Fluid Shear Force Regulates the Pathogenesis-Related Stress Responses of Invasive

Multidrug Resistant *Salmonella* Typhimurium 5579

by

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# ABSTRACT

The emergence of invasive non-Typhoidal *Salmonella* (iNTS) infections belonging to sequence type (ST) 313 are associated with severe bacteremia and high mortality in sub-Saharan Africa. Distinct features of ST313 strains include resistance to multiple antibiotics, extensive genomic degradation, and atypical clinical diagnosis including bloodstream infections, respiratory symptoms, and fever. Herein, I report the use of dynamic bioreactor technology to profile the impact of physiological fluid shear levels on the pathogenesis-related responses of ST313 pathovar, 5579. I show that culture of 5579 under these conditions induces profoundly different pathogenesis-related phenotypes than those normally observed when cultures are grown conventionally. Surprisingly, in response to physiological fluid shear, 5579 exhibited positive swimming motility, which was unexpected, since this strain was initially thought to be non-motile. Moreover, fluid shear altered the resistance of 5579 to acid, oxidative and bile stress, as well as its ability to colonize human colonic epithelial cells. This work leverages from and advances studies over the past 16 years in the Nickerson lab, which are at the forefront of bacterial mechanosensation and further demonstrates that bacterial pathogens are "hardwired" to respond to the force of fluid shear in ways that are not observed during conventional culture, and stresses the importance of mimicking the dynamic physical force microenvironment when studying host-pathogen interactions. The results from this study lay the foundation for future work to determine the underlying mechanisms operative in 5579 that are responsible for these phenotypic observations.

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1 INTRODUCTION

*Salmonella* Typhimurium belongs to the Gammaproteobacteria class of bacteria and is a member of the enterobacteriacae family. *Salmonella* is a Gram-negative rod shaped bacterium that is typically motile (Darwin and Miller 1999, Goburn *et al* 2007). It was first discovered in the late 1800's and has been extensively characterized and recognized as one of the best understood bacterial pathogens (Darwin and Miller 1999). For simplicity, it will be referred to in the remainder of this text as *Salmonella enterica* serovar Typhimurium, *Salmonella*, *S.* Typhimurium, or non-Typhoidal *Salmonella* (NTS). Many serovars belonging to *Salmonella enterica* cause bacterial infections in humans and animals (Brenner *et al* 2000, Haraga *et al* 2008). Many NTS serovars are the cause of foodborne illness all around the globe (Mead *et al* 1999, Hohmann 2001). There are over 1 million cases of NTS per year in the U.S. and the number of worldwide cases has been considered to be around 1 billion (Mead *et al* 1999, Brenner *et al* 2000, Goburn *et al* 2007). Non-Typhoidal *Salmonella* have adapted an opportunistic lifestyle that occupies the intracellular environment of host cells by surviving and replicating once inside (Goburn *et al* 2007, Haraga *et al* 2008). *S.* Typhimurium is able to reside inside of the human host and experiences multiple stressors such as low nutrients, high temperature, and a barrage of host defense systems (Ibarra and Steele-Mortimer 2009). *Salmonella* can experience a range of environments during its life cycle and is known to colonize various abiotic or biotic surfaces leading to contamination of food products and water (Foster and Spector 1995, Mead *et al* 1999, Hohmann 2001, Ellison and Brun

2015). In healthy humans, NTS infections typically cause self-limiting gastroenteritis, and do not usually require antibiotic treatment. Most NTS serovars have been shown not to be host restricted and can cause disease in multiple mammals such as humans, fowl, and cattle. The ability for this pathogen to survive contributes to the pathogenesis and needs to be better understood.

In sub-Saharan Africa, a dramatic increase in the number of NTS cases presenting an invasive phenotype has been observed. Following characterization by Multi Locus Sequence Typing (MLST), the majority of NTS isolates from the tropical African region were identified as sequence type (ST) 313 and were commonly isolated from patients co-infected with HIV (Kingsley *et al* 2009). Children are also affected by invasive NTS infections and are commonly associated with meningitis, malaria and malnutrition (Hohmann 2001, Kingsley *et al* 2009, Kariuki and Onsare 2015). While the case fatality rate is about 0.1% for classical *S.* Typhimurium infections in industrialized countries (Mead *et al* 1999), ST313 has been reported to cause lethal infections in anywhere from 25-45% of humans and is commonly associated with a multi drug resistant phenotype further complicating the disease outcomes (Gordon *et al* 2002, Gordon *et al* 2008, Kingsley *et al* 2009, Feasey *et al* 2012).  ST313 is recognized as a phylogenetically distinct clade and has been reported as the leading cause of blood borne infections in sub-Saharan Africa and needs to be further characterized to prevent disease outbreaks (Kingsley *et al* 2009, Okoro *et al* 2012, Kariuki and Onsare 2015). Colonization with NTS commonly results in self-limiting gastroenteritis and rarely leads to invasive disease in healthy individuals (Gordon *et al* 2002, Feasey *et al* 2012, Kingsley *et al* 2009). As early as 1990 there has been evidence of ST313 *S.* Typhimurium displaying an invasive

phenotype leading to significant mortality, and it has since become the most common bacterial blood isolate present in sub-Saharan Africa (Gilks *et al* 1990, Gordon *et al* 2008, Kingsley *et al* 2009, Reddy *et al* 2010).

There is evidence of chromosomal deletions present in ST313 indicating distinct phenotypic features first reported in 2009 that indicate microevolution is continuously occurring (Kingsley *et al* 2009). Whole genome sequencing of ST313 representative strain D23580, indicated a reduction of genomic content. This is similar to what has been observed for the human restricted pathogen *S.* Typhi; the cause of typhoid fever (House *et al* 2001, Kingsley *et al* 2009). Evidence of gene deletions and pseudogene formation was also discovered in ST313, similar to what had also been observed in *S.* Typhi (Baker and Dougan 2007). These characteristics may indicate the narrowing of host tropism to become a more human-restricted pathogen. ST313 serovars could provide evidence for becoming less adapted to the gut microenvironment and more suited to a systemic life style, which coincides with high rates of bacteremia in infected patients. (Kingsley *et al* 2009, Feasey *et al* 2012, Kariuki and Onsare 2015). The presence of pseudogenes and overall reduction of genome size is evidence of the ST313 clade presenting a phylogenetically distinct genotype from other *S.* Typhimurium isolates (Kingsley *et al* 2009). Classical ST19 and ST313 NTS serovars were profiled to see if there is any genetic relation, and they appear to have diverged from a common ancestor in the past (Kingsley *et al* 2009). The genomic content of ST313 was shown to be ~15 kb less than ST19 NTS (Kingsley *et al* 2009). There is an urgent need to better characterize NTS caused by ST313, the lack of information related to its pathogenesis and the high mortality rates suggest there are uncharacterized mechanisms contributing to

pathogenesis. ST313 NTS isolates present symptoms of gastroenteritis in fewer than 50% of cases and the lack of blood culture facilities in the region prevent rapid diagnosis and treatment options. Compounding the problem, ST313 isolates are often found to be resistant to multiple antibiotics.

There have been several previous studies indicating the correlation of severe bacteremia in sub-Saharan Africa and ST313 isolates (Kingsley *et al* 2009, Feasey *et al* 2012, Okoro *et al* 2012). A representative strain, known as D23580, has been fully sequenced by Kingsley *et al* and is a clinical isolate discovered in 2004 from the blood of a pediatric patient. Another closely related clinical isolate is known as 5579, a member of ST313, was chosen for this study. There is insufficient information regarding this genetically distinct ST313 isolate 5579 and the pathogenesis is not fully understood. The ST313 strain 5579 is a clinical isolate from the blood of a patient in 2003 from Nairobi, Kenya. It is closely related to D23580 based on genetic analysis (Kingsley *et al* 2009) and also presents a phenotype characterized by resistance to multiple antibiotics. When the genetic content of these two strains was compared, D23580 was shown to contain more deletions than 5579; a finding that was consistent with the isolation of 5579 a year prior to D23580. Studies by our laboratory have demonstrated D23580 has the ability to cause lethal disease in mice, thus demonstrating that the pathogen has not become host restricted to humans (Yang *et al* 2015a).

A down side of previous studies, which are aimed at better understanding the physiology of ST313, has overlooked an important aspect of the host-pathogen microenvironment known as physiological fluid shear. Fluid shear can be defined as the

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force exerted as it flows over a surface, and low fluid shear is be defined as >0.01 dyne/cm$^2$.
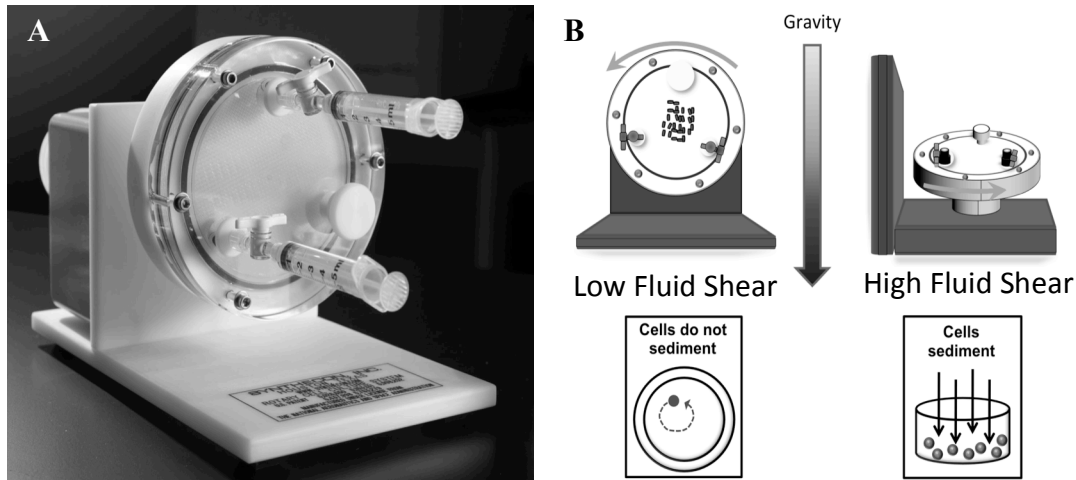


**Figure 1. Rotating Wall Vessel (RWV) bioreactor and operating orientations.** (Synthecon, Houston, TX). **(A)** Image of the NASA-designed RWV **(B)** The RWV bioreactor in the low fluid shear (left) and high fluid shear (right) orientation. The cylindrical vessel is completely filled with culture medium and air bubbles are removed to minimize fluid shear stress. In the low fluid shear orientation, cells are maintained in suspension and do not sediment. In the high fluid shear orientation, bacteria sediment to the bottom of the vessel and are subjected to higher shear conditions.
Image A, courtesy of NASA JSC; Image B, courtesy of Jennifer Barrila.

This mechanical force is always present *in vivo* and has been characterized previously by our lab via the use of the rotating wall vessel bioreactor (RWV) (Nickerson *et al* 2000, Wilson *et al* 2002a, Wilson *et al* 2002b, Nickerson *et al* 2004, Wilson *et al* 2007, Nauman *et al* 2007, Wilson *et al* 2008, Crabbeˊ *et al* 2010, Castro *et al* 2011, Crabbeˊ *et al* 2011). These findings have paved the way for a better understanding of a mechanical force of fluid shear and how it can alter pathogenic bacteria. The ability of microbes to sense and adapt to the environment is altered by mechanical forces such as fluid shear, and has been demonstrated in *S.* Typhimurium that fluid shear results in molecular

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genetic changes, alterations in pathogenesis, and virulence (Nickerson *et al* 2000, Wilson *et al* 2002a, Wilson *et al* 2002b, Nauman *et al* 2007). *Salmonella* experiences a range of fluid shear conditions during its voyage through a human host ranging from high in the circulatory system to low in regions of the small intestine such as between microvilli of epithelial cells (Nickerson *et al* 2003, Nickerson *et al* 2004, Nauman *et al* 2007). To better understand the disease-causing mechanisms, and to elucidate the effect of a physiologically relevant fluid shear experienced by 5579, the NASA designed RWV was introduced to test the effect of these mechanical fluid shear forces (Figure 1). The low fluid shear environment within the RWV provides an optimized suspension culture, in which cells experience a gentle fluid orbit to allow for the cells to respond in a physiologically relevant manner (Nickerson *et al* 2000, Wilson *et al* 2002a, Wilson *et al* 2002b, Nauman *et al* 2007). The effect of sedimentation is offset with the rotation of the RWV and cells remain suspended at a constant terminal velocity. Our laboratory previously demonstrated that culture of *S.* Typhimurium ST19 strain χ3339 (an animal-passaged derivative of the ST19 strain SL1344) in the RWV led to increased virulence, resistance to multiple pathogenesis-related phenotypes (including acid stress and macrophage killing) and a global change in gene expression (Nickerson *et al* 2000, Wilson *et al* 2002a, Wilson *et al* 2002b, Wilson *et al* 2007, Wilson *et al* 2008).  This observed global reprogramming of *S.* Typhimurium, when cultured in a low fluid shear environment, indicates the ability of *Salmonella* to alter its pathogenesis-related stress responses in a unique manner that is not yet fully understood, and not observed using conventional culture conditions. It is still unclear how *Salmonella* is able to transmit this

biomechanical signal from the outside of the cell to the inside in order to regulate its physiology.

It was hypothesized that culturing ST313 isolate 5579 in fluid shear levels commonly encountered during its natural progression through the host could provide insight as to how this pathogen can respond to the mechanical stimuli of fluid shear. By examining how fluid shear can alter the ability of this pathogen to replicate, survive pathogenesis-related stress, exhibit swimming motility and colonize human colonic epithelial cells, the physiology and phenotypic responses are characterized in response to the mechanical stimuli. There are a wide range of physiologically relevant forces experienced *in vivo* that can be generated in the RWV and they have provided evidence for bacteria to be "hardwired" in response to this fluid shear force. There have been previous studies performed with the RWV as a culture apparatus using a classical *Salmonella* serovar and this represents the first time ST313 isolate 5579 has been characterized under this physiological fluid shear force. The initial question was proposed to determine the effect of low fluid shear culture conditions on the pathogenesis of 5579, and how do phenotypic responses potentially provide clues for disease causing potential.

I hypothesized that low fluid shear levels generated in the RWV could alter the pathogenesis of *S.* Typhimurium 5579 by altering its physiology and phenotypic characteristics. Therefore we investigated the effects of low levels of fluid shear on the growth, motility, cell colonization ability, and pathogenesis related stress response using 5579 cultured to stationary phase. The purpose of this study was to determine the effect of a physiologically relevant fluid shear level on 5579 to better understand how pathogenesis and physiology can be altered. Characterizing bacteria and their ability to

regulate phenotypic responses in the physiological fluid shear condition can provide new

opportunities to target mechanisms that cause disease.

| *Salmonella* Typhimurium Sequence Type (ST) | Strain | Region of Isolation | Reference |
|---|---|---|---|
| **ST313 (iNTS)** | 5579 | Kenya (2003) | Kingsley *et al* 2009 |
| | D23580 | Malawi (2004) | Kingsley *et al* 2009 |
| **ST19 (NTS)** | SL1344 | U.S. (1982) | Hoiseth *et al* 1981 |
| | CAN 901 SL1344 Δ*flh*CD | | Radtke *et al* 2010 |

**Table 1. *Salmonella* Typhimurium strains used in this study.**

2  MATERIALS AND METHODS

*Bacterial Strains and Mammalian Cell Lines*

Bacterial strains are listed in Table 1. For all studies, stock cultures were streaked

onto Lennox Broth (LB) agar (supplemented with antibiotic if necessary) and incubated

at 37 °C. Isolated colonies were initiated in LB with aeration (250 rpm) overnight for 15

hours at 37 °C. To grow strains under conventional laboratory culture conditions,

overnight cultures were diluted 1:200 and incubated shaking (250 rpm) for 24 hours. For

RWV studies, overnight cultures were inoculated into 150 mL LB at a 1:200 dilution and

aseptically loaded into two bioreactors. The reactor was completely filled with culture

medium and bubbles removed. The RWV was oriented in the low fluid shear (LFS)

orientation and high fluid shear (HFS) for bacterial assays (Figure 1). All cultures in the

RWV were maintained at 37 °C and rotated at a speed of 25 rpm. Studies with

*Salmonella* were cultured in the RWV for 24 hours, which corresponds to late

log/stationary phase. The human colonic epithelial cell line, HT-29 (obtained from

American Type Culture Collection; HTB-38, Chen *et al* 1987) was used for bacterial

colonization studies. As described previously (Honer zu Bentrup *et al* 2006, Radtke *et al*

2010, De Weirdt *et al* 2012) the cells were cultured in GTSF-2 supplemented with 10%

fetal bovine serum, and incubated at 37 °C at a 10% $CO_2$ environment.

*24 Hour Growth Curve*

The growth profile of *S.* Typhimurium 5579 was determined during culture in the

RWV. The initial amount of bacteria determined prior to loading is T0. Bacteria were

sampled from the reactors at one-hour intervals for 12 hours and every 4 hours until the

24-hour time point. For reactors in the LFS orientation, samples were taken without

stopping the rotation of the RWV in order to maintain the low fluid shear condition

(LFS).  Prior to sampling the high fluid shear (HFS) culture, the RWV in HFS condition

was inverted to ensure the sample contained an even distribution of bacteria, since the

bacteria sediment in this orientation. Plates were incubated at 37 °C. In addition to

plating for viable CFU, the optical density at 600 nm ($OD_{600}$) was also measured for each

time point.

*Mammalian Cell Culture and Infection Assays*

HT-29 cells were cultured as a monolayer and maintained under 10% $CO_2$. To

initiate monolayers, approximately $1 \times 10^6$ - $2 \times 10^6$ cells from frozen stock were initiated

in a T75 flask and incubated in GTSF-2 media (2.25 g/L sodium bicarbonate) for 5-7

days until appropriate confluence was reached. Confluent HT-29 cells in T75 flasks were rinsed with Hanks Balanced Salt Solution (HBSS), trypsinized, and ~$2.75 \times 10^5$ cells per well were seeded in 24-well tissue culture plates in 1 ml of GTSF-2 media. The cells were grown for 24-48 hours at 37 °C and 10% $CO_2$ until reaching 100% confluency. Prior to infection with *Salmonella*, the media in each well was replaced with fresh media, with a final volume of 250 μL because the bacteria were though to be non-motile. Bacteria cultured either conventionally or in the RWV, for 24 hours, were inoculated with an m.o.i. of 10 to 1. LB media was used to dilute bacterial culture sample in order to achieve the desired m.o.i. in a 15 μL inoculation volume. The infection dose was confirmed by determining the CFU of initial inoculum (CFU/well) after plating on LB agar. Cell cultures were infected for 30 minutes at 37°C and 10% $CO_2$. Monolayers were then washed in triplicate with HBSS and a subset of the wells designated for the adherence time point were lysed with 0.1% sodium-deoxycholate. Samples were serially diluted and plated on LB agar to ascertain CFU/mL to determine the number of bacteria adhered to the host cells. Cells were then incubated an additional 2.5 hours with GTSF-2 containing gentamicin (50 mg/ml) to eliminate extracellular bacteria. To determine the number of invaded bacteria at 3 hours post-infection (h.p.i.) cells were washed twice with HBSS, lysed and plated as described above. Remaining wells designated for the survival time point were washed twice with HBSS and incubated with gentamicin-containing GTSF-2 media (10 μg/ml). At 24 h.p.i., cells were processed as described above and viable bacteria enumerated on LB agar.

*Motility Test*

*S.* Typhimurium strains were profiled for swimming motility on agar plates containing non-identical top and bottom layers of agar. The bottom layer consisted of LB with 1.5% agar and the top agar was comprised of either 0.26% or 0.3% agar, 1% tryptone, and 0.5% or 1% NaCl. Bacterial samples were diluted 1:1000 and stabbed into the agar plate by using a sterile pipette tip. The plates were incubated at 37 °C for 10-12 hours. The diameter of the motility pattern was measured in centimeters and <0.5 cm was considered no motility.

*Acid Stress Assay*

Stationary phase cultures of 5579 grown in the RWVs oriented in the LFS and HFS positions were exposed to acidic conditions through the addition of a pre-determined volume of 1M citrate buffer to adjust the pH to 3.5. The pH was verified before and after the experiment via electrode. Cultures were sampled, serially diluted and plated on LB agar prior to exposure of stress to determine T0. Bacterial samples exposed to acid stress were incubated statically at room temperature for 120 minutes, with cultures gently swirled and sampled periodically during incubation, to plate for CFU/mL. Samples were serially diluted in PBS and plated on LB agar in technical triplicate. Plates were incubated at 37 °C and the number of colonies were counted. A minimum of 3 biological replicates was performed.

*Oxidative Stress Assay*

Stationary phase cultures of 5579 grown in the RWVs oriented in the LFS and HFS positions were exposed to 0.12% Hydrogen Peroxide ($H_2O_2$). Oxidative Stress was achieved via the addition of 6% $H_2O_2$. Cultures were sampled, serially diluted and plated on LB agar prior to exposure of stress to determine T0. Bacterial samples exposed to

oxidative stress were incubated statically at room temperature for 40 minutes, with cultures briefly mixed and sampled periodically during incubation, to plate for CFU/mL. Samples were diluted serially in fresh 0.1 M sodium bicarbonate, to neutralize hydrogen peroxide stress, prior to plating on LB agar in technical triplicate. A minimum of 3 biological replicates was performed.

*Osmotic Stress Assay*

Stationary phase cultures of 5579 grown in the RWVs oriented in the LFS and HFS positions were exposed to 5M NaCl resulting in a final concentration of 4M. Cultures were sampled, serially diluted and plated on LB agar prior to exposure of stress to determine T0. Bacterial samples exposed to osmotic stress were incubated statically at room temperature for 24 hours, with cultures briefly mixed and sampled periodically during incubation, to plate for CFU/mL. Samples were diluted in PBS and plated on LB agar in technical triplicate. A minimum of 3 biological replicates was performed.

*Thermal Stress*

Stationary phase cultures of 5579 grown in the RWVs oriented in the LFS and HFS positions were exposed to thermal stress. A 2 mL sample of each culture was exposed to a temperature of 53 °C, with a heating block. Cultures were sampled, serially diluted and plated on LB agar prior to exposure of stress to determine T0. Bacterial samples exposed to thermal stress were incubated statically for 75 minutes, with cultures briefly mixed and sampled periodically during incubation, to plate for CFU/mL. Samples were diluted in PBS and plated on LB agar in technical triplicate. A minimum of 3 biological replicates was performed.

*Statistical Analysis*

The experimental results were analyzed using Microsoft Excel. A two-tailed Student's T test with equal variance was performed to determine P values. Standard error of mean (SEM) was determined as the standard deviation divided by the number of replicates performed. *** indicates p≤0.001, ** indicates p≤0.01, * indicates p≤0.05.

3  RESULTS

**S. Typhimurium 5579 exhibits similar growth profiles between low fluid shear and high fluid shear conditions**

5579 was monitored for its growth in RWVs oriented in the LFS and HFS positions for up to 24 hours (Figure 2A). Similar to previous findings by our laboratory with ST19 strain χ3339 (Nickerson *et al* 2000), LFS and HFS control cultures of 5579 showed minimal differences in growth profiles between the two fluid shear conditions. Absorbance values ($OD_{600}$) were measured and are shown in Figure 2B. A comparison of the 24 hour growth curve and absorbance values of 5579 cultured in LFS and HFS shows there are not significant differences between the fluid shear conditions. The CFU of 5579 can be followed through the stages of growth and can be characterized by an initial lag phase for the first two hours before entering log phase after 3 hours of culture in the RWV. At the 8 hour time point, 5579 cultures can be observed entering into stationary phase which is supported by Figure 2A. This provides evidence for the different phases of growth in 5579 according to the CFU. In Figure 2B, the absorbance values indicate that the HFS culture followed a more predictable pattern, which correlated to the CFU profile. The LFS culture presents a difference in absorbance values and displays a lower

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absorbance than that of the HFS culture from hour 8 until hour 12. This is during the late

log or early stationary phase of growth, and further studies should be performed to

determine what is causing the differences in absorbance values.
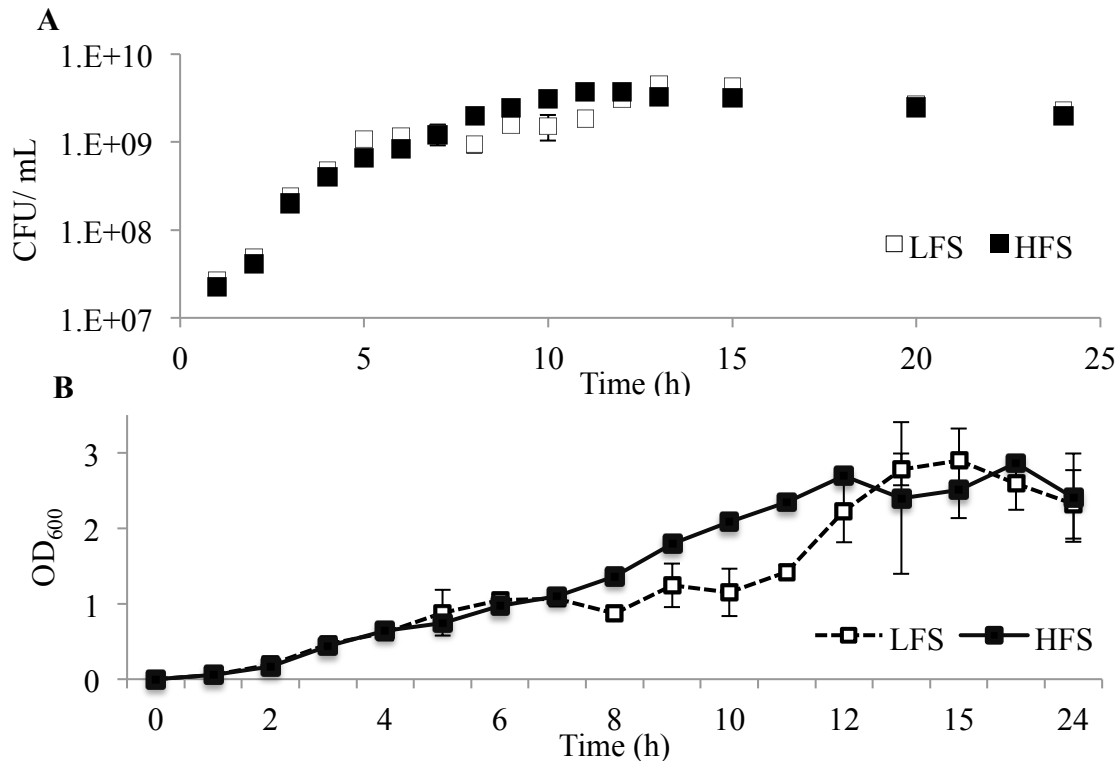


**Figure 2. Growth profile of *S.* Typhimurium 5579 in LFS and HFS conditions generated in the RWV.** Overnight cultures of 5579 were diluted in LB and grown under low fluid shear and high fluid shear conditions in the RWV for 24 hours. **(A)** Growth of 5579 was monitored for 24 hours and quantified by plating for viable colonies on LB to determine CFU. **(B)** Optical density at 600 nm. Experiments were performed in biological duplicate with technical triplicate and the error bars represent the standard deviation.

**Swimming motility of 5579 is induced by culture conditions generated in the RWV**

The ability of *Salmonella* to cause disease may be facilitated, at least in part, by

motility in order to allow for suitable sites of colonization to be reached by pathogens

(Falkow 1992, Macnab 1999, Wiersinga 2012). Previous studies using ST313 isolates

have demonstrated select flagellar proteins are expressed and also provided evidence of

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motility (Ramachandran *et al* 2015). The ST313 isolate D23580, which is closely related

to 5579, was profiled for swimming motility, compared to other *Salmonella* serovars, and

demonstrated a positive swimming motility phenotype (Yang *et al* 2015a). This is

uncharacteristic of ST313 isolates and previous studies have shown the swimming

motility is low or non-motile in this clade (Yang *et al* 2015a, Ramachandran *et al* 2015).

This seemingly non-motile phenotype of 5579 was further investigated with the use of
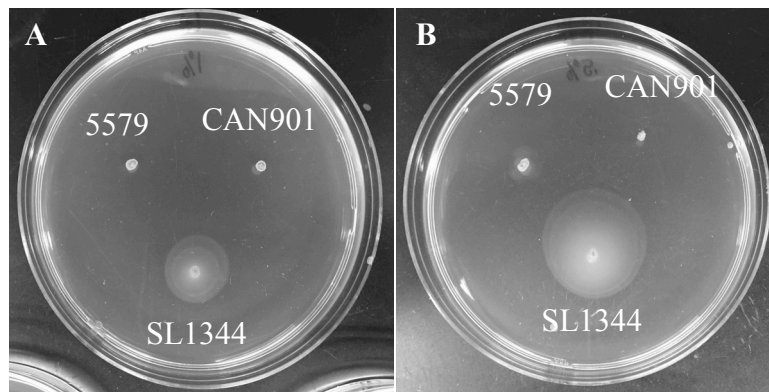


**Figure 3. No swimming motility following conventional shake flask conditions.** Classic non-Typhoidal *S.* Typhimurium strain SL1344 and an isogenic mutant SL1344 Δ*flh*DC (CAN901), were included as motile and non-motile controls, respectively, and compared to ST313 strain 5579 for swimming motility on plates containing 0.3% agar, 1% tryptone, and either **(A)**1% NaCl or **(B)** 0.5% NaCl. There is no swimming motility of 5579 in this culture condition.

physiologically relevant fluid shear levels generated in the RWV. *S.* Typhimurium 5579

was profiled for swimming motility following culture under conventional shaking culture

conditions, as well as following culture in the RWV under LFS and HFS conditions.

The findings in this study align with initial studies showing the phenotype of low

to no motility of 5579, and this was demonstrated using conventional culture conditions

(Figure 3A, Figure 3B, Yang *et al* 2015b). *S.* Typhimurium ST19 strains SL1344 and

isogenic mutant SL1344 Δ*flh*CD, CAN901, were used as motile and non-motile controls, respectively. Following conventional shaking culture 5579 showed no evidence of swimming motility (Figure 3A, Figure 3B). The swimming motility was also found to not be dependent on the salt concentration of the medium, under low levels (Figure 3, Figure 4). The question if 5579 was truly a non-motile *S.* Typhimurium ST313 isolate was investigated with the use of the RWV in order to expose the cells to a physiologically relevant fluid shear. Surprisingly, in contrast to what was observed for 5579 cultured under conventional shaking culture conditions, the swimming motility under LFS and HFS showed a positive swimming motility (Figure 4A, Figure 4C). The LFS swimming motility pattern was significantly higher when compared to the HFS control. There were not any differences in swimming motility at 0.5% NaCl and 1% NaCl (Figure 4). Collectively, these results provide evidence that *S.* Typhimurium 5579 is indeed motile, and that this motility can be modulated by differences in physiological fluid shear. To our knowledge, this work is the first to show that the motility of any bacterial pathogen can be modulated by physiological fluid shear in the RWV.

**Colonization ability of LFS and HFS grown 5579 using cultured human intestinal epithelial cells**

To investigate if colonization ability of 5579 is altered by culture in the RWV under physiological fluid shear levels, cell colonization studies were aimed at examining the ability to adhere and invade cultured HT-29 human colonic epithelial cells. (ATCC; HTB-38). The initial steps of adherence and invasion are important steps to initiate colonization of many intracellular pathogens (Finlay and Falkow 1989, Fields *et al* 1986, Wilson *et al* 2002c, Cossart and Sansonetti 2004, Goburn *et al* 2007, Ibarra and Steele-

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Mortimer 2009, LaRock and Miller 2015) and are necessary in the pathogenesis of

*Salmonella* Typhimurium interactions with the intestinal mucosa.
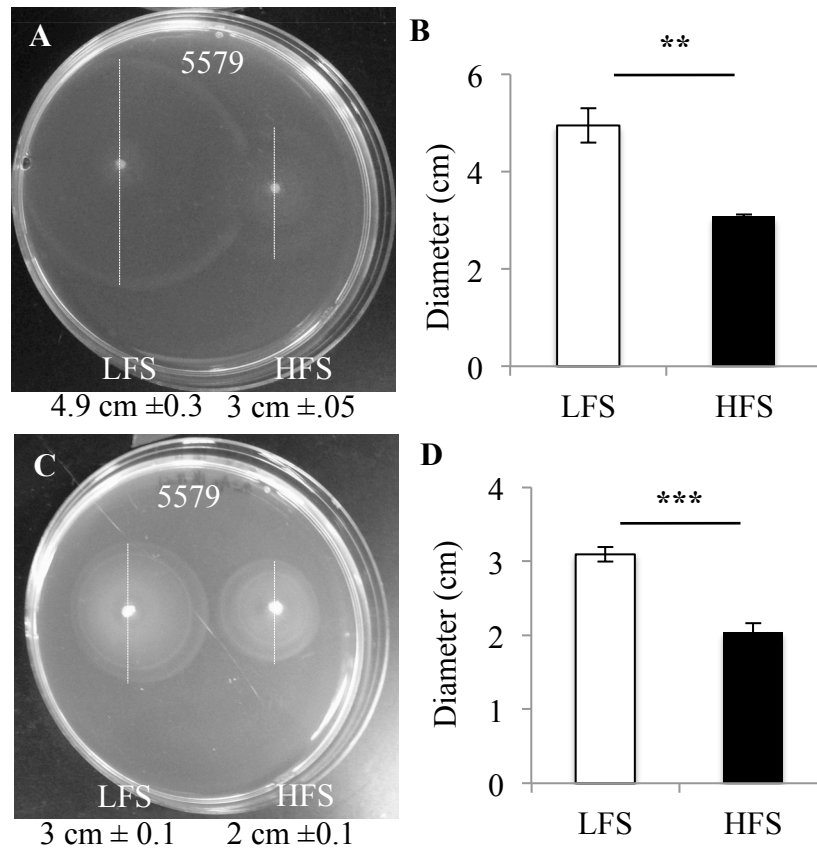


**Figure 4: Physiological fluid shear stimulates motility of 5579.**
ST313 strain 5579 was cultured under LFS and HFS conditions
and profiled for swimming motility. 5579 displays positive
swimming motility after culture in the RWV. **(A)** 0.26% Agar
containing 1% NaCl and 1% tryptone **(B)** Graphical representation
of LFS and HFS motility tested in A **(C)** 0.3% Agar containing 1%
NaCl and 1% tryptone  **(D)** Graphical representation of LFS and
HFS motility tested in C. The LFS culture shows significantly
more swimming motility when compared to HFS.
*** indicates p≤0.001, ** indicates p≤0.01

*Salmonella* Typhimurium 5579 cultured in LFS and HFS exhibited significantly higher

adherence and invasion profiles when cultured in LFS (Figure 5A, Figure 5B) (p≤0.001).

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LFS-cultured 5579 is able to more efficiently adhere and invade with cultured epithelial cells. There was a ~290 fold increase in adherence and ~7 fold increase in invasion for the LFS-cultured 5579 relative to the HFS control. Based on these results, LFS-cultured 5579 are able to adhere and invade cultured cells significantly higher than the HFS control condition, and flask conventional culture. This provides evidence for *S.* Typhimurium 5579 to sense and respond to fluid shear forces in a manner that could lead to increased pathogenicity. Since the motility of 5579 cultured under LFS was found to be greater than that of the HFS control, we wanted to assess whether this trend would correlate with an enhanced adherence and invasion of intestinal epithelial cells. To test this, we performed a gentamicin protection assay with RWV-cultured 5579. For comparison, 5579 was also grown under conventional shaking culture conditions to stationary phase. The intracellular survival was normalized to the initial inoculum (Figure 5C) and to the previous inoculum (Figure 6B). The data provides evidence that low fluid shear conditions allow for significantly ($p \leq 0.001$) more bacteria to be recovered when compared to the higher levels of fluid shear. There was a ~400 fold increase of bacterial survival when compared to the high fluid shear condition, which can be attributed to the low fluid shear condition by permitting increased survival ability or enhanced intracellular replication. There was evidence for bacterial replication at 24 hours when comparing to the number of invaded cells in all experimental groups tested. The intracellular survival compared to the number of invaded bacteria did not show statistical significance. The results presented here could be supported by previous work showing flagella could be important for adherence and invasion of human cells (Josenhans and Suerbaum 2002).
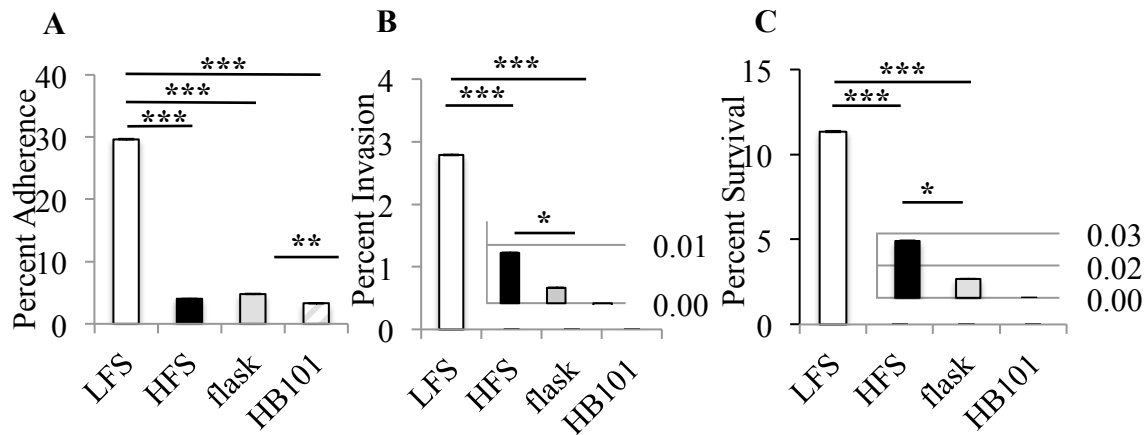
**Figure 5: Low fluid shear enhances colonization ability of 5579 within HT-29 monlayers.** Bacterial cells were cultured for 24 hours at 37°C in the RWV under low fluid shear (white bar) and high fluid shear conditions (black bar), and conventionally (grey bars). Cells were infected with a m.o.i. of 10:1 to HT-29 monolayers. Cell lysates recovered post infection were serially diluted and plated on LB agar in order to recover live bacteria. Survival was calculated to the initial inoculum **(A)** Bacterial adherence was calculated at 30 minutes post infection. **(B)** Bacterial invasion was calculated at 3 h.p.i. **(C)** Intracellular survival was calculated at 24 h.p.i. A minimum of 3 technical replicates were performed per experiment and at least 3 biological replicates were performed. *** indicates p≤ 0.001, ** indicates p≤ 0.01

**Low fluid shear-cultured *S.* Typhimurium 5579 is more resistant to acid stress when compared high fluid shear levels**

To determine the effect of low fluid shear on the acid stress resistance of 5579, cultures were grown in the RWV under LFS and HFS conditions for 24 hours. The ability of bacteria to survive a pH of 3.5 was investigated for 120 minutes by comparing low fluid shear and high fluid shear survival to determine if increased colonization of epithelial cells could be due to increased resistance to an acidic environment. LFS-cultured 5579 was more resistant to acid stress when compared to the HFS control at the 30 minute and 60 minute time points (p≤0.05).

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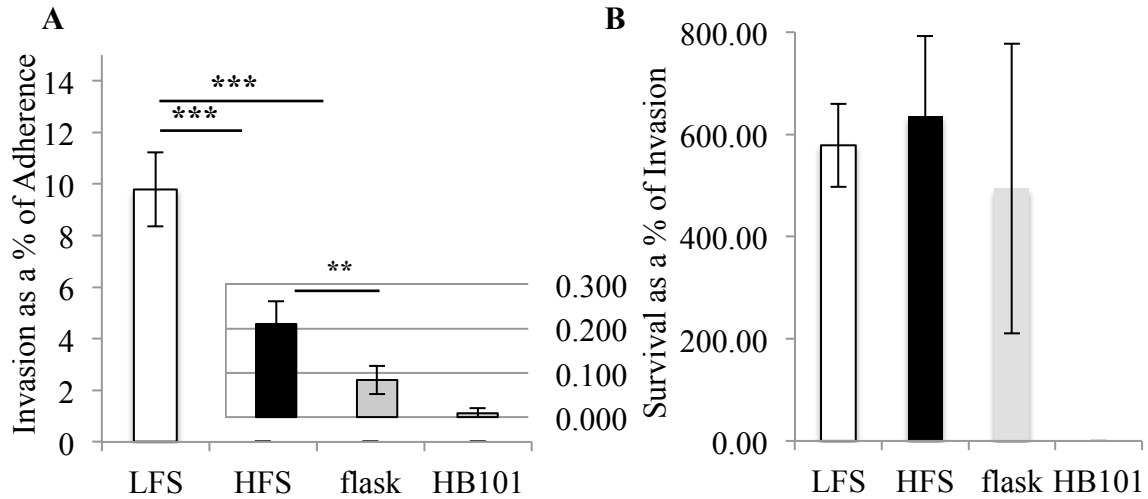
**Figure 6. Infection compared to previous inoculum.** *S.* Typhimurium 5579 percent survival was calculated as to adhered or invaded bacteria. **(A) Invasion as a percent of adhered bacteria** Bacterial invasion to HT-29 monolayers was calculated to number of adhered bacteria. **(B) Intracellular Survival as a percent of invaded bacteria** Bacterial intracellular survival to HT-29 monolayers was calculated to number invaded bacteria. A minimum of 3 technical replicates were performed per experiment and at least 3 biological replicates were performed. *** indicates p≤0.001, ** indicates p≤0.01

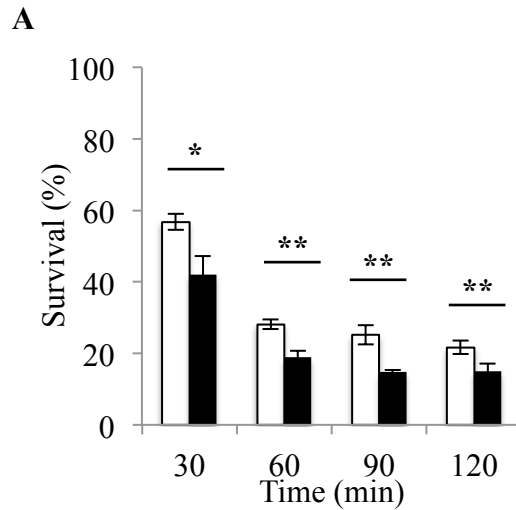The enhanced survival ability of LFS-grown 5579 was ~2 fold higher than that of the HFS-cultures (Figure 7). This supports the hypothesis that low fluid shear-cultured *S.* Typhimurium 5579 may be better adapted to survive the acidic conditions experienced during the natural course of *Salmonella* infection.

**A**



**Figure 7. Low fluid shear induced resistance to acid stress.**
Resistance of *S.* Typhimurium 5579 to acid stress is induced by low
fluid shear culture conditions. Bacteria was cultured under low fluid
shear (white bar) or high fluid shear (black bar) conditions and
subjected to an acidic condition. **(A) Acid stress response** (pH 3.5) To
evaluate the acid stress resistance of 5579 citrate buffer was added to
lower the pH of the culture to 3.5. Representative data from single
biological experiment and at least 3 biological replicates were
performed. Error bars represent the standard error of the mean.
 * indicates p≤0.05 ,** indicates p≤0.01


**Effect of low fluid shear on the resistance of *S.* Typhimurium 5579 to oxidative
stress**

To determine the effect of low fluid shear on the oxidative stress resistance of

5579, cultures were grown in the RWV under LFS and HFS-control conditions for 24

hours and subsequently exposed to hydrogen peroxide for up to 75 minutes (Figure 8A).

The ability of 5579 to survive an environment with elevated levels of hydrogen peroxide

was investigated since this is a stress that is encountered by *Salmonella* Typhimurium in

the intracellular environment of immune cells  (Foster and Spector 1995, Rychlik and

Barrow 2005). LFS-cultured 5579 was less resistant to oxidative stress when compared to

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high fluid shear conditions (p≤0.05). The increased survival ability of high fluid shear grown 5579 was ~14 fold higher then the low fluid shear conditions at the 30 minutes.

**Effect of low fluid shear on the resistance of *S*. Typhimurium 5579 to bile stress**

To determine the effect of low fluid shear and high fluid shear on the bile salt stress resistance of 5579, cultures were grown in the RWV under LFS and HFS control conditions for 24 hours and subsequently exposed to 10% bile salts for 180 minutes to determine if increased pathogenicity could be linked to enhanced environmental stress resistance (Figure 8B). LFS-cultured 5579 was less resistant to bile salt stress when compared to HFS-control (p≤0.01). The increased survival ability of high fluid shear grown 5579 was ~3.5 fold higher then the low fluid shear conditions for the duration of the experiment.
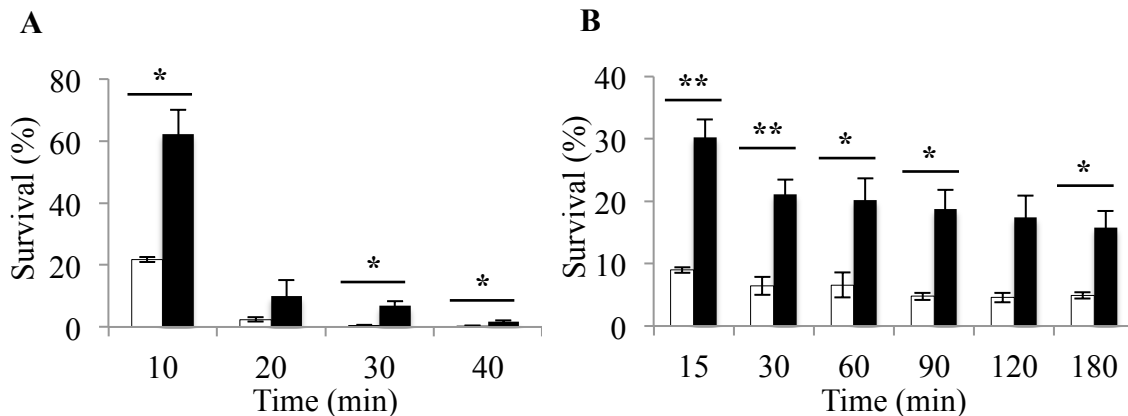


**Figure 8. Low fluid shear inducing sensitivity to oxidative and bile stress.** Sensitivity to environmental stress induced by low fluid shear culture conditions in *S*. Typhimurium 5579. Bacteria was cultured under low fluid shear (white bar) or high fluid shear (black bar) conditions and subjected to environmental stress as described in the materials and methods: **(A) Oxidative Stress** (0.12%) was achieved by the addition of 6% hydrogen peroxide. **(B) Bile salt stress** (10%) was achieved by the addition of 30% bile salts. Results represent averages of at least 3 biological replicates. Error bars represent the standard error of the mean.
** indicates p≤0.01, * indicates p≤0.05

**Effect of low fluid shear on the resistance of *S.* Typhimurium 5579 to thermal stress**

To determine the effect of low fluid shear and high fluid shear on the thermal stress resistance of 5579, cultures were grown in the RWV under LFS and HFS control conditions for 24 hours and subsequently exposed to 53°C for up to 75 minutes (Figure 9A). LFS-cultured 5579 did not show a significant difference in resistance to thermal stress relative to high fluid shear.

A                                                     B
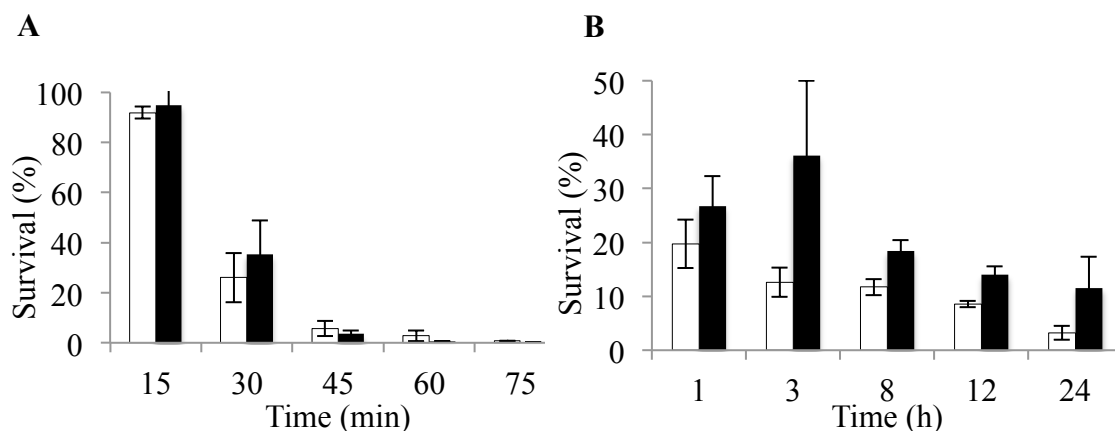


**Figure 9. Thermal and osmotic stress resistance of 5579.** Altered survival due to exposure of environmental stress after culture of *S.* Typhimurium 5579 in the RWV. Bacteria was cultured under low fluid shear (white bar) or high fluid shear (black bar) conditions and subjected to environmental stress as described in the materials and methods: **(A) Thermal stress** (53°C) **(B) Osmotic stress** (4M) was achieved by the addition of 5M NaCl. Results represent averages of at least 3 biological replicates. Error bars represent the standard error of the mean.

**Effect of low fluid shear on the resistance of *S.* Typhimurium 5579 to osmotic stress**

To determine the effect of low fluid shear and high fluid shear on the osmotic stress resistance of 5579, cultures were grown in the RWV under LFS and HFS control conditions for 24 hours and subsequently exposed to 4M NaCl for up to 24 hours (Figure 9B) to investigate resistance to a stress similar to what is encountered *in vivo*. LFS-cultured 5579 was less resistant to osmotic stress when compared to high fluid shear conditions.

4  DISCUSSION

Optimized suspension culture of pathogenic bacteria using RWV bioreactors can provide a physiologically relevant fluid shear environment to help characterize the effect of both low fluid shear and high fluid shear signals known to be present *in vivo.* The use of the RWV in a variety of investigations has accelerated the fields of tissue engineering and infectious disease research (Nickerson *et al* 2000, Wilson *et al* 2002a, Wilson *et al* 2002b, Pacello *et al* 2012, Wilson *et al* 2007, Wilson *et al* 2008, Nauman *et al* 2007, Honer zu Bentrup *et al* 2006, Radtke *et al* 2010, Castro *et al* 2011, Barrila *et al* 2010, Nickerson and Ott 2004). It has previously been shown by our laboratory, and others, that *S.* Typhimurium is hardwired in its ability to alter molecular genetic and phenotypic presentation in response to the low fluid shear condition generated in the RWV, including increased virulence, cross protection to multiple environmental stressors, and regulation of molecular genetic responses (Nickerson *et al* 2000, Wilson *et al* 2002a, Wilson *et al* 2002b, Pacello *et al* 2012, Wilson *et al* 2007, Wilson *et al* 2008).

The objective of this current study was to investigate the effect of physiologically relevant fluid shear levels on the motility, pathogenesis-related stressors (including acid, oxidative, bile, and thermal stress), and colonization profile of multi-drug resistant *S.* Typhimurium ST313 strain 5579. To our knowledge, this is the first study to investigate the impact of fluid shear on this particular ST313 isolate. Previous studies by our laboratory have shown that a closely related ST313 isolate, D23580, is able to sense and respond to fluid shear in ways that differ from the ST19 isolate χ3339 (an animal-

passaged derivative of the well-studied strain SL1344) (Gulig and Curtiss 1987, Yang *et al*. 2015b).

Initial studies characterizing *S.* Typhimurium in the RWV were carried out after a 10 hour incubation, which corresponds to late log phase of growth. The effect of physiological fluid shear on the environmental stress survival and *in vitro* colonization studies were investigated (Nickerson *et al* 2000, Wilson *et al* 2002b, Wilson *et al* 2002a). Exposing χ3339 to a low fluid shear environment generated in the RWV has shown to provide a cross resistance to multiple stressors; with increased survival after exposure to acid, thermal and osmotic stress, compared to high fluid shear (Nickerson *et al* 2000, Wilson *et al* 2002b, Wilson *et al* 2002a). This is consistent with LFS cultured 5579 and its survival ability in an acidic environment, as seen in Figure 7. The findings shown here demonstrate the LFS culture condition allows for a higher acid stress survival and these results are consistent with prior studies despite differences in strain and growth phase. The *in vitro* studies investigating ability of χ3339, cultured in the RWV, to survive within macrophage like cells resulted in LFS cultured bacteria being recovered in higher levels at select time points (Nickerson *et al* 2000, Wilson *et al* 2002b). In previous studies performed with RWV cultured χ3339 for 24 hours, the colonization ability and environmental stress responses were investigated. Interestingly, χ3339 was found to have a higher acid stress survival after culture in the high fluid shear condition (Wilson *et al* 2007, Wilson *et al* 2008). Collectively, these studies, using with a classical ST19 *S.* Typhimurium isolate, paved the way to study *Salmonella* under a physiological fluid shear level, which was further investigated with a multidrug resistant ST313 isolate. This strain, 5579 is one of the first reports of sequence type 313 to be studied in the RWV. To

further investigate the response of *Salmonella* under a physiological fluid shear condition, ST313 isolate 5579 was introduced which is a multidrug resistant strain causing invasive disease, and closely related to D23580. The differences in responses from previous findings in our lab using a classical *Salmonella* and ST313 isolate indicate the need to see if responses are conserved across *Salmonella* strains. Although D23580 and 5579 are closely related (Kingsley *et al* 2009) the responses were varied in response to environmental stressors and colonization assays. This is interesting in the fact that the ST313 isolates are closely related genetically, but present distinct phenotypic responses.

We observed that when 5579 was grown under conventional culture conditions (250 r.p.m. in a flask) and profiled for swimming motility, the strain appeared to be non-motile (This work, Yang *et al* 2015b). The conventional shaking culture condition is expected to maintain a higher fluid shear level when compared to the HFS condition, also considering there could be differences in aeration and other parameters. The un-naturally high level of fluid shear level generated under conventional shake flask conditions is not characteristic of an environment *Salmonella* would encounter *in vivo.* This could repress motility because it is not in an environment where it would be useful to survive. It has been previously found that this violent shaking condition could dislodge flagella from the bacterial cell (Stocker and Campbell 1959). Other findings regarding motility and fluid shear have presented peculiar findings with *Salmonella* in the fact that select flagellar genes appear to be down regulated the low fluid shear environment encountered during spaceflight (Wilson *et al* 2007). This could suggest that in a low fluid shear environment, swimming motility would be repressed but this was found to not be true with my study. Previous reports have characterized the motility of ST313 strains, which displayed low-

to-no motility (Ramachandran *et al* 2015). Interestingly, although the ST313 strain

D23580 is closely related to 5579, previous findings from our lab have shown that

D23580 is highly motile, distinguishing it from most ST313 strains, including 5579

(Yang *et al* 2015a). Interestingly, when we cultured 5579 in the RWV and profiled for

swimming motility, there was a positive phenotype. The swimming motility was found

to be dependent on the level of fluid shear, with the highest swimming motility observed

following low fluid shear culture, relative to the high fluid shear control (Figure 3A,

Figure 3B). To our knowledge, this report is the first that a physiological fluid shear can

uniquely modulate the motility of any bacterial pathogen.

A gentamicin protection assay using the human intestinal epithelial cell line HT-

29 was performed in order to determine whether there is a correlation between the LFS-

induced swimming motility, acid stress survival, and the ability of 5579 to colonize the

intestinal epithelium. When 5579 was cultured under LFS, it adhered, invaded and

survived at significantly higher levels than the HFS control (Figure 5). Previous studies

have indicated that flagella function as an important virulence factor in *Salmonella* by

assisting with motility to gain access to preferential colonization sites and can also be

important in adhesion and invasion of host tissues (Josenhans and Suerbaum 2002, Haiko

and Westerlund-Wikström 2013, Belas 2014). Although non-flagellated *Salmonella* are

still able to adhere and invade, a wild type *Salmonella* serovar would show enhanced

adherence and invasion indicating that flagella do play a role in pathogenesis, and is

shown to be host dependent (Lockman and Curtiss 1990, Josenhans and Suerbaum 2002,

Radtke *et al* 2010, Haiko and Westerlund-Wikstrom 2013). In work by Lockman and

Curtiss, there were no differences in virulence in a mouse model of infection when

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comparing motile and non-motile mutants and this could partially be explained by the route of infection that was used, which was intraperitoneal.

The increased ability of LFS to survive and replicate in the weakly acidic *Salmonella* containing vacuole (SCV) of intestinal epithelial cells could partially be attributed to an increase in acid stress resistance.  In work by Carden *et al*, the ability of 5579 to invade HeLa cells was compared to other classical NTS and ST313 isolates and they were observed to be similar to levels of other ST313 isolates tested (Carden *et al* 2015). It was also verified that ST313 isolate 5579 is able to produce flagella subunit proteins, although motility was not profiled (Carden *et al* 2015). Another study profiled select ST313 isolates for swimming motility and was observed to be positive, although 5579 was not included in the study (Ramachandran *et al* 2015). With this study, it was confirmed that 5579 displays a positive swimming motility and efficiently colonizes intestinal epithelial cells when cultured under a physiologically relevant fluid shear level.

We also evaluated the effect of low fluid shear on the ability of *S.* Typhimurium 5579 to survive potentially lethal stressors. The likelihood of *Salmonella* to establish an infection is largely determined by its ability to survive the defenses encountered inside of the human host, which serve as antibacterial agents.  *Salmonella* is able to adapt to multiple environmental stressors and can activate specific sets of genes in response to acid stress, osmotic stress, thermal fluctuations, and exposure to reactive oxygen species (Christman *et al* 1985, Leyer and Johnson 1993, Rychlik and Barrow 2005). There are also sets of genes that are altered in expression when the bacteria is exposed to a low fluid shear environment, termed the low fluid shear regulon (Wilson *et al* 2002a, Wilson *et al* 2002b, Wilson *et al* 2007, Wilson *et al* 2008). *Salmonella* infections are typically

initiated by the consumption of contaminated food or water. During transit through the gastrointestinal tract, bacteria are exposed to an acidic environment in the stomach and *Salmonella* Typhimurium will experience an acidic environment after penetration of epithelial cells and also inside of macrophages that contain a low pH (Foster and Hall 1990). The findings from my study, show that low fluid shear cultured 5579 has an increased resistance to acid stress, and this could provide evidence as to the increased colonization ability of cultured epithelial cells. The intracellular environment of the SCV is weakly acidic and enhanced resistance could assist *Salmonella* in colonization. The elevated susceptibility of *S.* Typhimurium 5579 to environmental stressors, such as oxidative and bile salt stress after a LFS culture condition is not fully understood. Previous studies examined *Salmonella* cultured in physiological fluid shear and the response to environmental stressors which demonstrated a higher pathogenesis related response and increased virulence after LFS culture (Nickerson *et al* 2000, Wilson *et al* 2002a, Wilson *et al* 2002b, Wilson *et al* 2007, Wilson *et al* 2008).

Within the broader research landscape, this work supports previously known paradigms that physiological fluid shear is important for pathogenesis and could aid in the colonization of host cells by providing distinct environmental signals (Nickerson *et al* 2000, Wilson *et al* 2002a, Wilson *et al* 2002b). Previous studies have shown the ability of the RWV to achieve biologically relevant responses that more accurately mimic the fluid shear environment encountered *in vivo* (Nickerson *et al* 2000, Wilson *et al* 2002a, Wilson *et al* 2002b, Nauman *et al* 2007). A low fluid shear environment has been shown to alter the molecular genetic response of *S.* Typhimurium via regulation by mechanisms that are

not completely understood (Nickerson *et al* 2004, Wilson *et al* 2002a, Wilson *et al* 2002b, Wilson *et al* 2007, Wilson *et al* 2008).

In previous work, our laboratory reported the discovery that ST313 strain D23580 causes lethal disease in a murine model of infection (Yang *et al* 2015a) and also found that ST313 isolates are able to responds to physiologically relevant levels of fluid shear in the RWV (This work, Yang *et al* 2015b). Strain 5579 is a ST313 isolate causing invasive disease, closely related to D23580, and was characterized under low fluid shear conditions to observe characteristics that are not observed using regular culture conditions. It is currently understood that *S.* Typhimurium is a motile pathogen and is propagated through the rotation of the flageller filament present on the outside of the cell (Macnab 1999, Belas 2014). The role of flagella has not been fully elucidated regarding its impact in human models, and there is evidence indicating results are model dependent using classical *S.* Typhimurium serovars. It is thought that flagella could be an important macromolecule aiding bacteria in sensing the extracellular environment, including fluid shear, and this has been shown to be true for multiple motile pathogens (Josenhans and Suerbaum 2002, Ellison and Brun 2015, Belas 2014).

The closely related ST313 strain D23580 indicated a positive swimming motility (Yang *et al* 2015a), which is closely related to 5579, being isolated only a year prior (Kingsley *et al* 2009). This provides evidence that there is production of flagella in ST313 isolates and they are able to display a phenotype characterized with swimming motility. Therefore, flagella are present and could be used in order to transmit the signal of a low fluid shear environment to *Salmonella* and can lead to changes in the

pathogenesis related phenotypic response. With further experiments the role of flagella could be fully elucidated in its role to sensing and responding to a force of fluid shear.

In summary, the data we present here show that *S.* Typhimurium ST313 5579 is able to sense and respond to a physiological fluid shear environment. There is evidence for this provided by unique regulation of swimming motility, enhanced colonization ability of intestinal epithelial cells, and by conferring a cross resistance to multiple environmental stressors, which could contribute to the pathogenesis of 5579. Although it was previously thought that 5579 is a non-motile ST313 isolate (This work, Yang *et al* 2015b), when cultured in low fluid shear levels commonly encountered *in vivo*, there is a swimming motility phenotype observed.  This indicates regulation of the pathogenesis related responses which could aid in the colonization ability of intestinal epithelial cells, significantly enhancing the ability to adhere, invade, and replicate inside of cultured cells after exposure to a low fluid shear environment. In addition, the low fluid shear environment was able to increase the survival ability of 5579 after exposure to an acid shock, and this could also contribute to the enhanced ability to survive and replicate within intestinal cells. This study provides evidence for the ability of *S.* Typhimurium 5579 to regulate its phenotypic responses when exposed to low fluid shear and could suggest that flagella is important in transmitting the signal of physiological fluid shear, and further studies should be performed as follow up to see if this is the case.  This work is, to my knowledge, the first report of 5579 being cultured in a physiologically relevant fluid shear level. It is also the first report, to my knowledge, that the environmental signal of fluid shear is able to modulate the swimming motility of any bacterial pathogen and

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stresses the need to use a more realistic culture condition when studying host-pathogen interactions.

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