The Effect of Fluid Shear on Pathogenesis-related Phenotypes of Non-typhoidal

Salmonella enterica serovar Typhimurium ST313 A130

by

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ABSTRACT

In sub-Saharan Africa, an invasive form of nontyphoidal *Salmonella* (iNTS) belonging to sequence type (ST)313 has emerged as a major public health concern causing widespread bacteremia and mortality in children with malaria and adults with HIV. Clinically, ST313 pathovars are characterized by the absence of gastroenteritis, which is commonly found in "classical" nontyphoidal *Salmonella* (NTS), along with multidrug resistance, pseudogene formation, and chromosome degradation. There is an urgent need to understand the biological and physical factors that regulate the disease causing properties of ST313 strains. Previous studies from our lab using dynamic Rotating Wall Vessel (RWV) bioreactor technology and "classical" NTS strain χ3339 showed that physiological fluid shear regulates gene expression, stress responses and virulence in unexpected ways that are not observed using conventional shake and static flask conditions, and in a very different manner as compared to ST313 strain D23580. Leveraging from these findings, the current study was the first to report the effect of fluid shear on the pathogenesis-related stress responses of *S.* Typhimurium ST313 strain A130, which evolved earlier than D23580 within the ST313 clade. A130 displayed enhanced resistance to acid, oxidative and bile stresses when cultured in the high fluid shear (HFS) control condition relative to the low fluid shear (LFS) condition in stationary phase using Lennox Broth (LB) as the culture medium. The greatest magnitude of the survival benefit conferred by high fluid shear was observed in response to oxidative and acid stresses. No differences were observed for thermal and osmotic stresses. Based on previous findings from our laboratory, we also assessed how the addition of phosphate or magnesium ions to the culture medium altered the acid or oxidative stress responses of A130 grown in the RWV. Addition of either

phosphate or magnesium to the culture medium abrogated the fluid shear-related differences observed for A130 in LB medium for the acid or oxidative stress responses, respectively. Collectively, these findings indicate that like other *Salmonella* strains assessed thus far by our team, A130 responds to differences in physiological fluid shear, and that ion concentrations can modulate those responses.

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INTRODUCTION

Salmonella are a genus of bacteria in the family of Enterobacteriaceae, which are commonly associated with gastrointestinal disease, and have the ability to spread through contaminated food and water. Depending on the strain, *Salmonella* can cause bacterial infection in both humans and animals (Brenner et al., 2000, Williams et al., 2010). *Salmonella* are closely related to other types of intestinal pathogens like *E. coli* and *Shigella* within the Enterobacteriaceae family (Williams et al., 2010). *Salmonella* are rod-shaped, typically motile, Gram-negative, facultative anaerobic bacteria with a biochemical profile that can vary among the thousands of isolates. Within the genus of *Salmonella* there are two species, *S. enterica* and *S. bongori*, with most human infections caused by *S. enterica* (Williams et al., 2010, Fookes et al., 2011). *Salmonella enterica* is a diverse species, with six subspecies and more than 2,500 serotypes (Brenner et al., 2000, CDC, Mar. 2015). The serotypes are based off of the Kauffmann-White scheme, which incorporates the O antigen (lipopolysaccharide) found on the bacterial surface and H antigen which is part of the flagella (Brenner et al., 2000, CDC, Mar. 2015). Of the six subspecies, *S. enterica* subsp. *enterica* I is the most common found in warm-blooded animals and contains over 1,500 serovars (Su & Chiu, 2007). One of the most medically relevant serovars of *S. enterica* that causes widespread mortality and morbidity in humans all over the world is serovar Typhimurium (referred to as *S.* Typhimurium from here forth) (Su & Chiu, 2007).

Salmonella enterica is one the leading causes of food-borne illness among intestinal pathogens in the United States (Scallan et al., 2015, CDC 2017). Symptoms occur about

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12 to 72 hours after exposure to contaminated food or beverage and include (but are not limited to) diarrhea, fever, and abdominal cramps (Ley et al., 2014, Scallan et al., 2015, CDC 2017). While infection in immunocompetent individuals is mainly limited to gastroenteritis, a systemic infection can occur in immunocompromised individuals and spread to other organs in the body via the bloodstream (Finlay & Falkow, 1989, Brent et al., 2006, Kingsley et al., 2009). Strains of *S.* Typhimurium have also been known to have a broad host range, causing infection in humans and many animals (Ley et al., 2014). The wide range of microenvironments encountered by *S*. Typhimurium within infected hosts, as well as the broad host-tropism, can provide continuing insight into the ever evolving pathogenic mechanisms of this bacterium.

In sub-Saharan Africa, emergence of an invasive form of non-Typhoidal *Salmonella* (iNTS) belonging to pathovar ST313 (sequence type 313) has been the cause of widespread bacteremia and mortality among children with malaria and adults with HIV (Kingsley et al., 2009). Unlike the classic NTS, ST313 have limited to no gastrointestinal symptoms with invasive salmonellosis leading to systemic infection and disease (Gilks et al., 1990, Graham et al., 2000a, Graham et al., 2000c, Gordon et al., 2008, Kingsley et al., 2009, Ley et al., 2014). The rapid microevolution of ST313 from classic NTS into a phylogenetically distinct clade must be studied to prevent further outbreak of disease.

ST313 can cause 20-25% fatality in children and up to 50% in adults in sub-Saharan Africa, which has elicited concern and classification of these iNTS diseases as emerging and neglected infectious diseases (Gilks et al., 1990, Graham et al., 2000b, Gordon et al., 2002, Gordon et al., 2008, Feasey et al., 2012, Ley et al., 2014). Most adults infected with

ST313 strains are HIV positive, while only 20% of infected children have association with HIV (Brent et al., 2006, Kingsley et al., 2009, Trong et al., 2010). The remaining percentage of children are susceptible to iNTS due to malaria, anemia and malnutrition as risk factors (Brent et al., 2006, Kingsley et al., 2009, Trong et al., 2010, Okoro et al., 2012). Abnormal levels of ions in the bloodstream is symptomatic of patients with malaria and HIV compared to healthy individuals due to renal failure (Szczech 2007, Akande 2016).

Infection by different ST313 serovars is further complicated by multidrug resistance (MDR), pseudogene formation, and chromosome degradation (Kingsley et al., 2009). These characteristics are commonly found in human-restricted pathogens like *S.* Typhi and *S.* Paratyphi A (the causal agents for typhoid and paratyphoid fever, respectively), which suggests that ST313 could be evolving towards host restriction (Kingsley et al., 2009, Ley et al., 2014, Feasey et al., 2012). The evolution of ST313 could provide information about why there was a shift of this gastrointestinal infection to a systemic infection. Since ST313 strains spread to the bloodstream, abnormal levels of ions in the bloodstream of patients with malaria and HIV could further contribute to proliferation of this pathogen into the blood. Additional research could provide information about why there is a shift of classical GI symptoms to systemic infection in these patients.

In 2009, ST313 isolates of *S.* Typhimurium were classified using multilocus sequence typing (MLST), which genetically differentiated the ST313 clade from the classic NTS ST19 clade, which causes limited gastroenteritis in healthy individuals (Kingsley et al.,

2009). Although both these clades are genetically different, it is important to note that the strains within both the clades are classified of S. Typhimurium. These strains share a common ancestor, and changes in genomic content of ST313 has led to close association of this clade with immunocompromised individuals with malaria and HIV (Kingsley et al., 2009, Feasey et al., 2012). There is an imperative need to elucidate the mechanisms related to pathogenicity of ST313 pathovars due to the high rates of morbidity and mortality in sub-Saharan Africa and threat for pandemic spread.

The iNTS ST313 clade are composed of two distinct lineages classified as lineage I and lineage II (Kingsley et al., 2009, Okoro et al., 2012). Within lineage I is representative strain A130 and in lineage II are representative strains D23580 and 5579 (Kingsley et al., 2009, Okoro et al., 2012). Both A130 and D23580 were isolated from Malawi in 1997 and 2004 respectively (Kingsley et al., 2009, Okoro et al., 2012). Lineage I was the primary source of iNTS from 1997 until there was an epidemic in 2004 from which lineage II emerged as the major source of iNTS. The iNTS epidemic in 2004, also led to expanded chloramphenicol resistance, which was due in part to the over-use of chloramphenicol in vulnerable populations with HIV, malaria, and other immunocompromising diseases. The location of emergence of iNTS infection, the migration of immunocompromised individuals and evolution of MDR due to overuse of antibiotics have all played a huge role in the expansion of STS313 infections in sub-Saharan Africa (Okoro et al., 2012). Kingsley and colleagues were able to identify ST313 as a distinct pathovar of iNTS Typhimurium due to changes in genomic content found on virulence plasmids compared to classic NTS ST19 (Kingsley et al., 2009). Interestingly,

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A130 contained none of the chromosomal deletions present in D23580 and 5579, which implied that A130 evolved earlier on the ST313 branch and that the deletions present in the D23580 and 5579 strains occurred after diverging from lineage I (Kingsley et al., 2009). Specifically, A130 is unique from the representative strains D23580 and 5579 in both evolutionary history and MDR profile as well as genetic make-up and phage typing, which could translate into different pathogenic strategies for infecting humans (Kingsley et al., 2009). The importance of studying A130 can clarify what occurred evolutionarily in the ST313 clade.

Salmonella encounters a variety of environmental signals during infection in the host. Since Salmonella is a food borne pathogen, infection begins by digesting contaminated food or beverage via the GI tract, where the initial site of infection is the distal small intestine (Rychlik & Barrow, 2005). As a facultative intracellular pathogen, Salmonella have the ability to adhere and invade intestinal epithelial cells, spreading through neighboring cells that line the small intestine causing gastrointestinal disease. This leads to inflammation and swelling within the intestine, which is responsible for the discomfort experienced during gastroenteritis (Rychlik & Barrow, 2005). Salmonella can infect macrophages and are able to survive inside a Salmonella containing vacuole (SCV) (Stokes et al., 1989, Ly & Casanova, 2007, Wilson et al., 2002c, Waterman & Holden, 2003). Since macrophages are constantly surveying the body for infection, this can allow Salmonella to spread to different tissues and cause systemic disease by spreading into the bloodstream. Systemic organs like the spleen, liver, and gall bladder can become infected by Salmonella (Ly & Casanova, 2007).

As soon as *Salmonella* gains entry in the human host, they are faced with environmental stresses that they need to overcome in order to cause disease. Some of these stresses broadly include physical barriers, chemical gradients, immune response, and physical forces (Aertsen & Michiels, 2004). Examples of stressors that the bacteria may encounter include fluctuations in oxygen levels, salt levels, nutrient limitations, pH, temperature changes, bile and oxidative stresses, stress from immune cells, and fluid movement found within the host (Rychlik & Barrow, 2005). *Salmonella* encounters stress in the highly acidic environment of the stomach. (Lee et al., 1994, Rychlik & Barrow, 2005). *Salmonella* then moves from the stomach to the duodenum of the small intestine (Alvarez-Ordóñez, et al., 2015), where the bacteria encounter bile salts released by the gall bladder that can break down their cellular membrane due to detergent-like actions (Aertsen & Michiels, 2004). *Salmonella* encounters oxidative stresses through reactive oxygen species as it gains entry into intestinal epithelial cells and macrophages. These environmental factors and stresses are present within the complex microenvironment of the human gut, bloodstream, and tissue. An important stress that is often overlooked is the physical force environment encountered by pathogens in infected host tissues. Our lab was the first to show that physical forces experienced by pathogens during the normal course of infection (specifically, fluid shear) globally alter bacterial gene expression, pathogenesis-related stress responses, and virulence in unexpected ways that have previously been overlooked or unappreciated using conventional culture approaches (Nickerson et al., 2000, Wilson et al., 2002a, Wilson et al., 2002b, Nauman et al., 2007, Wilson et al., 2007, Wilson et al., 2008). Indeed, our lab has shown there may be entire

classes of microbial genes/proteins involved in host interactions that have not previously been identified when bacteria are grown under conventional shaking and static culture conditions (Nickerson et al., 2000, Wilson et al., 2002a, Wilson et al., 2002b, Nauman et al., 2007, Wilson et al., 2007, Wilson et al., 2008). While conventional culture conditions have been crucial in advancing our understanding of bacterial pathogenesis they may not be the best models to mimic the physical force microenvironment encountered by pathogens *in vivo* which plays a key role in dictating infection and disease outcome. There is thus an urgent need to bridge the gap between the inherent limitations of traditional bacterial culture approaches in predicting physiological fluid-shear regulated infectious disease mechanisms.

Fluid shear stress is the tangential force of fluid across the surface of any object or organism per unit area, which for the purpose of this study is across the surface of bacterial cells (Tzima et al., 2005, Nauman et al., 2007, Hsiai & Wu, 2008). In order to study fluid shear in a laboratory setting, the rotating wall vessel (RWV; Figure 1) bioreactor designed at the NASA Johnson Space Center can be used as a culture tool since it creates a physiological low fluid shear environment (0.01 dynes/cm^2) (Wolf and Schwarz, 1991, Nickerson et al., 2000, Nauman et al., 2007). The RWV is a cylindrical culture vessel that is completely filled with culture media containing bacteria with the bubbles removed to minimize fluid shear stress (Nickerson et al., 2000). Figure 1 shows a picture of the RWV positioned in two different orientations. In the "low fluid shear" (LFS) position, a solid body rotation is created within the fluid by the vessel rotating on its axis parallel to the ground, which allows for bacteria to remain in suspension so that

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no sedimentation occurs. In the control orientation called "high fluid shear" (HFS), the rotational axis is perpendicular to the ground, resulting in sedimentation of the cells and higher levels of fluid shear stress. The design of the RWV in the LFS position allows for cells to grow in gentle suspension (often referred to as "optimized suspension culture"), thus minimizing the fluid shear stress on the suspended cells. In contrast, traditional shaking cultures impose a turbulent, high fluid shear stress environment on the cells that is likely not physiologically relevant to what is found *in vivo*, while traditional low fluid shear static cultures impose almost no fluid shear stress, which is also not physiologically relevant to what is found *in vivo*.
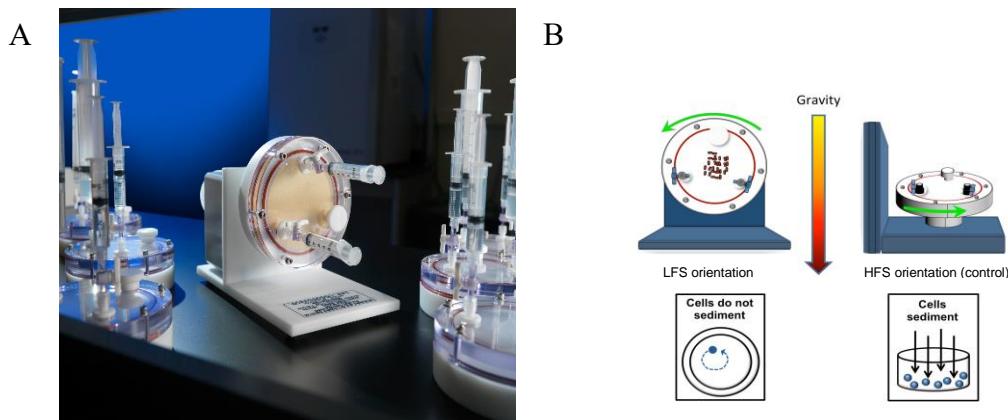
Figure 1. Rotating Wall Vessel (RWV) bioreactor (A) and the two physical orientations of the RWV under LFS and HFS conditions (B).
(A) Photo of the RWV bioreactor. (B) Schematic depicting two orientations of the RWV bioreactor that can be used, including the LFS orientation (left) and HFS control orientation (right).The RWV bioreactor allows for the modulation of fluid shear levels depending on its orientation. Cells remain in suspension and do not sediment when grown in the LFS condition. In the HFS orientation, the cells sediment and experience higher levels of fluid shear stress (Nauman et al., 2007). Image on right modified from Dr. Jennifer Barrila at Arizona State University.

One of the major physical forces that *Salmonella* encounters during *in vivo* infection is fluid shear in the intestinal tract. For example, *Salmonella* encounters low fluid shear between the brush border microvilli of epithelial cells within the small intestine as well as

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in the peristaltic movement of the intestinal tract (Nickerson et al., 2000, Nickerson et al., 2003, Nickerson et al., 2004, Nauman et al., 2007, Gayer & Basson, 2009), while they experience higher levels of fluid shear in the bloodstream (Nauman et al., 2007, Wilson et al., 2008, Nickerson et al., 2004). Because both low and high fluid shear are encountered by pathogens in the host, these responses may be important for bacterial reprogramming during transitions between environments of different physiological fluid shear levels, which could result in altered survival and infection. When *S*. Typhimurium has been cultured in a low fluid shear environment, widespread global reprogramming of virulence, gene expression and/or pathogenesis-related stress responses has been observed (Nickerson et al., 2000, Wilson et al., 2002a, Wilson et al., 2002b, Wilson et al., 2007). Understanding the response of *Salmonella* to the fluid shear encountered in the host may offer clues as to how this pathogen colonizes its host, and may provide new targets for novel strategies to fight infectious diseases.

Previous research on classic *S*. Typhimurium χ3339 (an animal-passage isolate of *S*. Typhimurium SL1344 wild-type) in the RWV bioreactors showed that low fluid shear (LFS) cultures exhibited increased virulence, increased resistance to pathogenesis-related stress responses, and global alterations in gene expression, as compared to the HFS control (Nickerson et al., 2000, Wilson et al., 2002a, Wilson et al., 2002b, Nauman et al., 2007). In 2000, Nickerson and colleagues investigated the effect of LFS on virulence and pathogenicity in χ3339 when grown to late-log phase. When *S*. Typhimurium was cultured in LFS, they observed increased virulence and colonization of the liver and spleen in an oral infection murine model. They also observed that LFS increased

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resistance to acid stress and ability to survive within macrophages, and elicited differences in protein expression (Nickerson et al., 2000). In 2002, Wilson and colleagues sought to find genes and regulatory factors related to the LFS signaling pathway. The authors discovered that the master stress response sigma factor RpoS, does not regulate the LFS response of *S.* Typhimurium grown to late log phase (Wilson et al., 2002a). Specifically, an *rpoS* mutant strain showed the same survival trends to wild type χ3339 when subjected to acid, thermal, osmotic and oxidative stresses as compared to high fluid shear during late log phase. This implied that the LFS response of *Salmonella* was novel and may account for the presence of a LFS regulon. The existence of an LFS regulon was subsequently demonstrated using global transcriptomic profiling, which identified 163 genes distributed throughout the chromosome and representing a variety of functionally diverse products (Wilson et al., 2002b). Surprisingly, while LFS culture increased the virulence of χ3339, none of the classical virulence genes were shown to be upregulated. This suggests the intriguing possibility that unknown virulence genes may be differentially regulated by LFS culture, which could lead to novel insights into infectious disease mechanisms and downstream translational opportunities for new treatment and prevention modalities. The previous studies on χ3339 and the influence of fluid shear in conjunction with environmental factors encountered during infection are essential for further characterization of the differences in response to fluid shear of other related *S.* Typhimurium strains like ST313.

Our laboratory has profiled the molecular genetic, phenotypic and virulence characteristics of representative ST313 strain D23580. It was discovered that D23580

responds to LFS in RWV bioreactors differently than the classic NTS χ3339. Specifically at late-log phase of growth, D23580 displayed increased resistance to bile salts and oxidative stress under HFS conditions, but no significant difference was observed between LFS and HFS under acid stress conditions. Further, D23580 demonstrated the ability to cause lethal disease in mice, thus validating that the pathogen was not host restricted (Parsons et al., 2013, Yang et al., 2015, Yang et al., 2016). In mice, D23580 spread more rapidly than SL1344 (χ3339 is the animal-passage of SL1344) into deep tissues, including the spleen and gall bladder at late log phase of growth (Okoro et al., 2015, Yang et al., 2015). It is important to note the differences in response to physiological fluid shear in the RWV among the classic non-invasive *S.* χ3339 and invasive NTS D23580. These phenotypic responses to stress and fluid shear could be conserved genetically, which could further explain the evolutionary relationship between these two strains and reveal novel pathogenesis mechanisms for the invasive nature of the ST313 strains. It is therefore crucial to investigate the stress response of the closely related strain iNTS ST313 A130 under LFS and HFS conditions. This is due, in part, to this strains earlier evolution within the ST313 clade and the genetic and MDR differences compared to D23580.

Like all of the ST313 strains, A130 causes systemic infection in immunocompromised patients with malaria and HIV. One characteristic of patients with malaria and HIV is either low or high levels of ions in the bloodstream compared to healthy patients. The combination of the differences in ion concentrations in the bloodstream and evidence of higher fluid shear could play a role in the stress response of

A130. Ions play a crucial role in the signaling of bacterial pathogenesis within a given environment by altering virulence mechanisms, energy metabolism, and signal transduction (Van Dien & Keasling, 1998, Hengge-Aroinis, 2002, Baek & Lee, 2006, Arribas-Bosacoma et al., 2007, Lamarche et al., 2007, Crépin et al., 2011, Ritzefeld et al., 2011). In particular, phosphate and magnesium ions have been linked to virulence genes in *Salmonella* as well as closely related enterobacteria like *E. coli* (Soncini, 1996, Moncrief & Maguire, 1998, Van Dien & Keasling, 1998, Baek & Lee, 2006, Alix & Blanc-Potard 2007, Arribas-Bosacoma et al., 2007, Lamarche et al., 2007, Crépin et al., 2011, Ritzefeld et al., 2011).

The combination of high and low fluid shear and differences in ion concentration in a media introduces a novel environment in which to further understand *S.* Typhimurium pathogenicity. In 2008, Wilson and colleagues examined how media ion composition affects *S.* Typhimurium χ3339 phenotypic response to acid stress in the RWV bioreactors. Using previous data related to media composition and χ3339 virulence cultured under extreme LFS conditions (in spaceflight), ion concentrations were analyzed in two types of media, LB (Lennox broth) and M9 (minimal media). It was found that significantly higher concentrations of phosphate (61-fold higher than the LB media), magnesium (18-fold higher than the LB media), sulfate (3.6-fold higher than the LB media), chloride (3-fold higher than the LB media), and potassium (2.4-fold higher than the LB media) were found in M9 media compared to LB, which resulted in changes in virulence of χ3339 cultured in spaceflight when used to infect mice. In order to test whether these ions could be playing a role in χ3339 virulence during spaceflight, LB

media was supplemented with the same concentration of ions found in M9 media (called LB-M9 salts) and ground-based post-flight studies were done in the RWV. The LB-M9 media was specifically supplemented with 25.18 mM NaH_2PO_4, 22 mM, KH_2PO_4, 18.68 mM NH_4Cl, 8.54 mM NaCl and 2 mM $MgSO_4$. In these studies, *S.* Typhimurium χ3339 was cultured in LB, M9, and LB-M9 salts, respectively, in both HFS and LFS culture conditions in the RWV and tested for changes in acid tolerance. There was an increased resistance of χ3339 to acid stress when cultured in HFS compared to LFS in LB media, but, on the contrary, when cultured in M9 and LB-M9 salts there was no difference in χ3339 survival between HFS and LFS conditions. The difference in concentrations of phosphate, magnesium, sulfate, chloride, and potassium in LB media were then tested for changes in acid stress response in HFS vs. LFS conditions. Increased concentrations of phosphate ions (25.18 mM or 77.96 mg/dL) had no effect on survival of χ3339 during acid stress under HFS and LFS conditions, while increased concentration of magnesium ions (2mM or 4.87 mg/dL) led to enhanced survival of HFS over LFS during acid stress. The results from this study concluded that modulating different ion concentrations controls the fluid shear response in *S.* Typhimurium χ3339, and specifically addition of phosphate negated the difference observed in survival observed during acid stress cultured in HFS and LFS conditions. This knowledge is essential for understanding the microenvironment that A130 experiences in the bloodstream of patients with malaria and HIV.

The levels of magnesium and phosphate in the blood of patients with HIV and malaria could be relevant to the progression of systemic disease of iNTS *S.*

Typhimurium. The role of magnesium and phosphate in the human body is related to maintenance of cellular activities, heart functioning, nutrient exchange, and nervous system operations (Szczech 2007). The normal levels of magnesium in the blood of adults without HIV or malaria range from 1.5-2.5 mg/dL (or 0.62-1.03 mM/L) (Resnick et al., 1984, Guerrero-Romero & Rodriguez-Moran, 2002, Häggström, 2010), while the normal levels of phosphate in the blood range from 2.5 to 4.5 mg/dL (or 0.81-1.45 mM/L) (Szczech 2007, Häggström, 2010, Akande 2016). There is a constant exchange of magnesium and phosphate ions in and out of cells, and a shift in these levels can cause hypermagnesemia/hypomagnesemia or hyperphosphatemia/hypophosphatemia (Resnick et al., 1984, Guerrero-Romero & Rodriguez-Moran, 2002, Szczech 2007, Häggström, 2010, Akande 2016). Hypermagnesemia/ hyperphosphatemia is characterized by high levels of either magnesium or phosphate in the blood over 2.77 mg/dL (over 1.14 mM/L) and 4.75 mg/dL (over 1.53 mM/L) respectively (Resnick et al., 1984, Romani 2013, Akande 2016), while hypomagnesemia/ hypophosphatemia is characterized by low level of magnesium or phosphate in the blood below 1.25 mg/dL (below 0.5 mM/L) and 2.25 mg/dL (below 0.9 mM/L), respectively (Guerrero-Romero & Rodriguez-Moran, 2002, Szczech 2007, Romani 2013, Akande 2016). Renal failure is a common occurrence in patients with HIV and malaria, and thus both hypermagnesemia/hypomagnesemia and hypermagnesemia/ hyperphosphatemia are present, since the function of the kidneys is to filter the blood (Szczech 2007, Akande 2016). These levels can depend on age, gender, how far along the patient is in disease progression, as well as medications for HIV or malaria (Szczech 2007, Akande 2016). The multifaceted reasons for why these ions are

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either high or low in the blood could potentially increase the virulence of invasive *S.* Typhimurium A130 in patients from sub-Saharan Africa. The possibility exists that *Salmonella* could be responding to differences in the levels of these ions that may be impacted by the levels of fluid shear in the bloodstream of the infected host. The interplay between fluid shear, ion concentrations, and the pathogenic-response of *S.* Typhimurium A130 is critical to consider given the ion concentrations of magnesium and phosphate in the blood of patients with iNTS associated with HIV and malaria.

The purpose of this study was to investigate the effect of fluid shear using the RWV bioreactors on the pathogenesis-related phenotypes of A130. It was hypothesized that culturing A130 under more physiologically relevant fluid shear conditions (as compared to conventional culture approaches) could advance our understanding of how this ST313 strain responds to its microenvironment during infection within the host. Additionally, there was an opportunity in this study to understand how A130 responds to environmental stresses compared to the other representative ST313 strains, D23580 and 5579, as well as the classic *S.* Typhimurium χ3339 under varying fluid shear conditions. Data taken from this study, may further elucidate the question of why this iNTS strain has evolved to become more host restricted compared to the classic NTS strain. Further, we wanted to investigate if addition of phosphate and magnesium ions affected the response of A130 to fluid shear under previously tested pathogenesis-related stresses. Using both the data found on the classic *S.* Typhimurium χ3339 for ion composition affecting response to fluid shear conditions under various stresses, as well as the known characteristic of patients with malaria and HIV having abnormal levels of ions in the

bloodstream, we sought to find out whether addition of phosphate in LB during acid stress and addition magnesium in LB during oxidative stress changed the response of A130 to fluid shear in the RWV bioreactors. In addition, it was observed if the response of classic NTS χ3339 to phosphate ion composition and fluid shear during acid stress was conserved in A130. Understanding how A130 regulates its phenotypic responses to pathogenesis-related stresses and ion concentrations under physiologically relevant fluid shear may provide urgently needed strategies to combat infectious disease caused by this (and perhaps other) ST313 strain/s.

MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

All bacterial strains (stock cultures kept at -80°C in 10% or 20% glycerol) were first

streaked on Lennox Broth (LB) agar or on specific antibiotic media that reflected the

antibiotic resistance profiles of strains as a phenotypic selection marker. Plates were

incubated overnight at 37°C and several well isolated colonies were inoculated into 5 mL

of Lennox Broth (LB) and allowed to grow for 15 hours at 37°C with aeration at 25

r.p.m. (rotations per minute) in the RWV bioreactor. This procedure was used for all

experiments.

2.2. Growth Curves

Growth curves were performed with LFS and HFS cultures to ensure that all assays were

performed at the same phase of growth. Overnight cultures were diluted 1:200 into 150

ml fresh LB medium and used to fill two RWV bioreactors, one for LFS and one for the

HFS control. Bubbles were removed and reactors were then incubated at 37° C and

rotated at 25 r.p.m. in the LFS or HFS positions. Cultures were sampled every hour or

every other hour (~ 0.2 ml) via syringe, serially diluted and plated by spreading in

triplicate of 10 ul drops per countable dilution to assess Colony Forming Units per mL

(CFU/mL). Cultures grown in the HFS bioreactor were inverted to mix three times in

order to ensure accurate sampling at each time point due to sedimentation. Cultures were

sampled from the LFS orientation while the reactor was rotating, since sedimentation is

not an issue. Growth curves were performed over a 24 hour period in biological

duplicate.

2.3. Stress Assays

Stress assays were performed in order to examine the influence of fluid shear on select pathogenesis-related stress responses for A130. Bacterial cultures were grown in RWVs positioned in the LFS and HFS orientations for 24 hours (to stationary phase) and stressors were applied immediately after RWVs were unloaded. Serial dilutions were performed in DPBS (Dulbecco's phosphate-buffer) for all stress assays except oxidative stress. In the oxidative stress assay, fresh 0.1M bi-carbonate solution was used as buffer, made from 0.1M Na_2CO_3 and 0.1M $NaHCO_3$. This was conducted in order to neutralize the oxidative stress, while plating on LB agar to obtain viable colonies. For all stress assays, the percent survival at each time point was obtained by plating serial dilutions of the cultures on LB agar in triplicate by spreading 10 ul of bacterial culture. The CFU/mL obtained at each time point was divided by the initial CFU/mL obtained at time zero (T0; prior to exposure to the stress) and multiplied by 100 to obtain percent survival. Graphs for combined trials shows the average CFU/mL and standard deviation (STDV). The triplicated experiments were combined and plotted in one graph using the average percent survival and standard error of the mean.

The five stress assays tested were acid (pH 3.5), oxidative (0.09% H_2O_2), bile (10% bile salts, approximately 50% sodium cholate and approximately 50% sodium deoxycholate), osmotic (4M NaCl), and thermal (52.5°C). For the acid stress assay, 1M citrate buffer was added to HFS and LFS culture to lower the pH to 3.5 immediately after emptying the RWVs. The volume needed to bring the culture to pH 3.5 was determined prior to the experiment. Percent survival was determined from static cultures of LFS and

HFS grown bacteria at room temperature over a series of kinetic time points for 120 minutes. After completion of acid stress, the final pH of the LFS and HFS cultures was recorded to ensure pH remained in the correct range during the experiment. LB plates were incubated at 37 °C until countable colonies were recovered. In the oxidative stress assay, prior to the experiment, fresh 0.09% H_2O_2 was made from 30% H_2O_2 stock solution (stored at 4°C, no older than month), which was added to LFS and HFS culture immediately after emptying RWVs. Percent survival was taken from static cultures of LFS and HFS at room temperature for 75 minutes and LB plates were incubated at 37 °C until countable colonies were recovered. Resistance to bile stress was tested by adding 10% bile salt from 30% bile salt solution stock to LFS and HFS cultures immediately after emptying RWVs. Percent survival was determined from static cultures of LFS and HFS at room temperature over a series of kinetic time points for 180 minutes and LB plates were incubated at 37 °C until countable colonies were recovered. Thermal stress was tested by taking 1.5 ml of LFS and HFS cultures immediately after emptying the RWVs and exposing to a heat block at a set temperature of 52.5°C. Percent survival was taken from static cultures of LFS and HFS at room temperature for 75 minutes and LB plates were incubated at 37 °C until countable colonies were recovered. In the osmotic stress assay, 4M NaCl was added to bacterial cultures immediately after emptying the RWVs and percent survival was taken from static cultures of LFS and HFS at room temperature over a series of kinetic time points for 24 hours. LB plates were incubated at 37 °C until countable colonies were recovered.

2.4. Statistical analyses

All experiments were performed in biological triplicates. Standard error of mean (SEM) was determined as the standard deviation divided by the number of replicates performed. The statistical significance (*$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$) was calculated with Microsoft Office Excel 2015 using Student's t-test.

RESULTS

3.1. Growth curve of A130 in the RWV bioreactor

The growth curves in Figure 2 show that the LFS and HFS cultures were in the same

phase of growth for all time points tested, with no statistical difference in CFU/mL. Lag

phase occurred between 0-2 hours, mid-log phase between 3-6 hours, late-log phase

between 7-8 hours, and stationary phase after 8 hours. All stress assays were performed at
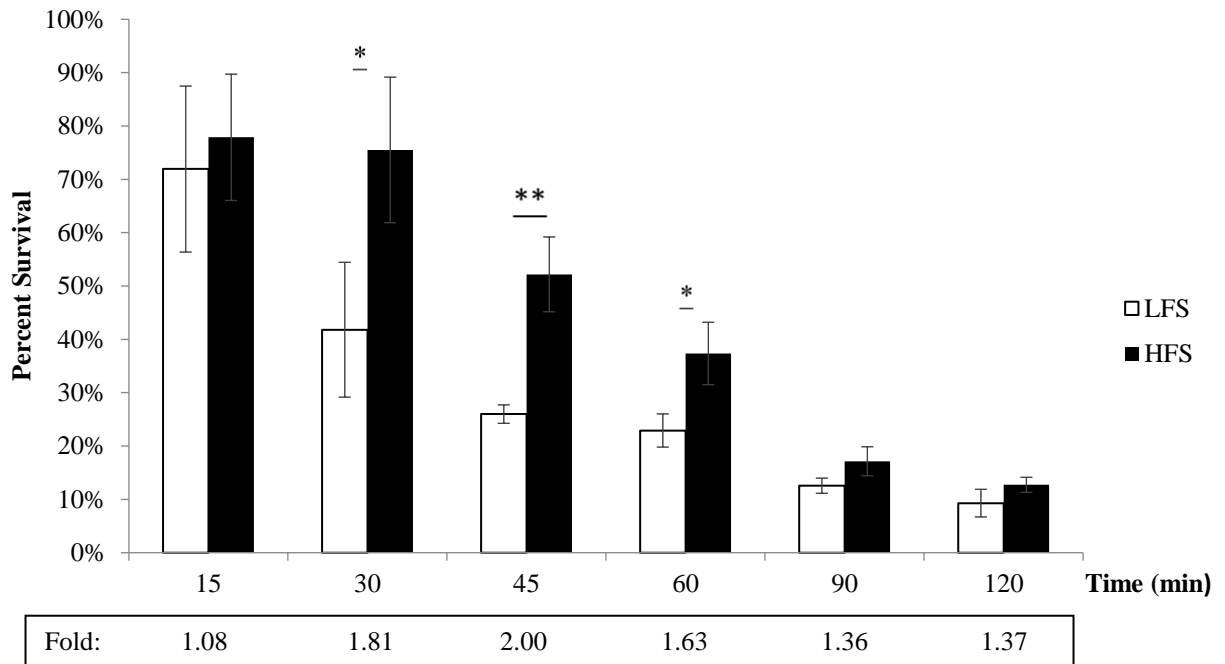
stationary phase (24 hours).
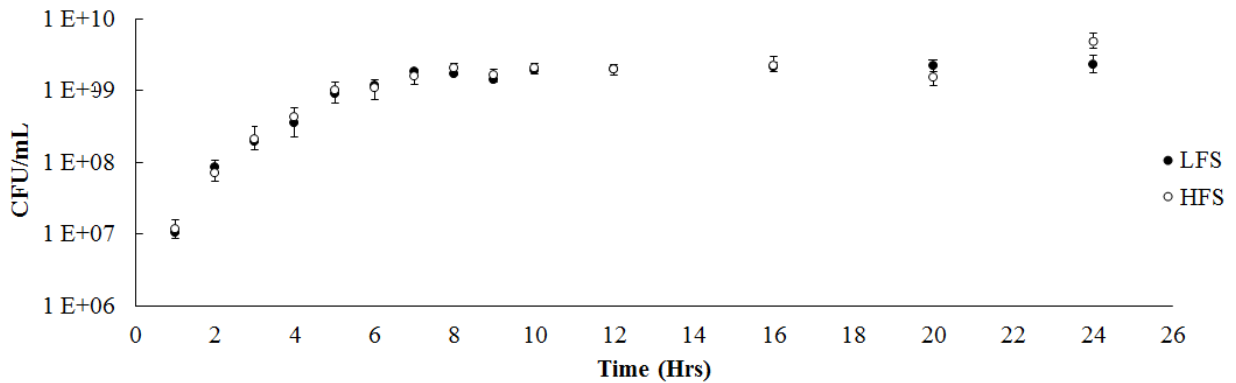
Figure 2. Growth curve of *S*. Typhimurium A130 in the RWV bioreactor under LFS and HFS conditions.
S. Typhimurium A130 was grown for 24 h in the RWV bioreactor in LFS (black circles, ●) and HFS control (white circles, ○) conditions. Results are expressed as the mean of values from two independent biological replicates and three technical replicates. Error bars indicate the standard deviations.

3.2. Increased resistance of A130 to acid stress when cultured in high fluid shear (HFS)

Increased resistance of A130 to acid stress (pH 3.5) was observed when cultured in the

HFS condition as compared to LFS, as shown in Figure 3. Significant differences in

percent survival were observed at 30, 45, and 60 min following addition of the acid stress

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(pH 3.5) (P ≤ 0.05). For both HFS and LFS cultures, a downward trend in survival was observed over the span of the two hour experiment. No differences in survival were observed at 15 min, 90 min, and 120 min. The fold differences for A130 between LFS and HFS for time points 30 min through 60 min under acid stress were generally consistent across these time points, ranging between 1.6-2 fold.

| Fold: | 1.08 | 1.81 | 2.00 | 1.63 | 1.36 | 1.37 |

Figure 3. Acid stress resistance (pH 3.5) of A130 following RWV culture.
Stationary phase A130 cultures grown in the RWV positioned in the HFS and LFS orientations were challenged with acid stress (pH 3.5) and plated for CFU/mL at the indicated time points. Results are expressed as the mean percent survival from three independent experiments. Error bars indicate standard deviation. Statistically significant differences (Student's t test) are indicated as follows: *P ≤ 0.05, **P ≤ 0.01.

3.3. Increased resistance of A130 to oxidative stress when cultured in HFS

Increased resistance of A130 to oxidative stress (0.09% hydrogen peroxide) was also observed when cultured in the HFS condition as compared to LFS, as shown in Figure 4

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(P < 0.05). The sensitivity of A130 grown under the LFS condition increased with time

and the ratio of percent survival doubled from 5-fold at 45 min to over 10-fold at 75 min
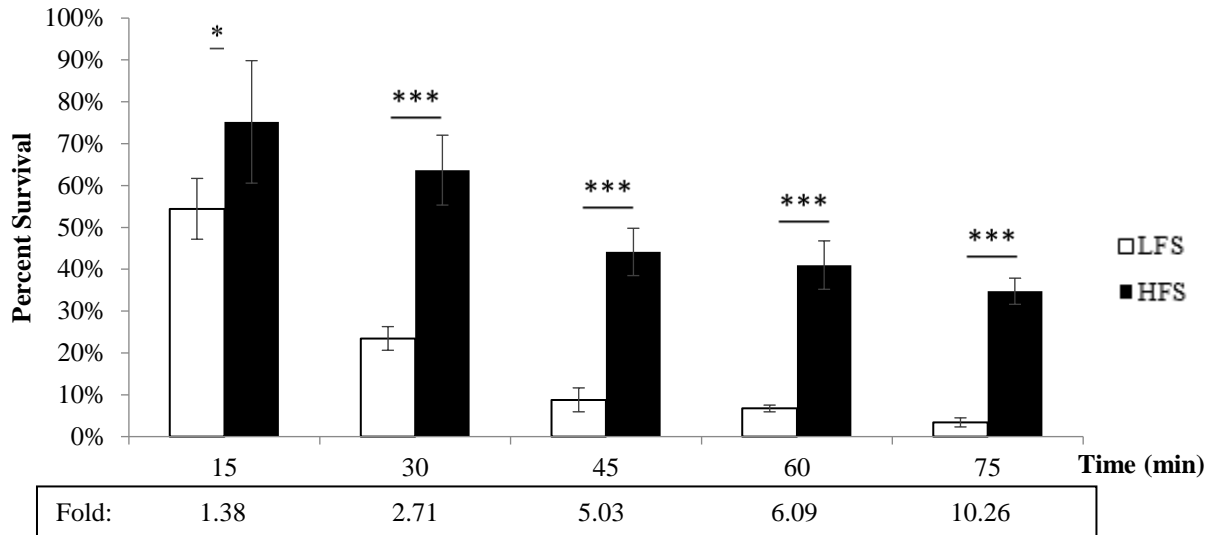
between the HFS and LFS conditions.

Figure 4. Oxidative stress resistance of A130 following RWV culture.
Stationary phase cultures of A130 grown in the RWV positioned in the HFS and LFS orientations
were challenged with 0.09% hydrogen peroxide and plated to assess CFU/mL at the indicated
time points. Results are expressed as the mean percent survival from three independent
experiments. Error bars indicate standard deviation. Statistically significant differences (Student's
t test) are indicated as follows: *P ≤ 0.05, ***P ≤ 0.001.

3.4. Increased resistance of A130 to bile stress when cultured in HFS

In line with the results obtained for acid and oxidative stresses, HFS culture also increased

increased the resistance of A130 to 10% bile salts solution (

Figure 5). Within the first 15 min of the experiment, the percent survival of bacteria

grown under LFS dropped to below 20% and continued to show a more gradual decrease

in survival then remained steady for the remainder of the time points tested (up to 3

hours). The bacteria grown under HFS also dropped in survival (to below 50%), however

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in contrast gradually decreased during the experiment. At the midpoint of the experiment, HFS survival was at 50% and only reduced by approximately 10% by the end of the experiment.

Figure 5. Bile stress resistance of A130 following RWV culture.
Stationary phase cultures of A130 grown in the RWV positioned in the HFS and LFS orientations were challenged with 10% bile salts and plated for CFU/mL at the indicated time points. Results are expressed as the mean percent survival from three independent experiments. Error bars indicate standard deviation. Statistically significant differences (Student's t test) are indicated as follows: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.
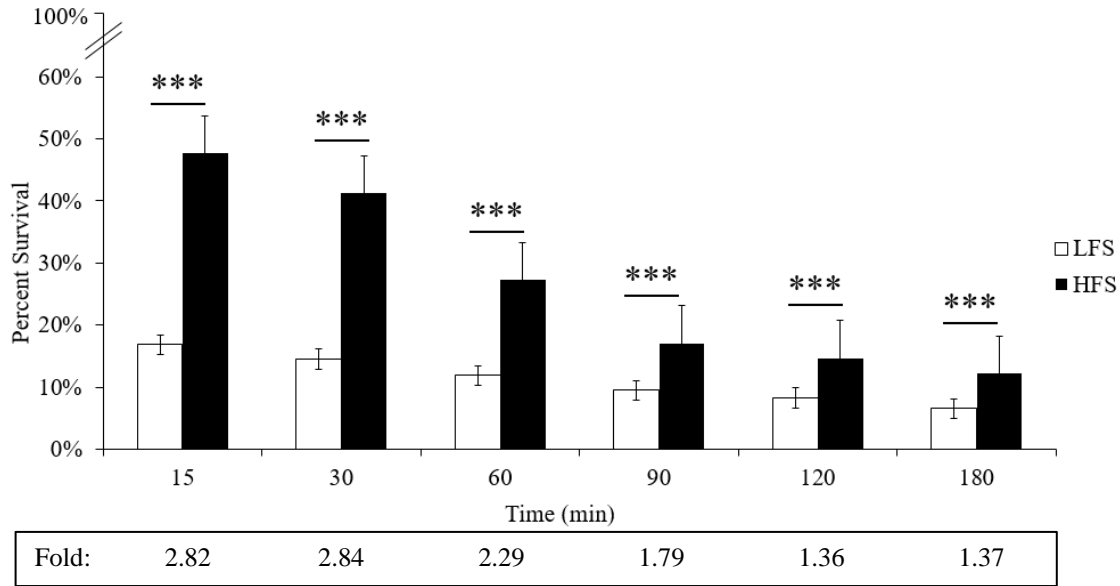
3.5. A130 displays no fluid shear-dependent differences in resistance to osmotic stress or thermal stresses

Figure 6 and Figure 7 show the results from experiments wherein A130 was challenged with osmotic stress using 4M NaCl or thermal stress at 52.5 °C, respectively. No significant differences between the HFS and LFS cultures were observed for either assay.

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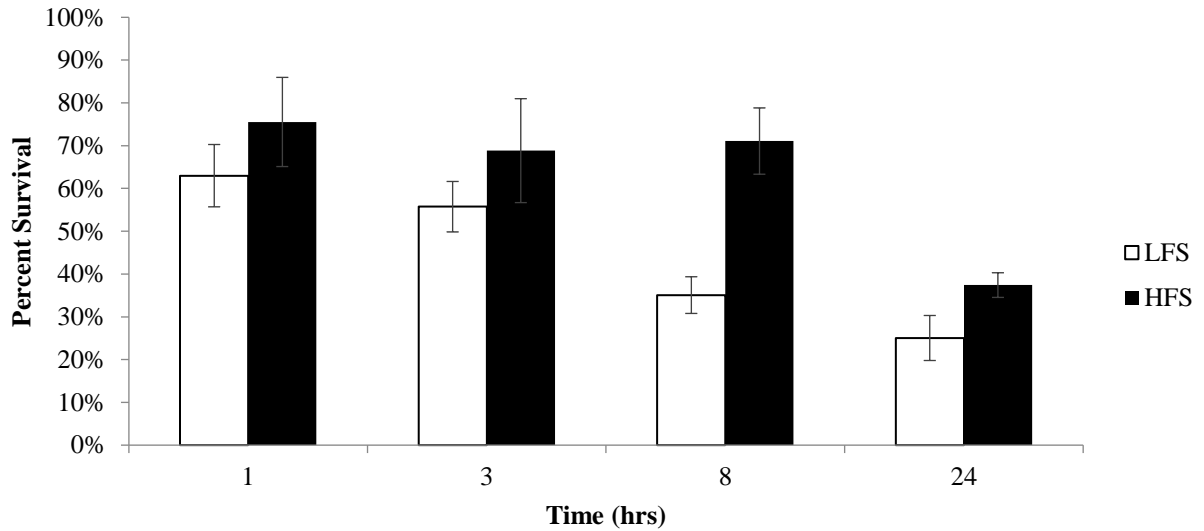
Figure 6. Osmotic stress (4M NaCl) assay of A130 after growth in the RWV bioreactor for 24 hours.
Stationary phase cultures of A130 grown in the RWV positioned in the HFS and LFS orientations were challenged with 4M NaCl and plated for CFU/mL at the indicated time points. Results are expressed as the mean percent survival from three independent experiments. Error bars indicate standard deviation. No statistically significant differences observed in osmotic stress when cultured in HFS and LFS.
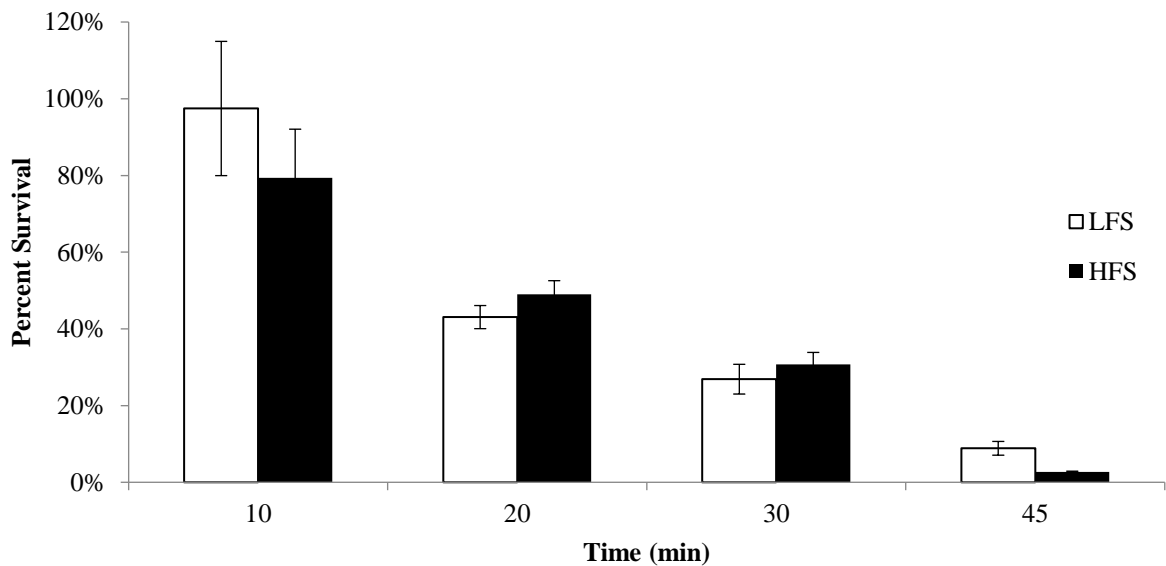
Figure 7. Thermal stress (52.5º C) assay of A130 after growth in the RWV bioreactor for 24 hours.
Stationary phase cultures of A130 grown in the RWV positioned in the HFS and LFS orientations were challenged with a temperature of 52.5º C and plated for CFU/mL at the indicated time

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points. Not all data is shown due to minimal survival at later time points. Results are expressed as the mean percent survival from three independent experiments. Error bars indicate standard deviation. No statistically significant differences were observed in thermal stress survival between A130 cultured in HFS and LFS.

3.6. Addition of MgSO$_4$ (2 mM) negates the altered oxidative stress response of A130 to HFS culture

Figure 8 shows that supplementing the LB media with additional 2 mM MgSO$_4$ resulted in a decreased resistance to oxidative stress of A130 grown under HFS and LFS conditions. Within the first 10 min of the experiment, the bacteria grown under both HFS and LFS conditions dropped to 50% survival and sensitivity of both strains increased over time to almost no survival by the end of the experiment. The data also showed no significant difference of A130 survival at each time point between the HFS and LFS conditions. When compared to the oxidative stress assay with no MgSO$_4$ supplementation (Figure 4), the addition of MgSO$_4$ (2 mM) negated the difference in survival observed during oxidative stress cultured in HFS vs. LFS conditions.
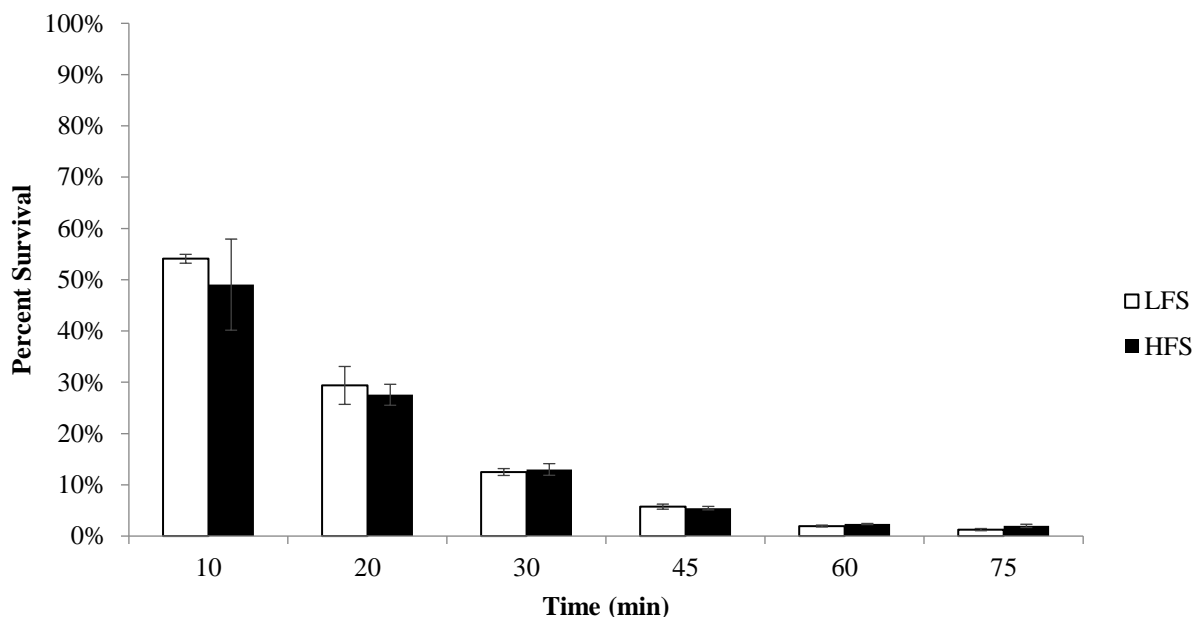
Figure 8. Oxidative stress resistance of A130 following RWV culture with LB media supplemented with MgSO$_4$ (2mM).
Stationary phase cultures of A130 grown in the RWV positioned in the HFS and LFS orientations were challenged with 0.09% hydrogen peroxide and plated to assess CFU/mL at the indicated time points. Results are expressed as the mean percent survival from three independent experiments.

3.7. Addition of Na$_2$HPO$_4$ (25 mM) negates the altered acid stress response of A130 to HFS culture.

Figure 9 shows that supplementing the LB media with additional 25 mM Na$_2$HPO$_4$, resulted in increased resistance to acid stress of A130 grown under both HFS and LFS conditions (Wilson et al., 2008). During the experiment, survival of A130 under both HFS and LFS remained similar with no significant difference of A130 survival at each time point between the HFS and LFS conditions. When compared to the acid stress assay with no Na$_2$HPO$_4$ supplementation (Figure 3), the addition of Na$_2$HPO$_4$,

(25 mM) negated the difference in survival observed during acid stress cultured in
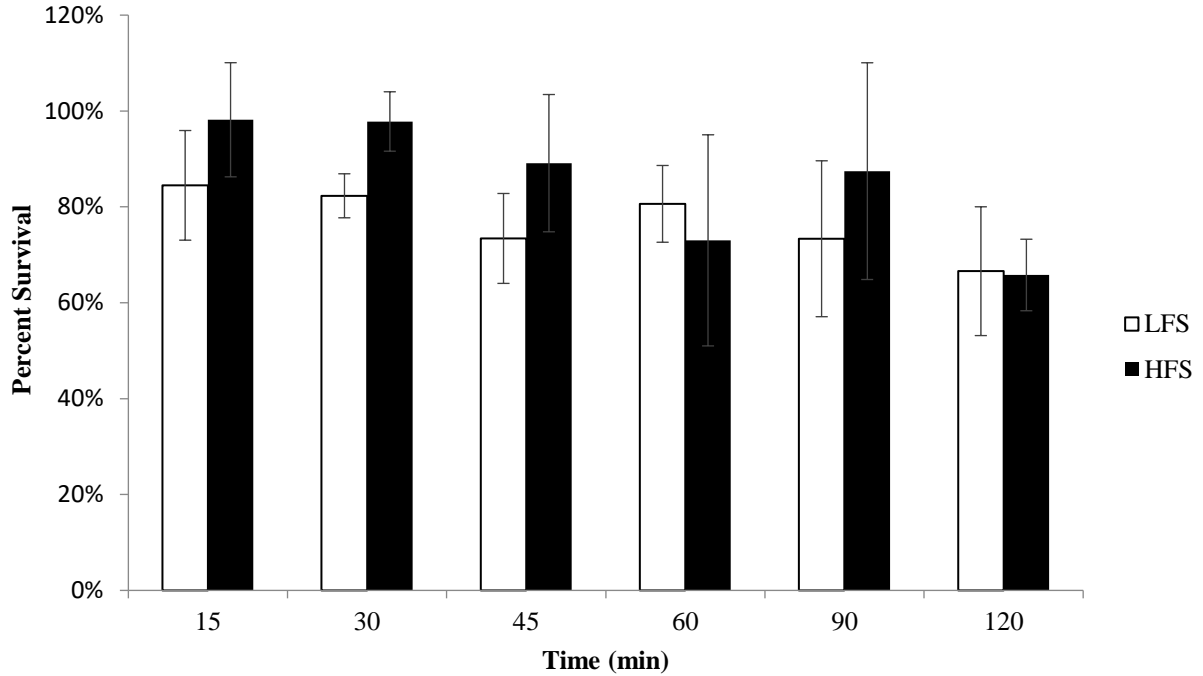
HFS vs. LFS conditions.

Figure 9. Acid stress resistance (pH 3.5) of A130 following RWV culture with media supplemented with Na₂HPO₄ (25 mM).
Stationary phase cultures of A130 grown in the RWV positioned in the HFS and LFS orientations were challenged with acid stress (pH 3.5) and plated for CFU/mL at the indicated time points. Results are expressed as the mean percent survival from three independent experiments.

Table 1. Comparison of stress survival ratios of *S*. Typhimurium strains cultured to stationary phase under LFS and HFS conditions. Data on D23580 from unpublished data Yang 2015, data on 5579 from unpublished data Castro 2016, and data on χ3339 from unpublished data Crenshaw 2016.

Strain	A130	D23580*	5579	χ3339
Acid Stress	HFS/LFS	No difference	LFS/HFS	HFS/LFS
Oxidative stress	HFS/LFS	HFS/LFS	HFS/LFS	HFS/LFS
(Final H₂O₂	(0.09%)	(0.06%)	(0.12%)	(0.24%)
concentration)				

Bile stress	HFS/LFS	HFS/LFS	HFS/LFS	HFS/LFS
Osmotic stress	No difference	No difference	HFS/LFS	–
Thermal stress	No difference	No difference	No difference	HFS/LFS

* Note that D23580 survival ratio to acid and oxidative stresses was due to increased level of fluid shear by the inclusion of beads. Due to difference in growth conditions, D23580 cannot be compared to the other *S.* Typhimurium strains. However, the trends potentially suggest that survival response to acid and oxidative stresses was conserved between A130, χ3339, and D23580.

In Table 1. Comparison of stress survival ratios of *S.* Typhimurium strains cultured to stationary phase under LFS and HFS conditions. Data on D23580 from unpublished data Yang 2015, data on 5579 from unpublished data Castro 2016, and data on χ3339 from unpublished data Crenshaw 2016., the stress survival ratios represent the fluid shear condition that had the greater survival over the fluid shear condition with the lesser survival throughout each of the stress assays. No differences indicates that there was no statistically significant difference in survival between the bacteria grown at LFS and HFS at that specific stress response.

DISCUSSION

The use of RWV bioreactor technology has allowed scientists to better understand how physiological fluid shear plays a role in regulating the initiation and progression of bacterial infection. In this current study, we used the RWV bioreactor to investigate the effect of physiological fluid shear on pathogenesis-related phenotypes of the multidrug resistant, non-typhoidal *S.* Typhimurium ST313 strain A130; a strain known to be associated with invasive bloodstream infections in children and HIV-infected adults in Sub-Saharan Africa. Our studies revealed that high fluid shear culture increased the resistance of A130 to several pathogenesis phenotypes, including acid, bile, and oxidative stress. In contrast, osmotic and thermal stress results showed no dependence on fluid shear under the conditions of this study. Interestingly, we also found that modulation of phosphate and magnesium ion concentrations in the medium altered the acid and oxidative stress responses. As these ions are known to be present at abnormal concentrations in patients with HIV and malaria (patient populations with known susceptibility to NTS infections with invasive ST313), this knowledge can be built upon in future studies to better ascertain the potential role of these ions in patients during co-infection. To our knowledge, this is the first report that physiological fluid shear regulates pathogenesis-related stress responses in A130, and the first to demonstrate that ions regulate the response of A130 to physiological fluid shear.

Previous studies by our laboratory were the first to demonstrate that differences in physiological fluid shear induced global changes in χ3339, a well-studied strain of *S.* Typhimurium belonging to sequence type ST19, including alterations in virulence, stress

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responses, and gene expression that were vastly different than when grown under conventional shaking culture (Nickerson et al., 2000, Wilson et al., 2002a, Wilson et al., 2002b, Wilson et al., 2007, Nauman et al., 2007, Wilson et al., 2008). It is well established that *S.* Typhimurium is challenged with an assortment of environmental stresses during infection, including acid, oxidative, bile, osmotic and thermal stresses - and the response of *Salmonella* to these stressors is critical for its pathogenesis and virulence (Wilson et al., 2002a, Wilson et al., 2002b, Aertsen & Michiels, 2004, Rychlik & Barrow, 2005, Ly and Casanova, 2007, Fabrega and Vila, 2013). Interestingly, previous studies by our laboratory also demonstrated that the ability of *S.* Typhimurium to survive these stresses was dependent on the level of fluid shear, further demonstrating the ability for this physical force to reprogram the pathogen.

Leveraging from these earlier studies, subsequent studies conducted by our laboratory profiled the virulence and pathogenesis-related stress responses of the ST313 strain D23580, which emerged at a later time than A130 (Yang et al., 2016). These studies demonstrated that following HFS culture at late-log phase of growth, D23580 displayed increased resistance to multiple pathogenesis-related stresses, and led to decreased time-to-death in orally infected mice relative to the same strain cultured under LFS culture (Yang et al., 2016). Furthermore, the inclusion of beads of differing sizes/densities in the RWV to incrementally increase fluid shear levels during culture led to corresponding progressive increases in the resistance of D23580 to pathogenesis-related stresses, enhanced colonization of macrophages and human intestinal cells, and

incremental alterations in gene expression at stationary phase of growth (unpublished

data Yang 2015; Manuscript in preparation).

Based on the collective body of data with both χ3339 and D23580, we

hypothesized that *S.* Typhimurium A130 would also possess the capability to respond to

varying fluid shear conditions. Due to the high level of genomic similarities between

A130 and D23580, we predicted that like D23580, A130 grown under HFS would also

exhibit higher resistance to pathogenesis-related stress responses. As shown in the results

section, this was indeed the case for two of the responses. Table 1 shows a comparison of

the results of five stress assays with χ3339 (*S.* Typhimurium ST19) and the three strains

of the ST313 clade that have been studied by our laboratory using RWV bioreactor

technology – D23580 (unpublished data Yang 2015; Manuscript in preparation, Yang et

al., 2016), A130 (this study), and 5579 (unpublished data, Castro 2016), the latter of

which is closely related to D23580, both of which evolved later than A130. The results

listed are for cultures of these strains analyzed in the RWV under HFS and LFS at the

stationary phase of growth (24 hours) for A130, χ3339, and 5579 (Unpublished data,

Castro 2016, unpublished data, Crenshaw 2016). As mentioned, the only published

results with D23580 in the RWV were done in late log phase, which is a different phase

of growth than used in the current study. However current work by our group with

D23580 has looked at the effects of stresses on stationary phase cultures with

incrementally increasing levels of fluid shear in the RWV (provided by the addition of

beads with different densities). The results for D23580 showed enhanced resistance to

oxidative and acid stresses when grown under incremental increases of fluid shear using

addition of beads (Unpublished data, Yang 2015; Manuscript in preparation). Although no direct comparison can be made due to differences in protocol with D23580, the trends that were observed during these experimental conditions were mimicked in A130 and χ3339. Since the phenotypic stress response of D23580 does not change due to variation in high fluid shear it could be assumed that this response is conserved evolutionarily among the three strains.

These comparisons indicate that the osmotic stress and thermal stress responses are the least conserved responses across the *S.* Typhimurium strains tested in this study, while acid, oxidative, and bile stress responses seemed to be more conserved among all of the strains (Unpublished data, Yang 2015; Manuscript in preparation; unpublished data, Castro 2016, unpublished data, Crenshaw 2016). For A130, D23580, and χ3339, high fluid shear culture increased the resistance of the pathogen to acid stress compared to low fluid shear culture, while for ST313 strain 5579 (that evolved later than A130), the opposite trend (LFS/HFS) was observed. This may indicate an evolutionary change which either led to a mutation or deletion in a gene in 5579, which is important for the acid stress response (Gordon et al., 2008, Kingsley et al., 2009). Interestingly, strain 5579 displayed the opposite trend for the acid stress response as compared to the three other *S.* Typhimurium strains (A130, D23580, χ3339), with LFS cultures displaying enhanced resistance. It was hypothesized due to the high level of genetic similarity between D23580 and 5579 that these strains would exhibit a conserved response to acid stress; however, this was not the case. Instead the response of 5579 to acid stress was more consistent with previous studies of χ3339 grown to late log phase under low fluid shear,

where there was increased resistance to acid stress. (Nickerson et al., 2000, Wilson et al., 2002a, Wilson et al., 2002b, unpublished data Castro, 2016).

In response to bile stress, A130, 5579, and χ3339 all displayed higher survival in the HFS compared to LFS cultures, implying that this phenotypic response is conserved across all of these strains (Unpublished data Castro, 2016, Unpublished data Crenshaw, 2016). There is no information currently available regarding the response of D23580 to bile stress at stationary phase of growth under low and high fluid shear. Bile is made and released by the liver and stored in the gallbladder, where it aids with digestion due to its detergent-like properties in the duodenum of the small intestine (Lee et al., 1994, Aertsen & Michiels, 2004, Rychlik & Barrow, 2005, Alvarez-Ordóñez, et al., 2015). It breaks down fats into fatty acids, which can be taken into the body by the digestive tract (Shen & Fang, 2012). The microenvironment of the small intestine is influence by a mixture of both low and high fluid shear. Specifically, in the duodenum, the physical forces of peristalsis contractions are more rapid in frequency compared to the distal ileum for propulsive function (Gayer & Basson, 2009). Known membrane components related to bile resistance include LPS, *tol* genes, and PhoQ of the PhoP/Q two component system (TCS), which could imply that the defense of *S.* Typhimurium to bile salt reduces the ability of bile salts to bind to the outer membrane, which are found in the three ST313 strains and χ3339 (Ramos-Morales et al., 2003, Prouty et al., 2004, Kingsley et al., 2009). Understanding how these *S.* Typhimurium strains respond to bile stress and fluid shear can better convey what they experience *in vivo* during the course of infection in the small intestine.

All *S.* Typhimurium strains, including classic NTS χ3339 and iNTS ST313

strains, displayed increased resistance to oxidative stress when cultured under HFS

relative to LFS cultures (Table 1). Examining the results of A130 from the acid,

oxidative, and bile stress assays, the assay that seemed to have the largest difference in

survival between HFS and LFS was oxidative stress. Specifically, 45 minutes post-

addition of oxidative stress, the fold difference between HFS and LFS was about 5-fold,

which then doubled by 75 minutes (Figure 4). The conserved response to fluid shear

across all of the *S.* Typhimurium strains to oxidative stress could imply that genes related

to oxidative response to also be involved in response to changes in fluid shear.

RpoS is the master regulator of stress responses in stationary phase for *S.*

Typhimurium, which provides resistance for oxidative stress (Hengge-Aronis, 2002,

Wilson et al., 2002a, Aertsen & Michiels, 2004, Rychlik & Barrow, 2005, Chiang &

Schellhorn, 2012). Previous studies by our laboratory, and those of Pacello *et al.*, found

conflicting responses of S. Typhimurium to fluid shear and its relationship to RpoS

(Pacello et al., 2012, Unpublished data Crenshaw, 2016). Specifically, Pacello *et al.*,

found the LFS condition had increased survival to oxidative stress compared to the HFS

condition for *S.* Typhimurium 14028 during stationary phase of growth, and that this

response was independent of RpoS (Pacello et al., 2012). However, in studies by

Crenshaw *et al.*, during stationary phase of growth, it was observed that the HFS

condition increased the resistance of *S.* Typhimurium χ3339 during oxidative stress

compared to the LFS condition, which confirmed RpoS dependent stress regulation.

However for A130, the response to oxidative stress and RpoS regulation under different levels of fluid shear is unknown (Unpublished data Crenshaw, 2016).

Other implications of the large fold trend observed in response to oxidative stress between HFS and LFS for A130, could suggest that this ST313 strain may have evolved to respond to the HFS in the bloodstream, an environment that it would routinely encounter due to systemic disease presentation (Feasey et al., 2008, Gordon et al., 2008, Kingsley et al., 2009). The presence of reactive oxygen species in phagocytic cells could also have allowed adaptation for greater survival in these environments (Wilson et al., 2002c, Aertsen & Michiels, 2004, Rychlik & Barrow, 2005, Ly and Casanova, 2007, Fabrega and Vila, 2013). RpoE is a commonly found sigma factor that allows for oxidative stress tolerance in cells at stationary phase (Rychlik & Barrow, 2005). It has been shown in a strain of *S.* Typhimurium that an *rpoE* mutant was hypersusceptible to oxidative stress (Testerman et al., 2002). As all of the experiments in this study were conducted at stationary phase, where RpoE plays an important role in oxidative stress tolerance, one future experiment could explore the impact of an *rpoE* mutation in A130 on the fluid shear-dependent stress response. It would be essential to look at RpoE, since it plays a large role in the regulation of RpoS.

In the second half of this study, we explored whether supplementation of phosphate or magnesium ions to the media abrogate the phenotypic differences observed in stress responses between HFS and LFS for A130, as it had been shown previously for χ3339 (Wilson et al., 2008). Figure 8 and Figure 9 show that the addition of 25mM Na_2HPO_4 or 2 mM $MgSO_4$ negated the phenotypic difference observed for A130 during

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acid stress and oxidative stress, respectively. In Figure 8, the addition of magnesium decreased resistance to oxidative stress of A130 grown under HFS condition. The periplasmic concentration of magnesium in the environment is detected by the PhoP/PhoQ two component system (Soncini, 1996, Moncrief & Maguire, 1998). *S.* Typhimurium encounters magnesium within the phagosome during intracellular infection, where PhoP/Q activates transcription of genes *mgtA* and *mgtCB*, which encode magnesium transporters when magnesium concentrations are low (Spinelli, et al., 2008, Thompson et al., 2011). The implication of this result physiologically, could imply that areas of the body where A130 could cause infection with high levels of $MgSO_4$ could deter infection.

Supplementation of LB media with additional 25 mM Na_2HPO_4 negated the difference observed during acid stress in the absence of Na_2HPO_4 supplementation in A130, wherein bacteria grown under HFS were more resistant to acid stress than LFS. In addition, phosphate supplementation increased the resistance of A130 to acid stress for both HFS and LFS cultures as compared to when it was grown under identical conditions with no Na_2HPO_4 supplementation. The PhoR/B regulon is involved with the management of bacterial phosphate, particularly during nutrient deprivation, and has been shown in previous studies with *E. coli* to play a role in response to environmental stress of bacteria (Baek & Lee, 2006, Beier & Gross, 2006, Lamarche et al., 2007, Crepin et al., 2011, Marzan & Shimizu, 2011). Specifically, a phosphate containing molecule called guanosine tetraphosphate (ppGpp) is responsible for the "stringent stress response" (Spira & Yagil, 1998, Magnusson et al., 2005, Lamarche et al., 2007). The ppGpp nucleotide

sequesters sigma factor RpoS, which is a global response regulator to many environmental stresses (including acid and oxidative stresses) and induced during stationary phase to encode genes related a nutrient-limiting environment (Magnusson et al., 2005, Lamarche et al., 2007). In order to explore how concentrations of magnesium and phosphate in the blood of patients with iNTS associated with HIV and malaria – fluid shear, ion concentrations, and pathogenic-response to physiological stress must be investigated.

In summary, to our knowledge this is the first report that physiological fluid shear regulates pathogenesis-related stress responses in A130, and the first to demonstrate that ions regulate the response of A130 to physiological fluid shear. Specifically, stationary phase cultures of A130 displayed enhanced resistance to acid, oxidative and bile stresses when cultured in HFS control condition compared to LFS condition when LB was used as the culture medium. Supplementation of either phosphate or magnesium ions to the culture medium abrogated the fluid shear-related differences observed for A130 in LB medium for the acid or oxidative stress responses, respectively. Comparisons made across three ST313 strains and a classic *S.* Typhimurium strains showed that the osmotic and thermal stress responses were the least evolutionarily conserved, while acid, bile, and oxidative stresses were conserved with most of the four strains displaying increased resistance of HFS cultures over LFS cultures.

Understanding the effects of fluid shear and phosphate and magnesium concentrations on stress assay responses (specifically acid and oxidative) may provide insight for therapy treatments for patients with HIV and malaria who suffer from

hypermagnesemia/hypomagnesemia or hypermagnesemia/ hyperphosphatemia, and are inflicted with iNTS Typhimurium. The levels of fluid shear that are found within the microenvironment of a host where these pathogens cause infection and the ability of these physical forces to alter pathogenesis-related stress responses and virulence may aid in suppression of this systemic disease and improve the lives of these patients with already severe complications from immunocompromised conditions. Understanding the influence of physiologically relevant fluid shear and its effect on environmental stress responses and virulence characteristics of ST313 pathovars thus holds promise to provide urgently needed strategies to combat the devastating public health crisis in Sub-Saharan Africa.

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