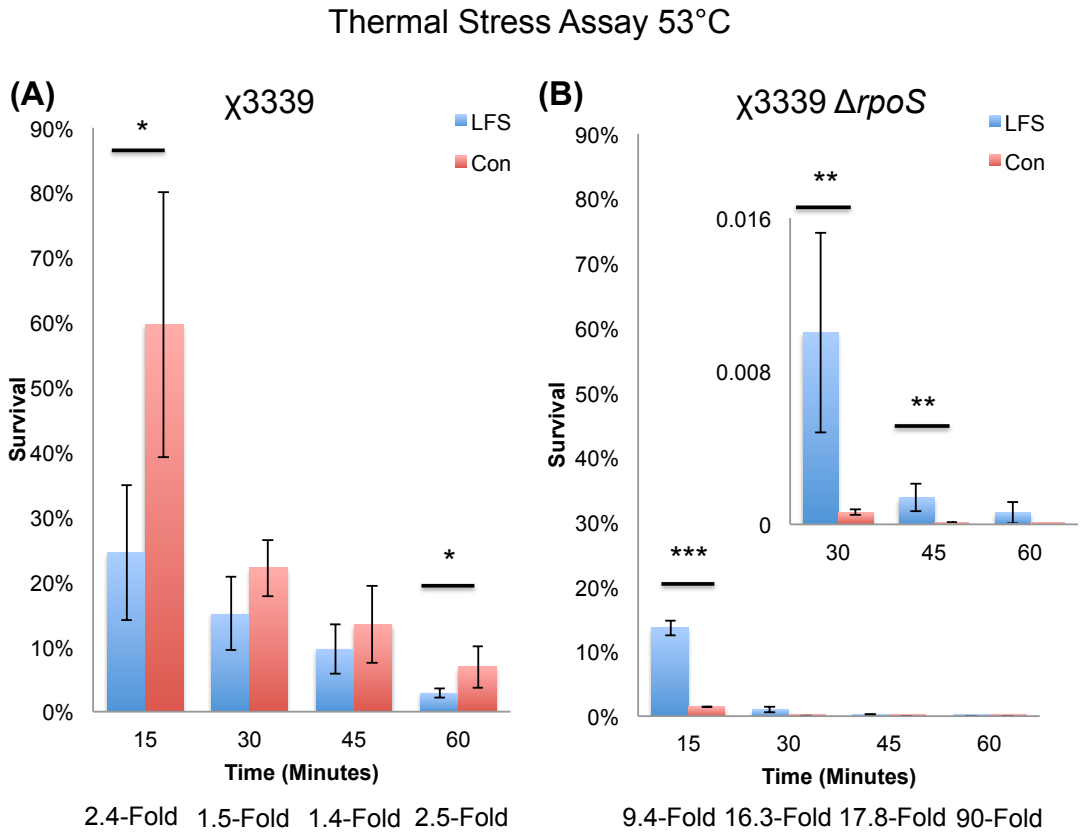

Figure 8. Thermal Stress Assay
Salmonella strains (A) χ3339 (CAN968) and (B) *ΔrpoS* (CAN672) were cultured in the RWV oriented in the LFS (blue bars) and control (Con, red bars) orientations for 24 hours at 37°C, and then subjected to thermal stress at 53°C. Percent survival was assessed at each time point by sampling each culture, performing serial dilutions and plating on LB agar to determine CFU/ml. Bacterial counts at each time point were normalized to the counts obtained prior to the addition of the stress. Results shown represent three biological replicates, with three technical replicates per experiment. The p value was calculated using a Student's t-test. P-value is considered significant if $p < 0.05$. The error bars correspond to the standard error of the mean.

CHAPTER 4

DISCUSSION

The purpose of this study was to investigate the relationship between fluid shear and RpoS regulation of the stationary phase *S*. Typhimurium stress response. RpoS is the master regulator of the general stress response and provides cross-protection against different environmental stresses (Hengge-Aronis, 2000, 2002a). *S.* Typhimurium encounters a variety of environmental stresses during its normal lifecycle including acid, bile, oxidative, and thermal stress (Hengge-Aronis, 2000, 2002a; Hofmann A. F., 1998, Rychlik et al. 2005). The ability of the bacterium to survive these and other stressors dictates the outcome of infection, and as such, RpoS has been shown to regulate genes involved in virulence and pathogenesis-related stress resistance (Allen et al. 2008; Bang et al. 2005; Coynault et al. 1996; Fang et al. 1992; Hengge-Aronis, 2000, 2002a; Nickerson et al. 2000, 2004; Robbe-Saule et al. 1995; Wilmes-Riesenberg et al. 1997; Wilson et al. 2002a, 2002b).

It is essential to study the response of *Salmonella* within the context of physiologically relevant forces found *in vivo,* including low fluid shear, which is encountered by *Salmonella* in several regions of the body, including between brush border microvilli of the intestinal epithelium (Guo et al. 2000; Nickerson et al. 2004). Previous studies by our lab using wild type *S.* Typhimurium χ3339 cultured in the RWV to late log phase were the first to demonstrate that the mechanical force of fluid shear can alter the virulence, global gene expression and stress response profiles of the pathogen (Nickerson et al. 2000; Wilson et al. 2002a, 2002b). Follow-up studies by our lab using an isogenic *rpoS* mutant strain found that during late log phase of growth LFS regulation of acid, osmotic, thermal and oxidative stress was independent of RpoS (Wilson et al. 2002a). These studies suggested that LFS culture pre-adapts *S*. Typhimurium to respond to acid, thermal, osmotic and oxidative stress using an alternative pathway(s) (Wilson et al. 2002a). These studies formed the basis for the current set of experiments presented here. This study is the first to look at the correlation between LFS and RpoS regulation of stress in response to a broad spectrum of pathogenesis-related stresses in stationary phase *S.* Typhimurium χ3339.

In this study, it was determined that the LFS condition displayed increased sensitivity to acid and bile stress relative to the control condition for both the wild type and isogenic *rpoS* mutant, indicating that under the conditions tested in this work, RpoS is not involved in regulating the stationary phase LFS-response of *S*. Typhimurium to those stresses, but does provide cross-protection that is observed as the increased sensitivity of the *rpoS* mutant. The underlying reason for LFS-associated sensitivity of *S*. Typhimurium relative to the control during stationary phase is not known, although Hfq a global regulator has been shown previously by our lab to be a likely regulator of the expression of a large group of genes in response to the LFS stimulus (Wilson et al. 2002b, 2007). Hfq is a RNA chaperone protein that activates small regulatory RNAs that have been shown to directly regulate RpoS stability (Muffler et al. 1997b).

In previous studies by other laboratories, it was determined that *E. coli* and *S*. Typhimurium strain 14028 grown to stationary phase displayed an RpoS-dependent phenotype to acid stress (Lynch et al. 2004; Pacello et al. 2012). This is in contrast to the findings of this study, which showed an RpoS-independent phenotype to acid stress (**Fig. 4**). In late log phase, *S.* Typhimurium χ3339 expressed an enhanced survival when cultured in LFS as compared to the control condition after being subjected to acid stress (Wilson et al. 2002a). Although S. Typhimurium χ3339 cultures grown to log phase and stationary phase share acid stress regulation independent of RpoS, they do not share trends in survival profiles when cultured in the LFS orientation. RpoS is but one of many regulators that act to induce acid shock proteins (ASP). The existence of other regulators (discussed below) could explain the RpoS independent regulation of *S*. Typhimurium χ3339 in stationary phase under LFS conditions.

In addition to RpoS, the PhoP and Fur proteins can regulate the acid tolerance response (ATR), by induction of ASP (Baik et al. 1996; Bearson et al. 1996, 1997, 1998; Foster, J., & Spector, M. 1995; Rychlik et al. 2005). In an RpoS independent response to acid stress, *S.* Typhimurium can induce ATR by using the PhoP protein, the regulator of the PhoP/PhoQ two-component system (PhoP/Q) and stabilizer of RpoS in the acid stress response (Bearson et al. 1998; Ruiz and Silhavy, 2003; Zwir et al. 2005). The PhoP/Q system has been shown to be

important in macrophage survival and virulence (Galan, J., & Curtiss, R. 1989; Mahan et al. 1996). It is also responsible for sensing Mg^{+2}, and can regulate genes for pH-resistance (Vescovi et al. 1994; Zwir et al. 2005). Another ATR regulator is the ferric uptake regulator (Fur), known for its regulation of iron dependent genes, and has been shown to also regulate ASP through an iron independent manner (Bearson et al. 1996; Hall et al. 1996). Both PhoP and Fur mutants express increased acid sensitivity, which suggests their importance in the ATR (Hall et all 1996; Vescovi et al. 1994). PhoP, Fur, or other proteins may be contributing to the acid stress response of *S*. Typhimurium χ3339 under the LFS conditions (Wilson et al. 2002a, 2002b, 2007, 2008).

Other studies have indicated a role for RpoE, the alternative sigma factor responsible for membrane and periplasmic homeostasis during extracytoplasmic stress, in resistance to low pH *in vitro* (Humphreys et al. 1999; Muller et al. 2009; Testerman et al. 2002). RpoE may also be playing a role in *S*. Typhimurium resistance to acid stress in LFS conditions. Hfq regulates PhoP, Fur, RpoE, and RpoS proteins (Wilson et al. 2002b, 2007, 2008). Hfq regulates the PhoP/Q system with the sRNA, GcvB, which expresses the gene *rstB* (Jin et al. 2009; Wilson et al. 2002b). The putative membrane sensory kinase *rstB* gene is up regulated during LFS culture in addition to Hfq and is able to regulate the Fur protein **(**Jeon et al. 2008; Wilson et al. 2002b, 2007). Along with Fur, Dps is a ferritin and stress response protein that is capable of providing resistance to stress (Nair and Finkel, 2004). Furthermore, Hfq is known to directly and indirectly regulate Dps expression, a DNA binding protein that is responsible for acid tolerance, oxidative tolerance and thermal tolerance by altering DNA topography (Nair and Finkel, 2004).

S. Typhimurium encounters bile salts when entering the duodenum, were the bile salts that are stored in the gall bladder and synthesized by liver hepatocytes are released (Hofmann A. F., 1998). Detergent-like bile salts can cause damage to the cell membrane, proteins, and DNA (Begley et al. 2005; Merritt and Donaldson, 2009; Prieto et al. 2004). To our knowledge this study is the first study to examine the effects of bile salt in stationary phase cultured *S.* Typhimurium χ3339 under LFS conditions (**Fig. 5**). The results from this study show an RpoS independent regulation of bile stress in response to LFS. RpoS independent genes have been recognized as

bile resistance factors, such as those that encode outer membrane proteins and the PhoP/Q regulon (Prouty et al. 2002; Prouty et al. 2004; Pucciarelli et al. 2002; Rychlik et al. 2005; van Velkinburgh and Gunn, 1999). PhoP/Q is important in both acid and bile stress resistance and could be a mechanism by which the LFS resistance is regulated. In addition, due to the expression of outer membrane proteins during exposure to bile stress, RpoE the extracytoplamic sigma factor may be involved with bile resistance in LFS conditions of *S.* Typhimurium (Hernandez et al. 2012; Mecsas et al. 1993; Prouty et al. 2002; Pucciarelli et al. 2002). Again, the global regulator Hfq could play a regulatory role for the RpoS independent response to bile stress and has been shown to regulate outer membrane proteins (Wilson et al. 2002b, 2007, 2008).

As *S.* Typhimurium interacts with the host within the brush border microvilli of the intestinal epithelium, it is subjected to active oxygen species, phagocytic bursts by host cells, and oxidative stress (Farr and Kogoma, 1991). Previously examined *S*. Typhimurium *rpoS* mutants have shown increased sensitivity to oxidative stress, providing evidence of RpoS regulation (Fang et al. 1992; Robbe-Saule et al. 1995; Wilmes-Riesenberg et al. 1997; Wilson et al. 2002a). *S*. Typhimurium cultured in LFS conditions and subjected to oxidative stress has been shown to be RpoS independent in late-log and stationary phase (Wilson et al. 2002a; Pacello et al. 2012). The findings from this study represent RpoS dependent stress regulation of oxidative stress for *S*. Typhimurium χ3339 cultured in LFS to stationary phase of growth. The results between the *S.* Typhimurium 14028 and χ3339 strains also differed in respect to the survival profile trends. The survival profile for *S*. Typhimurium 14028 exhibited enhanced resistance in the LFS condition (Pacello et al. 2012), as *S*. Typhimurium χ3339 showed no difference in resistance between the LFS and control conditions at 0.09% H_2O_2 (**Fig. 6**), and an increased sensitivity in LFS when the wild type was subjected to 0.24% (**Fig. 7**), which confirms previous findings (Soni et al. 2014). The rpoS mutant was subjected to 0.24% H_2O_2 concentration and showed no survival (data not shown). LFS culture orientation provided increased sensitivity to *S*. Typhimurium χ3339 cultured in late-log phase, compared to the enhanced resistance observed in response to other stresses (Wilson et al. 2002a). Although, RpoS has been identified as a regulator of the oxidative stress

response, Hfq and the alternative sigma factor RpoE also regulate genes and proteins responsible for oxidative stress tolerance, were Hfq regulates the oxidative stress response independent of RpoS (Figueroa-Bossi et al. 2006; Guisbert et al. 2007; Sittka et al. 2007; Wilson et al. 2007; Zhang et al. 1998). RpoE mutants have shown to be sensitive to oxidative stress providing a role for RpoE in the regulation of this stress response (Testerman et al. 2002). RpoE has been shown to play a role in regulating RpoS and assists RpoS in response to oxidative stress in stationary phase (Muffler et al. 1997a; Rychlik et al. 2005; Testerman et al. 2002). Therefore, the RpoS dependent stress response may be occurring either in addition to the global regulator Hfq or activity of RpoE when exposed to oxidative stress.

This study revealed the LFS condition induced RpoS dependent stress response to increased temperature in stationary phase *S*. Typhimurium χ3339 (**Fig. 8**). The findings from this study support the RpoS dependent regulation and response to thermal stress in LFS conditions. A previous study by our lab indicated that LFS culture enhanced the resistance to thermal stress of late log *S*. Typhimurium χ3339, independent of RpoS (Wilson et al. 2002a). Interestingly, in this study the wild type strain expressed increased sensitivity when it was cultured in LFS orientation. However, the *rpoS* mutant exhibited enhanced resistance when cultured in LFS condition (Wilson et al. 2002a). The down regulation of RpoS in LFS conditions may occur as a regulatory system that decreases expression of RpoS for the increased expression of the heat shock alternative sigma factors RpoH and RpoE. In addition, studies using *E. coli* and the regulation of RpoH and heat shock proteins (HSP), indicated high expression levels as temperature increased rapidly, called the induction phase and lasts until adaptation phase occurs 20-30 minutes after exposure (Arsene et al. 2000; Guisbert et al. 2004; Herman, 2000; Rouviere et al. 1995; Straus et al. 1987; Yura et al. 2000). RpoE and RpoH have also been shown to provide heat tolerance in *Salmonella* (Rychlik et al. 2005). These findings suggest a role for LFS condition effecting the regulation of heat shock proteins using RpoH, and a possible explanation for enhance *rpoS* mutant resistance to wild type levels.

In summary, this study suggests that when pre-adapted in LFS conditions, stationary phase, the response of *S*. Typhimurium χ3339 to acid and bile stressors is independent of the master regulator of the general stress response, RpoS. In addition, *S*. Typhimurium χ3339 regulates oxidative and thermal stress responses dependent of RpoS when cultured in LFS to stationary phase.

In the LFS condition, the master regulator of the general stress response provides protection to the cell through cross-protection and not in response to acid or bile stress. Although stationary phase *S*. Typhimurium χ3339 expressed RpoS independent regulation of the stress response to acid and bile stress, the complete loss of tolerance was not observed and therefore suggests that other key pathway(s) are being utilized to respond to environmental stress. The regulation of Hfq has been previously examined by our lab, and can globally alter gene expression, virulence and pathogenesis related stress responses when exposed to LFS conditions (Wilson et al. 2007).

Future investigations should also look into the RpoS dependent gene expression of oxidative and thermal stresses when *S*. Typhimurium χ3339 is cultured in LFS. Interestingly, the RpoS dependent regulation in the LFS condition does not enhance the resistance of *S*. Typhimurium χ3339, but instead increases the sensitivity of the wild type. This provides a platform for further investigation to identify the way stationary phase *S*. Typhimurium regulates RpoS in LFS and may provide new insight for regulation of pathogenesis-related stress responses.

In conclusion, this study has examined the relationship between LFS and RpoS stress response regulation by stationary phase *S*. Typhimurium χ3339. The stress assays provided evidence of both RpoS independent (acid and bile) and RpoS dependent (oxidative and thermal) stress response regulation by stationary phase *S*. Typhimurium χ3339 in LFS cultures. The role of RpoS in regulating the general stress response to oxidative and thermal stress in LFS conditions by *S*. Typhimurium χ3339 has presented a platform for future investigation into the effects of LFS and RpoS regulation. Future studies should investigate the concentration of RpoS

in the LFS and control growth conditions in stationary phase *S*. Typhimurium χ3339 after being subjected to oxidative and thermal stress, to identify how RpoS is being regulated. To distinguish the concentration of protein between LFS and control, the Bardford protein assay can be utilized to identify whether LFS is affecting overall protein expression in stationary phase *S*. Typhimurium χ3339. Following the Bradford protein assay, it will be important to address whether there is differences at the transcriptional level. Regulation of *rpoS* during transcription can be observed by performing a quantitative PCR analysis. A quantitative western blot analysis can identify whether there is a difference in the translational regulation of RpoS between LFS and control conditions. These potential future studies will provide information about the regulation of RpoS in LFS conditions at the transcriptional and translational levels, to provide insight into the regulation of RpoS in stationary phase *S.* Typhimurium χ3339 oxidative and thermal stress response.

The results generated from this study establish that the physiological LFS globally alters the response to acid and bile stress independent of RpoS in stationary phase *S.* Typhimurium χ3339, while RpoS was shown to provide cross-protection. Providing evidence for the LFS condition capable of inducing RpoS independent response to stress in stationary phase *S.* Typhimurium χ3339 through an alternative pathway(s). To our knowledge, this is the first report that the general stress response to acid and bile stressors in stationary phase LFS-cultured *S*. Typhimurium χ3339 is regulated independently of RpoS, while oxidative and thermal stress response was regulated dependently on RpoS. Future studies will aim to further understand known and unknown mechanisms of the LFS regulatory circuit. Results from this study further highlight how *S.* Typhimurium integrates the process of mechanosensation in its regulatory paradigm to uniquely pre-adapt the organism to survive pathogenic stresses in a manner that is different from that observed during conventional culture, and which may lead to characterization of previously unidentified virulence strategies.

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